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Combinatorial Regulation by MrpC2 and FruA Involves Three Sites in the *fmgE* Promoter Region during *Myxococcus xanthus* Development[▽]

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Starvation causes cells in a dense population of *Myxococcus xanthus* to change their gliding movements and construct mounds. Short-range C-signaling between rod-shaped cells within mounds induces gene expression that promotes differentiation into spherical spores. Several C-signal-dependent genes have been shown to be regulated by cooperative binding of two transcription factors to the promoter region. These FruA- and MrpC2-regulated genes (designated *fmg*) each exhibit a different arrangement of binding sites. Here, we describe *fmgE*, which appears to be regulated by three sites for cooperative binding of FruA and MrpC2. Chromatin immunoprecipitation analysis showed that association of MrpC2 and/or its longer form, MrpC with the *fmgE* promoter region, depends on FruA, consistent with cooperative binding of the two proteins *in vivo*. Electrophoretic mobility shift assays with purified His_{10}-MrpC2 and FruA-His_6 indicated cooperative binding *in vitro* to three sites in the *fmgE* promoter region. The effects of mutations on binding *in vitro* and on expression of *fmgE-lacZ* fusions correlated site 1 (at about position −100 relative to the transcriptional start site) with negative regulation and site 2 (just upstream of the promoter) and site 3 (at about position +100) with positive regulation. Site 3 was bound by His_{10}-MrpC2 alone, or the combination of His_{10}-MrpC2 and FruA-His_6, with the highest affinity, followed by site 1 and then site 2, supporting a model in which site 3 recruits MrpC2 and FruA to the *fmgE* promoter region, site 1 competes with site 2 for transcription factor binding, and site 2 occupancy is required to activate the promoter but only occurs when C-signaling produces a high concentration of active FruA.

Myxococcus xanthus is a Gram-negative bacterium that provides an attractive model to study signaling and gene regulatory mechanisms (59). Flexible, rod-shaped cells move over solid surfaces, forming swarms that seek prey bacteria on which to feed (3). Upon nutrient limitation, cells alter the frequency with which they reverse their direction of gliding and coordinate their movements to construct mounds containing approximately 10^5 cells (14). During this process of aggregation, some cells undergo programmed cell death (33), others remain outside the nascent fruiting bodies as peripheral rods (36), and others differentiate into dormant, stress-resistant, spherical spores. The mature fruiting body is a mound of spores capable of producing a swarm of cells when a nutrient source becomes available.

Fruiting body development is coordinated by intracellular and extracellular signals. Nutrient limitation triggers a stringent response that leads to production of intracellular (p)ppGpp and induction of early developmental genes (10, 47), including *dnaA* (perhaps only in cells destined to become spores), which encodes the initiator protein of DNA replication (41). Cells secrete proteases that produce a mixture of amino acids and peptides known as the A-signal (23, 38), which appears to function as a quorum signal, allowing aggregation to begin and additional genes to be expressed if cells are at a sufficiently high density (15, 22, 24). A second extracellular signal called the C-signal is unusual for bacterial signals in that it functions only at short range (2), perhaps only when cells are in contact. Efficient C-signaling requires that cells become aligned (18), as they do in the outer domain of a nascent fruiting body (43). Many studies support a model in which an increasing level of C-signaling governs mound formation and ensures that spores form within mounds (reviewed in references 12, 14, 45, and 49). The mechanism of C-signaling is only partly understood. It involves CsgA (46), a protein associated with the outer membrane, where it is cleaved by a secreted protease to a 17-kDa form that appears to be the C-signal (17, 26, 40); however, a receptor has not been identified. In any case, a *csgA* mutant fails to form stable aggregates, exhibits reduced or abolished expression of many developmental genes, and forms very few spores (19, 46). Genes normally expressed shortly after early mound formation exhibit reduced expression in a *csgA* mutant, and genes normally expressed later fail to be expressed (19).

The C-signal-dependent changes in motility behavior and gene expression that lead to aggregation and sporulation are mediated by FruA, which is similar to the response regulators of two-component signal transduction systems (6, 37). It has been proposed that FruA is phosphorylated in response to C-signal (6), but a cognate histidine protein kinase has not been identified, and some evidence suggests that FruA might function without being phosphorylated (29). The C-terminal DNA-binding domain of FruA has been shown to bind to sites in the promoter regions of genes that fail to be expressed in a *fruA* mutant, suggesting that FruA is a transcriptional activator (52–53, 58, 62). Recently, FruA was shown to bind coopera-

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tively with MrpC2 to the promoter region of the C-signal-dependent *fmgA* gene (29).

MrpC2 is a truncated form lacking the 25 N-terminal amino acid residues of MrpC (54). MrpC is similar to the proteins in the cyclic AMP receptor protein (CRP) family (50). Its production is inhibited during growth by phosphorylation (34–35). The phosphorylated form of MrpC binds weakly to DNA compared with the unphosphorylated form. Starvation inhibits phosphorylation of MrpC, and some of the unphosphorylated form may be cleaved to MrpC2 or a slightly shorter form lacking 7 more residues at its N terminus (54). MrpC2 binds better than MrpC to sites upstream of the *mrpC* and *fruA* promoters, activating transcription so that the concentrations of MrpC, MrpC2, and FruA increase in starving cells (35, 54). MrpC also binds to the *mazF* promoter region and activates expression of MazF, a toxin that causes programmed cell death during development (33). Interestingly, MrpC binds to MazF and inhibits its activity, allowing some cells in the population to escape programmed cell death and eventually form spores. MrpC2 has not yet been tested for binding to MazF and to the *mazF* promoter region.

The finding that MrpC2 and FruA bind cooperatively just upstream of the *fmgA* promoter to activate transcription suggests that positional information through C-signaling and FruA is integrated with starvation sensing and cell death control through MrpC and MrpC2 (29). Such combinatorial regulation was subsequently demonstrated for other C-signal-dependent genes, although different arrangements of MrpC2 and FruA binding sites were discovered. The two proteins bind cooperatively just upstream of the *fmgBC* and *fmgD* promoters, but FruA binds proximal to these promoters (25, 30), whereas MrpC2 binds proximal to the *fmgA* promoter (29). In the *fmgD* promoter region, a second MrpC2 binding site partially overlaps the FruA binding site and the promoter (25). It was proposed that binding of MrpC2 to the downstream site represses *fmgD* transcription until C-signaling causes the concentration of active FruA to increase sufficiently to outcompete the downstream MrpC2 for cooperative binding with the upstream MrpC2. The model would explain why *fmgD* transcription begins later during development and is more dependent on C-signaling than transcription of *fmgA* and *fmgBC*.

Here, we report that a second gene in the late class that depends completely on C-signaling is under combinatorial control of MrpC2 and FruA, which bind cooperatively to three sites in the promoter region. The gene, herein designated *fmgE*, was first identified as a developmentally regulated gene by an insertion of the transposon Tn*5 lac* at the Ω4406 locus (21). Developmental *lacZ* expression was abolished in a *csgA* mutant (19). The gene at the Ω4406 locus (MXAN3464) (8) is predicted to code for a protein annotated as hypothetical (27), which a BLAST search (1) shows is similar to hypothetical proteins in diverse bacteria and a few eukaryotes, and all of which share a conserved domain found in zinc-dependent metalloproteases. *fmgE* is likely monocistronic (27). The Tn*5 lac* insertion in *fmgE* delays and reduces aggregation and reduces sporulation by about 8-fold (S. Mittal and L. Kroos, unpublished data). Deletion analyses revealed unusual regulation of *fmgE*. A negative regulatory element was localized between positions −533 and −100, relative to the transcriptional start site, and a positive regulatory element was localized between +50 and +140 (55). On the other hand, mutational analysis highlighted the importance of sequences just upstream of the promoter, as in other C-signal-dependent promoter regions (56, 57, 60, 61), including two 5-bp elements (consensus sequence GAACA) at positions −69 to −65 and positions −64 to −60 and a C-box-like sequence, CATCGTG, at positions −55 to −49 (the C-box consensus sequence is CAYYCCY, in which Y means C or T) (55). Using a combination of *in vitro* and *in vivo* approaches, we found that MrpC2 and FruA bind cooperatively not only immediately upstream of the *fmgE* promoter but also slightly farther upstream at about position −100 and downstream at about +100, accounting for the negative and positive regulatory elements, respectively. The different affinities of the three sites for the combination of MrpC2 and FruA support a model in which a rising concentration of active FruA produced in response to C-signaling during development results in occupancy of the downstream site first, followed by occupancy of the upstream site, and finally, occupancy of the site just upstream of the promoter, which activates transcription.

MATERIALS AND METHODS

Bacterial strains and plasmids. Strains and plasmids used in this study are listed in Table 1. To construct plasmids, pKV45 was used as the template for PCR amplification of *fmgE* DNA segments, using upstream primers each containing an XhoI site and downstream primers each containing a BamHI site (Table 2 lists primers). Each PCR product was cloned using pCR2.1-TOPO and *Escherichia coli* strain TOP10, as described by the manufacturer. Each DNA insert was sequenced at the Michigan State University Genomics Technology Support Facility to ensure that the correct sequence was obtained. Each pCR2.1-TOPO derivative was digested with XhoI and BamHI, the DNA insert was gel purified, and it was cloned into XhoI-BamHI-digested pREG1727 using *E. coli* strain DH5α and standard methods (44).

Growth and development. *M. xanthus* strains were grown at 32°C in CTT (1% Casitone, 10 mM Tris-HCl [pH 8.0], 1 mM KH_2PO_4-K_2HPO_4, 8 mM $MgSO_4$ [final pH, 7.6]) medium (11) or on CTT agar (1.5%) plates. When required, 40 μg of kanamycin sulfate per ml was added. Fruiting body development was performed on TPM (10 mM Tris-HCl [pH 8.0], 1 mM KH_2PO_4-K_2HPO_4, 8 mM $MgSO_4$ [final pH, 7.6]) agar (1.5%) plates, as described previously (21).

ChIP. *M. xanthus* strains DK1622 and DK5285 were used for chromatin immunoprecipitation (ChIP) as described previously (29, 62), except Dynabeads protein G (100 μl/ml of cell extract; Invitrogen) were used instead of protein A-Sepharose beads for preclearing and immunoprecipitation. The primers used for PCR of the *fmgE* promoter region were LK1328 and LK1034, and those used for PCR of the *rpoC* coding region were LK1861 and LK1862 (Table 2).

Preparation of His_{10}-MrpC2 and FruA-His_6. Recombinant proteins were expressed in *E. coli* and purified as described previously (29, 35).

Preparation of ^{32}P-labeled DNA fragments. DNA fragments from the *fmgE* promoter region were generated by PCR using a wild-type or mutant plasmid (Table 1) as the template and the primers listed in Table 2. For electrophoretic mobility shift assays (EMSAs), ^{32}P-labeled DNA was synthesized by PCR after labeling the primers with [γ-^{32}P]ATP using T4 polynucleotide kinase (New England Biolabs), and the DNA fragment was purified after 15% PAGE (44).

EMSAs. EMSAs were performed as described previously (62), except that binding reaction mixtures were incubated at 25°C for 15 min.

Construction of *M. xanthus* strains and determination of *lacZ* expression during development. Strains containing a plasmid integrated at the Mx8 phage attachment site *attB* were constructed by electroporation (16). Transformants were selected on CTT agar plates containing kanamycin sulfate and screened on TPM agar plates containing 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside per ml in order to avoid rare transformants with unusual developmental *lacZ* expression (56). Three transformants were typically chosen for further analysis, and β-galactosidase activity was measured as described previously (21).

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
E. coli strains		
TOP10	λ⁻ F⁻ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*ΔM15 Δ*lacX74 recA1 araD139* Δ(*ara-leu*)*7697 galU galK rpsL* (Str^r) *endA1 nupG*	Invitrogen
DH5α	λ⁻ ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17*(r_K^- m_K^-) *supE44 thi-1 gyrA relA1*	9
BL21(DE3)	F⁻ *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* with DE3, a λ prophage carrying the T7 RNA polymerase gene	Novagen
SMhisMrpC2	BL21(DE3) containing pET16b/His$_{10}$-MrpC2	29
SMFruAhis	BL21(DE3) containing pET11km/FruA-His$_6$	29
M. xanthus strains		
DK1622	Wild type	13
DK5285	*fruA*::Tn*5 lac* Ω4491	20
MKV6	*attB*::pKV6 (pREG1727 with 150-bp XhoI-BamHI fragment from pKV3)[a]	55
MKV24	*attB*::pKV24 (pREG1727 with 351-bp XhoI-BamHI fragment from pPV251)	This study
MKV54	*attB*::pKV54 (pREG1727 with 398-bp XhoI-BamHI fragment from pKV45)	This study
MBS03	*attB*::pBS08 (pREG1727 with 197-bp XhoI-BamHI fragment from pBS02)	This study
MBS04	*attB*::pBS07 (pREG1727 with 216-bp XhoI-BamHI fragment from pBS01)	This study
MBS06	*attB*::pBS21 (pREG1727 with 193-bp XhoI-BamHI fragment from pBS15)	This study
MBS07	*attB*::pBS18 (pREG1727 with 170-bp XhoI-BamHI fragment from pBS13)	This study
MBS08	*attB*::pBS19 (pREG1727 with 160-bp XhoI-BamHI fragment from pBS14)	This study
Plasmids		
pET16b/His$_{10}$-MrpC2	pET16b carrying a gene encoding His$_{10}$-MrpC2 under the control of a T7 RNA polymerase promoter	35
pET11km/FruA-His$_6$	pET11km carrying a gene encoding FruA-His$_6$ under the control of a T7 RNA polymerase promoter	S. Inouye
pREG1727	Ap^r Km^r; P1-*inc attP 'lacZ*	7
pCR2.1-TOPO	Ap^r Km^r; *lacZ*α	Invitrogen
pKV3	pCR2.1-TOPO with *fmgE* DNA from positions −100 to +50	55
pKV4	pREG1727 with 1.0-kb XhoI-BamHI fragment from pPV4406-1.0	55
pKV12	pCR2.1-TOPO with *fmgE* DNA from positions −100 to + 320	55
pKV28	pKV12 with CATCGTG-to-ACGATGT mutation from positions −55 to −49	55
pKV29	pKV12 with GGACA-to-TTCAC mutation from positions −69 to −65	55
pKV30	pKV12 with GAACC-to-TCCAA mutation from positions −64 to −60	55
pKV45	pCR2.1-TOPO with *fmgE* DNA from positions −150 to +251, generated by PCR using LK0926 and LK1245 as primers and pKV4 as template	This study
pKV47	pKV12 with A-to-C mutation at position −54	55
pPV251	pCR2.1-TOPO with *fmgE* DNA from positions −100 to +251	55
pBS01	pCR2.1-TOPO with *fmgE* DNA from positions −100 to +116, generated by PCR using LK1328 and LK2527 as primers and pKV45 as template	This study
pBS02	pCR2.1-TOPO with *fmgE* DNA from positions −150 to +50, generated by PCR using LK0926 and LK1034 as primers and pKV45 as template	This study
pBS13	pCR2.1-TOPO with *fmgE* DNA from positions −120 to +50, generated by PCR using LK2577 and LK1034 as primers and pKV45 as template	This study
pBS14	pCR2.1-TOPO with *fmgE* DNA from positions −110 to +50, generated by PCR using LK2578 and LK1034 as primers and pKV45 as template	This study
pBS15	pCR2.1-TOPO with *fmgE* DNA from positions −100 to +93, generated by PCR using LK1328 and LK2606 as primers and pKV45 as template	This study

[a] Where possible, the plasmid description is given in parentheses after the strain description.

RESULTS

MrpC and FruA associate with the *fmgE* promoter region *in vivo*. ChIP assays using polyclonal antibodies to MrpC, which also recognize MrpC2 (35), and polyclonal antibodies to FruA were performed to determine whether these transcription factors associate with the *fmgE* promoter region during *M. xanthus* development. Cells were collected after 18 h and subjected to ChIP with affinity-purified anti-MrpC immunoglobulin G (IgG) (or IgG from a nonimmunized rabbit as a control) or anti-FruA serum (or preimmune serum from the same rabbit as a control). The ChIP DNA was analyzed by PCR with primers designed to amplify the *fmgE* promoter region (positions −100 to +50) or, as a control, the *rpoC* coding region (+1780 to +1905) (62). The PCR analysis showed enrichment of the *fmgE* promoter region by ChIP with anti-MrpC rather than control IgG (Fig. 1A, lanes 5 and 6) and with anti-FruA compared to control preimmune serum (Fig. 1B, lanes 5 and 6). Similar results were observed in two additional experiments. As expected, the PCR analysis with primers designed to amplify the *rpoC* coding region showed no enrichment with anti-MrpC or anti-FruA compared to with controls. We conclude that MrpC and/or MrpC2 and FruA are present in the vicinity of the *fmgE* promoter at 18 h into development.

Association of MrpC and/or MrpC2 with the *fmgE* promoter region depends on FruA. It was shown previously that associ-

TABLE 2. Primers used in this study

Primer	Sequence[a]	Description or reference[b]
LK0926	GGCTCGAGGTTTTTCTCTGTGAAAGAGGAC	Position −150 forward with XhoI site
LK1034	CCGGATCCGTTGTTACCGGCATTGGTGC	Position +50, reverse with BamHI site
LK1245	CCGGATCCACTGGCGCCGGCTGTAGGC	Position +251, reverse with BamHI site
LK1328	GGCTCGAGCATTCTGTGCGGCGTTTCAG	Position −100, forward with XhoI site
LK1861	CCTTGAGCGCGATGGAGATA	60
LK1862	CTCGGCGGCCTCATCGAC	60
LK2442	GAGGGCGCACGAATCGTCC	Position −25, reverse
LK2464	CAATCCCATGTCCTCATCTG	Position −25, forward
LK2487	TTTCTCTGTGAAAGAGGACCC	Position −147, forward
LK2512	CCTGAAACGCCGCACAGAATG	Position −80, reverse
LK2527	GGATCCCGATTGCGTTCCTGCTCCATG	Position +116, reverse with BamHI site
LK2577	GGCTCGAGCATTAACGGGCGATCTTCCTC	Position −120, forward with XhoI site
LK2578	GGCTCGAGCGATCTTCCTCATTCTGTGCG	Position −110, forward with XhoI site
LK2606	GGATCCGGTGAGGACAGCGGTCAG	Position +93, reverse with BamHI site

[a] Restriction sites are underlined.
[b] The position number is relative to the start site of *fmgE* transcription, and the orientation (forward or reverse) is relative to the direction of *fmgE* transcription.

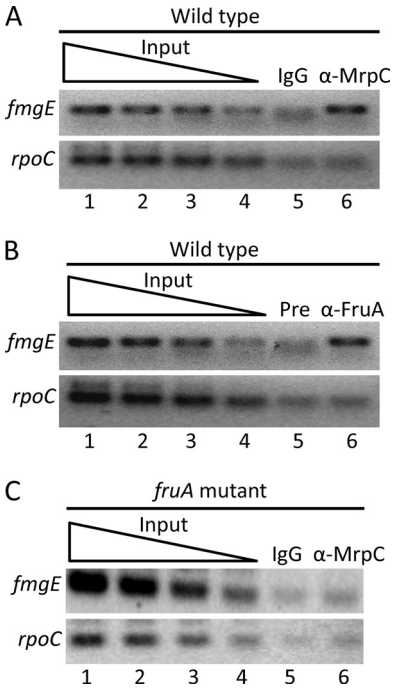

FIG. 1. Association of MrpC and/or MrpC2 and FruA with the *fmgE* promoter region during development. ChIP analysis of *M. xanthus* at 18 h into development. Cells were treated with formaldehyde and lysed. Cross-linked chromatin was immunoprecipitated with antibodies. DNA was amplified with primers for the *fmgE* promoter region (positions −100 to +50 relative to the start site of transcription) or for the *rpoC* coding region (positions +1780 to +1905 relative to the predicted translation start) as a control. A 2-fold dilution series of input DNA purified from 0.025, 0.0125, 0.00625, or 0.003125% of the total cellular extract prior to immunoprecipitation was used as a template in parallel PCRs to show that the PCR conditions allow detection of differences in DNA concentration for each primer set. (A) Wild-type strain DK1622 with affinity-purified IgG antibodies against MrpC (α-MrpC) or, as a control, with total IgG (IgG) from nonimmunized rabbits. (B) Wild-type strain DK1622 with antiserum against FruA (α-FruA) or, as a control, preimmune antiserum (Pre). (C) *fruA* mutant strain DK5285 with antibodies as shown in panel A.

ation of MrpC and/or MrpC2 with the *fmgA* and *fmgBC* promoter regions depends on FruA, presumably due to cooperative binding of the two proteins just upstream of the promoters (29–30). In contrast, association of MrpC and/or MrpC2 with the *fmgD* promoter region did not depend on FruA, as determined by ChIP-PCR analysis of a *fruA* mutant at 18 h into development (25). Although MrpC2 and FruA bind cooperatively just upstream of the *fmgD* promoter *in vitro*, a second MrpC2 binding site overlaps the FruA binding site, and two molecules of MrpC2 can bind cooperatively to the *fmgD* promoter region in the absence of FruA *in vitro*. Presumably either because of this cooperative binding or simply due to the presence of a higher-affinity binding site than those present in the *fmgA* and *fmgBC* promoter regions, MrpC and/or MrpC2 was found to be associated with the *fmgD* promoter region of a *fruA* mutant. To test whether association of MrpC and/or MrpC2 with the *fmgE* promoter region depends on FruA, we performed ChIP-PCR analysis of a *fruA* mutant at 18 h into development. Reproducibly, the PCR analysis showed no enrichment of the *fmgE* promoter region by ChIP with anti-MrpC compared to control IgG (Fig. 1C, lanes 5 and 6). Likewise, there was no enrichment of the *rpoC* coding region compared to the control, as expected. Therefore, association of MrpC and/or MrpC2 with the *fmgE* promoter region at 18 h into development depends on FruA, as seen previously for the *fmgA* and *fmgBC* promoter regions (29–30), suggesting that the two proteins bind cooperatively to the *fmgE* promoter region.

MrpC2 and FruA bind near the *fmgE* promoter to sequences important for promoter activity. Previous studies showed that MrpC2 and FruA bind cooperatively just upstream of the *fmgA* promoter, with MrpC2 proximal to the promoter (29), and the two proteins bind cooperatively just upstream of the *fmgBC* and *fmgD* promoters, with FruA proximal to the promoter (25, 30). Since multiple-base-pair changes just upstream of the *fmgE* promoter were shown to increase or decrease promoter activity (55) (Fig. 2A, top), we tested whether MrpC2 and/or FruA bind near the *fmgE* promoter. EMSAs were performed with *fmgE* promoter region DNA from positions −100 to −25 and purified His_{10}-MrpC2 and FruA-His_6. Each protein produced a single shifted complex, although the complex produced by His_{10}-MrpC2 was barely detectable (Fig. 2A, lanes 1

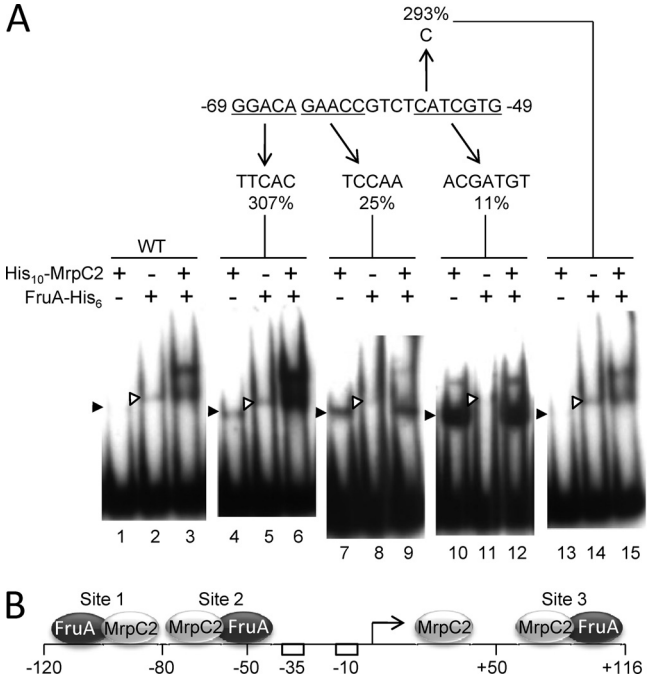

FIG. 2. Binding of MrpC2 and FruA to the *fmgE* promoter region. (A) Effects of mutations in the *fmgE* promoter region on *fmgE-lacZ* expression *in vivo* and on MrpC2 and FruA binding *in vitro*. (Top) Summary of the effects of four mutations on developmental *fmgE-lacZ* expression (55). The mutations are shown, and the numbers indicate the maximum β-galactosidase activity during development, expressed as a percentage of the maximum activity observed for the wild-type promoter. (Bottom) EMSAs with ^{32}P-labeled *fmgE* DNA (2 nM) spanning from positions −100 to −25 and His_{10}-MrpC2 (1 μM), FruA-His_6 (3 μM), or both His_{10}-MrpC2 (1 μM) and FruA-His_6 (3 μM), as indicated. The probe DNA had the wild-type (WT) sequence or the indicated mutation. The filled arrowhead points to the shifted complex produced by His_{10}-MrpC2 alone, and the open arrowhead points to the complex produced by FruA-His_6 alone. (B) Summary of binding sites for MrpC2 and FruA in the *fmgE* promoter region. The approximate location and relative positions of MrpC2 and FruA at sites 1, 2, and 3 are deduced from the effects of 5′-end deletions, the mutations shown in panel A, and 3′-end deletions, respectively, on binding *in vitro*, as explained in the text. The position of MrpC2 binding alone, depicted upstream of position +50, is less precisely known but lies between positions −25 and +50.

and 2). Together, the two proteins produced two shifted complexes, and the total amount of shifted complexes was greater than that expected for additive binding (Fig. 2A, lane 3). This pattern was shown previously to be indicative of cooperative binding (29).

To test whether mutations that affect *fmgE* promoter activity also affect the binding of MrpC2 and FruA, EMSAs were performed with *fmgE* promoter region DNA from positions −100 to −25 containing multiple-base-pair changes. The GGACA-to-TTCAC change, which increased promoter activity by about 3-fold *in vivo* (55), increased *in vitro* binding of His_{10}-MrpC2 alone and greatly increased the apparent cooperative binding of the two proteins (Fig. 2A, lanes 4 to 6), suggesting that the increased promoter activity is due to increased binding of MrpC2 and FruA. In contrast, the GAACC-to-TCCAA change, which decreased promoter activity by about 4-fold (55), decreased binding of FruA-His_6 alone, and cooperative binding was no longer apparent (Fig. 2A, lanes 8 and 9). Likewise, the CATCGTG-to-ACGATGT change, which decreased promoter activity by about 9-fold (55), decreased binding of FruA-His_6 alone, and cooperative binding was no longer apparent (Fig. 2A, lanes 11 and 12). Therefore, for all three mutations, promoter activity *in vivo* was correlated with cooperative binding of the two transcription factors *in vitro*. We conclude that cooperative binding of MrpC2 and FruA just upstream of the *fmgE* promoter is important for promoter activity. Interestingly, both mutations that decreased promoter activity not only decreased the binding of FruA-His_6 alone but also increased the binding of His_{10}-MrpC2 alone (Fig. 2A, compare lanes 1, 7, and 10). In the case of the CATCGTG-to-ACGATGT change, not only did binding of His_{10}-MrpC2 alone increase markedly but also a second shifted complex was observed (Fig. 2A, lane 10). We propose that this mutation creates a second MrpC2 binding site and that the other two mutations alter the normal MrpC2 binding site (see Discussion). Since the most upstream mutation tested (GGACA to TTCAC) affected MrpC2 binding but not FruA binding, we infer that MrpC2 binds upstream of FruA (Fig. 2B, site 2), an arrangement similar to that just upstream of the *fmgBC* and *fmgD* promoters (25, 30). The binding sites for the two proteins might partly overlap, given that both downstream mutations (GAACC to TCCAA and CATCGTG to ACGATGT) affected the binding of both of the proteins. In previous work, it appeared that MrpC2 and FruA bind to partially overlapping sites just upstream of the *fmgA* promoter, and it was suggested that the two proteins interact with opposite faces of the DNA in a region of overlap (29).

One mutation that affected *fmgE* promoter activity did not detectably alter the binding of MrpC2 or FruA, as determined by EMSAs. Changing A to C at position −54 increased promoter activity by about 3-fold (55) but had no effect on shifted complex formation (Fig. 2A, lanes 13 to 15). There are several possible explanations for this (see Discussion).

MrpC2 and FruA bind to a downstream positive regulatory element. In previous work, deletion of downstream DNA between +50 and +140 reduced *fmgE* promoter activity by 3-fold, indicating the presence of a positive regulatory element (55). To test whether MrpC2 and/or FruA bind to the downstream region, EMSAs were performed with several DNA fragments. His_{10}-MrpC2 produced a single shifted complex with DNA from positions −25 to +50, but FruA-His_6 did not bind detectably (Fig. 3A, lanes 1 to 3). Likewise, FruA-His_6 did not bind detectably to DNA from positions −25 to +93; however, His_{10}-MrpC2 produced two shifted complexes, suggesting the presence of two MrpC2 binding sites on this fragment (Fig. 3A, lanes 4 to 6). In contrast, DNA from positions −25 to +116 produced a single shifted complex with FruA-His_6, and two shifted complexes with His_{10}-MrpC2, and when both proteins were added, the majority of DNA was shifted to an upper complex, suggestive of cooperative binding (Fig. 3A, lanes 7 to 9). Taken together, the EMSA results suggest that MrpC2 and FruA bind cooperatively between +50 and +116, with MrpC2 proximal to the *fmgE* promoter (Fig. 2B, site 3), and MrpC2 binds weakly between positions −25 and +50 (Fig. 2B).

Since deletion of DNA between +50 and +140 previously reduced *fmgE* promoter activity by 3-fold (55), we hypothesized that the positive regulatory element implicated in this

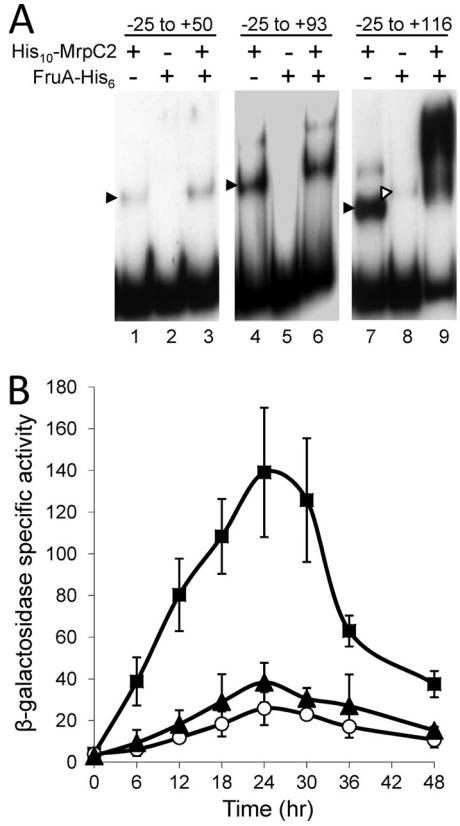

FIG. 3. MrpC2 and FruA bind to a downstream positive regulatory element. (A) Binding of MrpC2 and FruA to site 3. EMSAs with ^{32}P-labeled *fmgE* DNA (2 nM) spanning from position −25 to the indicated 3′ ends and His_{10}-MrpC2 (1 μM), FruA-His_6 (3 μM), or both His_{10}-MrpC2 (1 μM) and FruA-His_6 (3 μM), as indicated. The filled arrowhead points to the shifted complex produced by His_{10}-MrpC2 alone, and the open arrowhead points to the complex produced by FruA-His_6 alone. (B) Effects of 3′-end deletions on developmental *fmgE-lacZ* expression. β-Galactosidase specific activity during development was measured for *lacZ* fused to *fmgE* spanning from positions −100 to +50 (▲), −100 to +93 (○), or −100 to +116 (■). The units of activity are nanomoles of *o*-nitrophenyl phosphate per minute per milligram of protein. Points show the average values of three transformants, and each error bar depicts 1 standard deviation of the data.

region involves cooperative binding of MrpC2 and FruA to site 3, as depicted in Fig. 2B. To test this hypothesis, we compared the *fmgE* promoter activities of DNA fragments with different 3′ ends. Each fragment was cloned into the transcriptional fusion vector pREG1727, which has the *E. coli lacZ* gene as a reporter and integrates in the *M. xanthus* chromosome via site-specific recombination at the Mx8 phage attachment site (7). Consistent with the hypothesis that site 3 is a positive regulatory element, DNA from positions −100 to +116 exhibited higher developmental *lacZ* expression than DNA from positions −100 to +93 or from −100 to +50 (Fig. 3B). We conclude that MrpC2 and FruA bind cooperatively to a positive regulatory element located about 100 bp downstream of the *fmgE* promoter.

MrpC2 and FruA bind to an upstream negative regulatory element. In previous work, deletion of upstream DNA between positions −533 and −100 increased *fmgE* promoter activity by 4-fold, indicating the presence of a negative regulatory element (55). Comparison of *fmgE* promoter activity of DNA fragments spanning from positions −150 to +251 and from positions −100 to +251, using the *lacZ* transcriptional fusion vector described above, localized the negative regulatory element to the region between positions −150 and −100 (data not shown). To test whether MrpC2 and/or FruA binds to this upstream region, EMSAs with several DNA fragments were performed. Although a 5′-end deletion to position −100 eliminated negative regulation, we reasoned that a binding site might include sequences slightly farther downstream, so we tested fragments with different 5′ ends and the same 3′ end at position −80 (to avoid the MrpC2 and FruA binding sites located just upstream of the *fmgE* promoter) (Fig. 2B, site 2). His_{10}-MrpC2 and FruA-His_6 each produced a single shifted complex with DNA from positions −147 to −80, and in combination, the two proteins produced at least two shifted complexes in greater abundance, suggestive of cooperative binding (Fig. 4A, lanes 1 to 3). With DNA from positions −120 to −80, His_{10}-MrpC2 produced a single shifted complex, but binding of FruA-His_6 was barely detectable; however, in combination, the two proteins produced an upper complex, suggestive of cooperative binding (Fig. 4A, lanes 4 to 6). In contrast, DNA from positions −110 to −80 appeared to bind His_{10}-MrpC2 weakly, binding of FruA-His_6 was not detected, and there was no evidence of cooperative binding (Fig. 4A, lanes 7 to 9). These results suggest that MrpC2 and FruA bind cooperatively between positions −120 and −80, with MrpC2 proximal to the *fmgE* promoter (Fig. 2B, site 1).

To see if negative regulation correlated with MrpC2 and FruA binding to site 1, as depicted in Fig. 2B, DNA fragments with 5′ ends at the −150, −120, or −110 position, and the same 3′ end at +50, were tested for *fmgE* promoter activity using the *lacZ* transcriptional fusion vector described above. The two fragments with 5′ ends at −150 or −120 exhibited much lower developmental *lacZ* expression than the fragment with its 5′ end at position −110 (Fig. 4B). Therefore, the same 5′-end deletion to position −110 that eliminated cooperative binding of His_{10}-MrpC2 and FruA-His_6 to site 1 (Fig. 4A) also eliminated or greatly reduced negative regulation (Fig. 4B), correlating the upstream negative regulatory element with site 1.

Sites 1, 2, and 3 exhibit different affinities for MrpC2 and for the combination of MrpC2 and FruA *in vitro*. Since the 5′-end deletions tested for *fmgE-lacZ* expression as shown in Fig. 4B had the same 3′ end at +50 and therefore lacked site 3, we conclude that site 1 functions negatively in the absence of site 3 *in vivo*. Also, site 3 functions positively in the absence of site 1, since the 3′-end deletions tested for *fmgE-lacZ* expression, as shown in Fig. 3B, as well as others tested previously (55), had the same 5′ end at position −100 and therefore lacked site 1. To explain how sites 1 and 3 function negatively and positively, respectively, we considered the possibility that site 2 must be occupied by MrpC2 and FruA to activate the *fmgE* promoter and that sites 1 and 3 independently influence the occupancy of site 2 in different ways. Specifically, because our comparison of His_{10}-MrpC2 and FruA-His_6 binding to site 1 (Fig. 4A, lane 3), site 2 (Fig. 2A, lane 3), and site 3 (Fig. 3, lane 9) suggested that site 3 exhibits the highest affinity, followed by site 1 and then site 2, we hypothesized that site 3 acts positively by recruiting MrpC2 and FruA to the *fmgE* promoter region

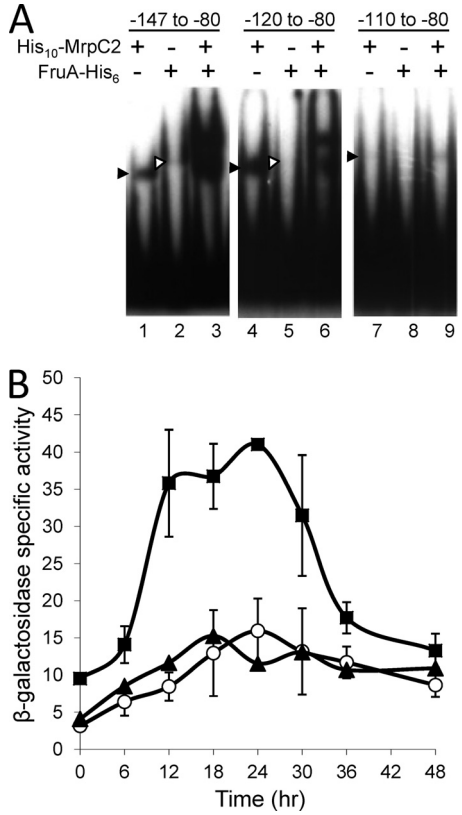

FIG. 4. MrpC2 and FruA bind to an upstream negative regulatory element. (A) Binding of MrpC2 and FruA to site 1. EMSAs with ^{32}P-labeled *fmgE* DNA (2 nM) spanning from the indicated 5′ ends to position −80 and His_{10}-MrpC2 (1 μM), FruA-His_6 (3 μM), or both His_{10}-MrpC2 (1 μM) and FruA-His_6 (3 μM), as indicated. The filled arrowhead points to the shifted complex produced by His_{10}-MrpC2 alone, and the open arrowhead points to the complex produced by FruA-His_6 alone. (B) Effects of 5′-end deletions on developmental *fmgE-lacZ* expression. β-Galactosidase specific activity during development was measured for *lacZ* fused to *fmgE* spanning from positions −150 to +50 (▲), −120 to +50 (○), or −110 to +50 (■). The units of activity are nanomoles of *o*-nitrophenyl phosphate per minute per milligram of protein. Points show the average values of two or three transformants, and each error bars depicts 1 standard deviation of the data.

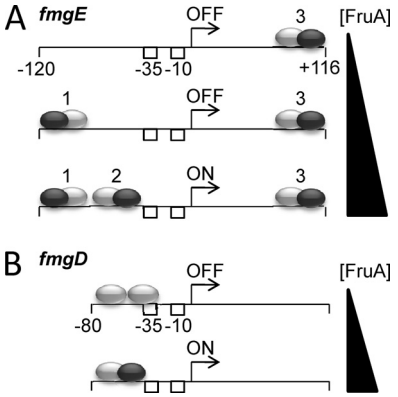

FIG. 5. Models for C-signal-dependent regulation of *fmgE* and *fmgD*. As development proceeds, C-signaling causes the concentration of active FruA to rise (triangles). (A) In the *fmgE* promoter region, three sites for cooperative binding of MrpC2 (light gray ovals) and FruA (dark gray ovals) are occupied in the order of their affinities for the two transcription factors, first site 3, then site 1, and finally site 2, resulting in activation of transcription. (B) In the *fmgD* promoter region, cooperative binding of two MrpC2 initially represses transcription, but eventually FruA outcompetes the downstream MrpC2 for binding to the upstream MrpC2, activating transcription. Activation of *fmgD* might occur at a slightly lower concentration of active FruA than activation of *fmgE* (see Discussion).

and that site 1 acts negatively by competing with site 2 for binding of the transcription factors. According to this model, the three sites would be occupied in the order of their affinities for the transcription factors, as depicted in Fig. 5A. We further noted that His_{10}-MrpC2 alone appeared to bind site 3 with the highest affinity (Fig. 3A, lane 7), followed by site 1 (Fig. 4A, lane 1) and then site 2 (Fig. 2A, lane 1), whereas FruA-His_6 alone appeared to bind each site with similarly low affinity (compare Fig. 2A, lane 2, Fig. 3A, lane 8, and Fig. 4A, lane 2). To confirm these observations, we compared the binding of His_{10}-MrpC2 alone at different concentrations to sites 1, 2, and 3. As expected, His_{10}-MrpC2 bound to site 3 with the highest affinity, followed by site 1 and then site 2 (Fig. 6A). Likewise, we compared the binding of His_{10}-MrpC2 at different concentrations in combination with FruA-His_6 and found the same order of binding affinities for sites 1 to 3 (Fig. 6B). We conclude that the affinities of sites 1, 2, and 3 for the combination of MrpC2 and FruA *in vitro* are correlated with the affinities of the three sites for MrpC2 alone *in vitro* and that the affinities are consistent with a model in which site 3 would be occupied first *in vivo* and act positively by recruiting MrpC2 and FruA to the *fmgE* promoter region, site 1 would be occupied second and act negatively by competing with site 2 for binding of the transcription factors, and finally, site 2 would be occupied when the transcription factors reach a sufficient concentration, activating transcription of *fmgE* (Fig. 5A).

DISCUSSION

The novel contribution of this study is that multiple cooperative binding sites for MrpC2 and FruA can be used to regulate a gene during *M. xanthus* development. Not only do the two transcription factors bind immediately upstream of the *fmgE* promoter, as observed for other *fmg* genes (25, 29–30), they bind slightly farther upstream to regulate negatively and downstream at about position +100 to regulate positively. It is interesting that *fmgE* exhibits complex combinatorial control by MrpC2 and FruA. This could be a general characteristic of genes that are induced late during *M. xanthus* development and depend strongly on C-signaling for expression, since *fmgD* falls into this category, and its regulation was more complex than those of *fmgA* and *fmgBC*, which are expressed earlier and depend only in part on C-signaling. Whereas MrpC2 and FruA bind cooperatively just upstream of the *fmgA*, *fmgBC*, and *fmgD* promoters to activate transcription (25, 29–30), a second MrpC2 binding site partially overlaps both the FruA binding site and the *fmgD* promoter −35 region (25) (Fig. 5B). The position of the downstream MrpC2 binding site and the effects of mutations on binding *in vitro* and on promoter activity *in vivo* suggest that binding of MrpC2 to the downstream site represses *fmgD* transcription. Derepression presumably de-

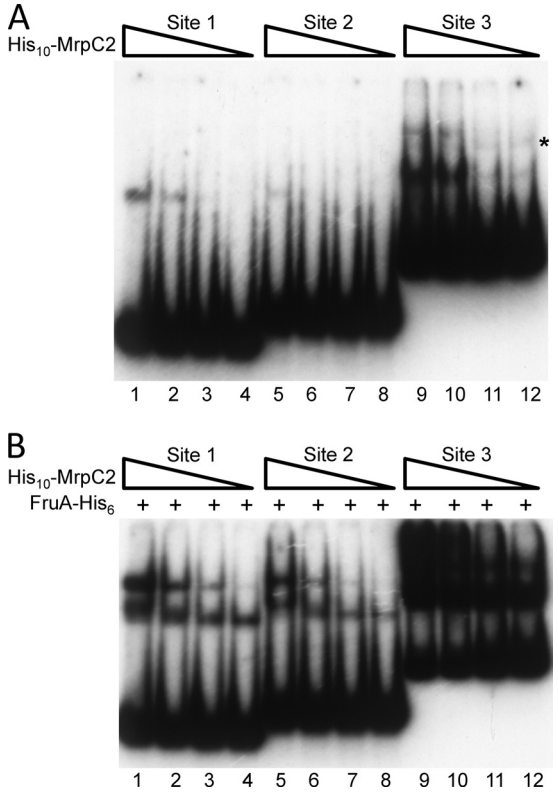

FIG. 6. Relative affinities of MrpC2, and the combination of MrpC2 and FruA, for sites 1, 2, and 3 in the *fmgE* promoter region. (A) Binding of MrpC2 to sites 1, 2, and 3. EMSAs with ^{32}P-labeled *fmgE* DNA (2 nM) spanning from positions −147 to −80 (site 1), from positions −100 to −25 (site 2), or from positions −25 to +116 (site 3) and His_{10}-MrpC2 at decreasing concentrations (1, 0.5, 0.25, and 0.125 μM). The asterisk denotes a band observed in lanes 9 to 12, which was also observed in the absence of His_{10}-MrpC2 (data not shown), and therefore appears to be a minor contaminant of this probe preparation rather than a shifted complex. (B) Binding of MrpC2 and FruA to sites 1, 2, and 3. EMSAs with ^{32}P-labeled *fmgE* DNA and His_{10}-MrpC2 at decreasing concentrations, as shown in panel A, and with FruA-His_6 (3 μM).

pends on C-signal-dependent activation of FruA, which outcompetes the downstream MrpC2 for cooperative binding with the upstream MrpC2. Likewise, we propose that cooperative binding of MrpC2 and FruA to site 2 (Fig. 2B) just upstream of the promoter activates *fmgE* transcription but that occupancy of site 2 occurs only when C-signaling produces a high enough concentration of active FruA (Fig. 5A). According to our model, site 1 acts negatively by competing with site 2 for cooperative binding of MrpC2 and FruA. Consistent with the model, not only did loss of site 1 (due to a 5′-end deletion to position −100) increase promoter activity, but also activity no longer depended on C-signaling (55). Further, we propose that the high-affinity site 3 acts positively by recruiting MrpC2 and FruA to the vicinity of sites 1 and 2, effectively increasing the local concentration of the two transcription factors as they dynamically bind and are released from many sites in the chromosome.

Our ChIP analysis provides evidence that association of MrpC and/or MrpC2 with the *fmgE* promoter region depends on FruA (Fig. 1), consistent with the cooperative binding of the two proteins *in vivo*. Sites 1, 2, and 3 exhibited enhanced binding by the combination of His_{10}-MrpC2 and FruA-His_6, relative to the binding of either protein alone in EMSAs (Fig. 2A, 3A, and 4A), a pattern shown previously to be indicative of cooperative binding (29). EMSAs also provided evidence for binding of His_{10}-MrpC2 alone between positions −25 and +50 (Fig. 3A); however, the binding was weak *in vitro*, and ChIP analysis of a *fruA* mutant suggested that none of the MrpC2 sites in the *fmgE* promoter region are occupied appreciably in the absence of FruA, at least at 18 h into development. In contrast, MrpC and/or MrpC2 are associated with the *fmgD* promoter region in a *fruA* mutant at 18 h into development, based on a comparable ChIP experiment (25). This difference between *fmgD* and *fmgE* supports the notion that the two genes are regulated differently (Fig. 5), despite their similar late induction during development and strong dependence on C-signaling.

The effects of mutations in site 2, just upstream of the *fmgE* promoter, showed a correlation between the cooperative binding of His_{10}-MrpC2 and FruA-His_6 *in vitro* and *fmgE-lacZ* expression during development (Fig. 2A). The GGACA-to-TTCAC mutation that increased cooperative binding also increased *fmgE-lacZ* expression, and the GAACC-to-TCCAA mutation that decreased cooperative binding also decreased expression. These results indicate that cooperative binding of MrpC2 and FruA to site 2 is important for *fmgE* promoter activity. Interestingly, both mutations increased the binding of His_{10}-MrpC2 alone. A possible explanation is that both mutations create a sequence that matches the (A/G)TTT(C/G)A (A/G) consensus sequence for MrpC2 binding (35) at 5 out of 7 positions (i.e., the GGACA-to-TTCAC mutation creates AC TTCAC from positions −71 to −65, and the GAACC-to-TCCAA mutation creates ACTTGGA at positions −58 to −64 on the opposite strand), but the GAACC-to-TCCAA mutation positions MrpC2 inappropriately for cooperative binding with FruA. The wild-type sequence does not match the consensus noted above, but it does match a second consensus for MrpC2 binding, GTGTC(N_8)GACAC (35), at 6 out of 10 positions (i.e., GGGAA(N_8)GACAG at positions −81 to −64). Like the GAACC-to-TCCAA mutation, the CATCGTG-to-ACG ATGT mutation reduced cooperative binding and promoter activity, and it increased binding of His_{10}-MrpC2 alone, but in this case, two shifted complexes were observed, as if a second MrpC2 binding site was created by the mutation (Fig. 2A). Indeed, the mutation creates part of the sequence ATGTC(N_8)GATTC from positions −52 to −35, which matches the second MrpC2 binding consensus sequence noted above at 7 out of 10 positions. If MrpC2 binds to this sequence, it might repress *fmgE* transcription by blocking the binding of FruA and/or the RNA polymerase, analogous to the proposed role of the downstream MrpC2 binding site in the *fmgD* promoter region (25) (Fig. 5B). The A-to-C mutation at position −54 did not affect binding of His_{10}-MrpC2 and/or FruA-His_6 to site 2 in the *fmgE* promoter region (Fig. 2A, lanes 13 to 15), yet the mutation increased *fmgE-lacZ* expression by nearly 3-fold (55). A simple explanation for the discrepancy is that the binding reaction conditions present *in vitro* do not reflect the conditions present *in vivo*. The mutation occurs within the sequence CATCG(N_5)CGGG from positions −55 to −42, which matches the consensus sequence for binding of the FruA DNA-binding do-

main, GGG(C/T)(A/G)($N_{4–6}$)(C/T)GGG (58), at 6 out of 9 positions, but the mutation does not improve the match to the consensus. Perhaps the mutation alters the interaction of FruA with its site in a subtle way that does not increase affinity but, nevertheless, permits greater activation of transcription. We cannot rule out the possibility that the mutation affects the binding of a protein other than FruA and/or MrpC2; however, there is no evidence for such a protein.

Cooperative binding of MrpC2 and FruA to site 3, located about 100 bp downstream of the *fmgE* transcriptional start site, appears to upregulate *fmgE* transcription (55) (Fig. 3). Positive regulation by transcription factors that bind downstream of promoters is unusual in bacteria. Other instances have been described in *M. xanthus*, but the mechanisms are not understood (28, 58). Downstream positive regulators in other bacteria have been proposed to function by a variety of mechanisms, such as antagonizing a repressor (4), recruiting an activator (48), or directly facilitating productive interaction of the RNA polymerase with the promoter (32, 39, 51). Our results do not exclude any of these potential mechanisms to explain how cooperative binding of MrpC2 and FruA to site 3 might activate *fmgE* transcription. However, ChIP followed by sequencing (ChIP-seq) indicates that MrpC and/or MrpC2 binds to a large number (>1,700) of sites in the *M. xanthus* chromosome (M. Robinson, B. Son, and L. Kroos, unpublished data), and site 3 exhibits high affinity for His_{10}-MrpC2 alone or the combination of His_{10}-MrpC2 and FruA-His_6 (Fig. 6), so we favor the simple model that site 3 acts positively by increasing the local concentration of the two transcription factors in the vicinity of the *fmgE* promoter. We note that the complement of the sequence CAGAACT from positions +71 to +77 (i.e., AGTTCTG) matches the (A/G)TTT(C/G)A(A/G) consensus sequence for MrpC2 binding (35) at 5 out of 7 positions. Also, the complement of the sequence GCTG(N_4)CACCC from positions +83 to +95 (i.e., GGGTG(N_4)CAGC) matches the GGG(C/T)(A/G)($N_{4–6}$)(C/T)GGG consensus sequence for the binding of the FruA DNA-binding domain (58) at 6 out of 9 positions, and the loss of 2 bp from the end of this sequence might explain why FruA-His_6 failed to bind to DNA from positions −25 to +93 (Fig. 3A).

Another unusual feature of *fmgE* regulation is the presence of an upstream *cis*-regulatory element that acts negatively (55), which we found is located about 100 bp upstream of the transcriptional start site and is correlated with site 1 for cooperative binding of MrpC2 and FruA (Fig. 4). Typically, upstream negative regulation involves cooperative binding of a transcription factor to an upstream site and to a site near or within the promoter, forming a repression loop (5, 31). We considered the possibility that MrpC2 bound to site 1 forms a repression loop with MrpC2 bound somewhere between positions −25 and +50 and that C-signal-dependent activation of FruA leads to cooperative binding with MrpC2 at site 1, breaking the repression loop. However, our ChIP analysis does not support this model since MrpC and/or MrpC2 does not appear to be associated with the *fmgE* promoter region in the absence of FruA (Fig. 1C). Since site 1 exhibits slightly higher affinity for His_{10}-MrpC2 alone or for the combination of His_{10}-MrpC2 and FruA-His_6 than site 2 (Fig. 6), we favor the idea that site 1 acts negatively by competing with site 2 for cooperative binding of the two transcription factors. We note that the sequence GGGCG(N_4)TCCT from positions −113 to −101 matches the GGG(C/T)(A/G)($N_{4–6}$)(C/T)GGG consensus sequence for binding of the FruA DNA-binding domain (58) at 6 out of 9 positions and could account for the loss of cooperative binding of FruA-His_6 and His_{10}-MrpC2 to DNA from positions −110 to −80 (Fig. 4A). Also, the sequence GTT TCAG from positions −87 to −81 matches the (A/G)TTT(C/G)A(A/G) consensus sequence for MrpC2 binding (35) at all 7 positions.

Our results demonstrate that multiple cooperative binding sites for MrpC2 and FruA are used to regulate *fmgE* during *M. xanthus* development, raising the question of why such elaborate control is observed. Like *fmgD*, expression of *fmgE* depends strongly on C-signaling and occurs late during development. However, regulation of *fmgD* appears to be simpler, involving cooperative binding of two MrpC2 proteins just upstream of the promoter in the off state and FruA outcompeting the downstream MrpC2 to activate the promoter (25) (Fig. 5B). We note two differences between *fmgD* and *fmgE* that might explain the more streamlined regulation of *fmgD*. First, *fmgE* appears to be a recent addition to the *M. xanthus* genome (27). Its coding region does not exhibit the strong bias toward usage of guanine or cytosine at the third codon position typical of *M. xanthus* genes. Also, the overall G+C content of *fmgE* and its upstream intergenic region is 59% (27), much lower than that of the whole genome (68.9%) (8). In contrast, *fmgD* exhibits third-codon-position G+C bias typical for *M. xanthus* coding regions, so it likely has resided longer in the genome than *fmgE*, perhaps allowing more streamlined regulation of *fmgD* to evolve. A second difference between *fmgD* and *fmgE* is their spatial regulation. *fmgD-lacZ* is expressed in the outer domain of nascent fruiting bodies (42), where rod-shaped cells become aligned and presumably engage in efficient C-signaling (43). *fmgE-lacZ* is expressed primarily in the inner domain of fruiting bodies (42), where spores are observed (43), and β-galactosidase specific activity reaches about 8-fold-higher levels in spores than in rod-shaped cells, whereas it reaches only 2-fold higher levels in spores than in rods for *fmgD-lacZ* (21). Therefore, the more elaborate control of *fmgE* might ensure that it is expressed predominantly after cellular differentiation. This raises the intriguing possibility that the concentration of active FruA continues to rise in cells that are forming spores. These cells lose their ability to move independently and presumably lose their ability to engage in C-signaling. It has been proposed that sporulating cells are passively transported to the inner domain of nascent fruiting bodies by the circling movements of undifferentiated rod-shaped cells in the outer domain (42). In any case, our finding that *fmgE* is under combinatorial control of MrpC2 and FruA, and involves cooperative binding to three sites, underscores the importance and versatility of this regulatory mechanism. As proposed for other *fmg* genes (25, 29–30), positional information through C-signaling and FruA is presumably integrated with starvation sensing and cell death control through MrpC and MrpC2 to govern *fmgE* expression, and the particular arrangement and affinity of binding sites for the two transcription factors dictate the spatiotemporal pattern of expression.

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