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Combinatorial Regulation by a Novel Arrangement of FruA and MrpC2 Transcription Factors during *Myxococcus xanthus* Development[▽]

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Myxococcus xanthus **is a gram-negative soil bacterium that undergoes multicellular development upon nutrient limitation. Intercellular signals control cell movements and regulate gene expression during the developmental process. C-signal is a short-range signal essential for aggregation and sporulation. C-signaling regulates the *fmgA* gene by a novel mechanism involving cooperative binding of the response regulator FruA and the transcription factor/antitoxin MrpC2. Here, we demonstrate that regulation of the C-signal-dependent *fmgBC* operon is under similar combinatorial control by FruA and MrpC2, but the arrangement of binding sites is different than in the *fmgA* promoter region. MrpC2 was shown to bind to a crucial *cis*-regulatory sequence in the *fmgBC* promoter region. FruA was required for MrpC and/or MrpC2 to associate with the *fmgBC* promoter region in vivo, and expression of an *fmgB-lacZ* fusion was abolished in a *fruA* mutant. Recombinant FruA was shown to bind to an essential regulatory sequence located slightly downstream of the MrpC2-binding site in the *fmgBC* promoter region. Full-length FruA, but not its C-terminal DNA-binding domain, enhanced the formation of complexes with *fmgBC* promoter region DNA, when combined with MrpC2. This effect was nearly abolished with *fmgBC* DNA fragments having a mutation in either the MrpC2- or FruA-binding site, indicating that binding of both proteins to DNA is important for enhancement of complex formation. These results are similar to those observed for *fmgA*, where FruA and MrpC2 bind cooperatively upstream of the promoter, except that in the *fmgA* promoter region the FruA-binding site is located slightly upstream of the MrpC2-binding site. Cooperative binding of FruA and MrpC2 appears to be a conserved mechanism of gene regulation that allows a flexible arrangement of binding sites and coordinates multiple signaling pathways.**

Myxococcus xanthus is a rod-shaped bacterium that glides on solid surfaces, forming a single-species biofilm that provides an attractive model to study how signaling couples gene expression to environmental and cellular cues (64). *M. xanthus* cells in the biofilm grow and divide when nutrients are available but, upon starvation, a multicellular developmental process ensues, during which the cells move into aggregates and form mound-shaped structures called fruiting bodies. Approximately 10^5 cells participate in forming a fruiting body, in which a portion of the cells differentiate into dormant, stress-resistant, spherical spores. Other cells undergo programmed cell death (37) or autolysis caused by siblings in the developing biofilm (41, 66), and some cells remain outside of fruiting bodies as peripheral rods (40). These fates are met by different proportions of cells in the biofilm, depending on genetic and environmental factors (3, 41). The spores in a fruiting body can germinate and resume growth and division when nutrients become available.

Signals act at different times during the developmental process to coordinate cell behavior and determine cell fate. Nutrient limitation causes a stringent response that results in production of (p)ppGpp and the induction of early developmental genes (12). A mixture of amino acids and peptides, known as the A-signal, is generated by secreted proteases and is believed to allow quorum sensing (27). A-signal-dependent genes are expressed, and cells alter their pattern of movement so that aggregates begin to form. Subsequent gene expression, and the maturation of aggregates into spore-filled fruiting bodies, depends on C-signaling, which is mediated by the product of the *csgA* gene (51). CsgA is associated with the outer membrane of the cell, where it is processed by a secreted protease to a 17-kDa form that appears to act as a short-range signal (21, 30, 45). C-signal transduction requires cell alignment (19) and possibly end-to-end contact between cells (46), so it communicates positional information. Cells become aligned as aggregates transform into nascent fruiting bodies, and the resulting high level of C-signaling has been proposed to trigger the expression of genes required for sporulation (48). Indeed, the expression of C-signal-dependent genes that are important for sporulation is restricted to nascent fruiting bodies (16, 47), and many studies support a model in which an increasing level of C-signaling controls gene expression to coordinate aggregation and sporulation during development (10, 20, 26, 29).

How does C-signaling regulate expression of target genes? FruA plays a key role in the C-signal transduction pathway (5, 42). It is similar to response regulators of two-component signal transduction systems and has been proposed to be phosphorylated in its N-terminal regulatory domain in response to C-signal and perhaps other signals (5, 15), but a cognate his-

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tidine protein kinase has not been identified, and several types of evidence suggest that FruA might function without being phosphorylated (35). The C-terminal domain of FruA is similar to the C-terminal DNA-binding domain of the NarL/FixJ subfamily of response regulators (63). The C-terminal domain of FruA has been shown to bind to sites in the promoter regions of developmentally regulated genes that fail to be expressed in *fruA* mutant cells, suggesting that FruA is a transcriptional activator (56, 57, 62, 68). Recently, FruA was shown to bind cooperatively with MrpC2 to the promoter region of the C-signal-dependent *fmgA* (for FruA- and MrpC2-regulated gene A) gene (35), revealing a novel mechanism of combinatorial control, as cooperative binding of a response regulator (FruA) and a distinct transcription factor (MrpC2) had not been observed previously.

MrpC2 is a smaller form of MrpC (58), which is similar to the cyclic AMP receptor protein (CRP) family of transcriptional regulators (54). MrpC is expressed during vegetative growth and is phosphorylated by a cytoplasmic serine/threonine protein kinase (STPK) called Pkn14 (38, 39). Pkn14 is in turn phosphorylated by a membrane STPK called Pkn8. Phosphorylation of MrpC by the Pkn8/Pkn14 cascade results in weaker binding of MrpC to DNA and also appears to inhibit proteolytic cleavage of MrpC to MrpC2 (39), which lacks the 25 N-terminal residues of MrpC (58). The STPK cascade is counteracted by an unknown mechanism early in development, allowing MrpC and MrpC2 concentrations to rise. MrpC2 binds to DNA with higher affinity than MrpC, and appears to play a key role as a transcriptional activator during development (39). Recently, MrpC was shown to function as an antitoxin by interacting directly with the toxin MazF, an mRNA interferase that mediates programmed cell death during development (37). MrpC also binds to the *mazF* promoter region and activates expression. Binding of MrpC2 to the *mazF* promoter region and MazF has not been tested. The dual functions of MrpC, and possibly MrpC2, as an antitoxin and a transcription factor make it an important determinant of cell fate. The finding that MrpC2 and FruA bind cooperatively to crucial *cis*-regulatory sequences upstream of the *fmgA* promoter suggests that these transcription factors coordinate starvation signaling and cell death with positional information via short-range C-signaling to govern gene expression and cell fate during *M. xanthus* development (35). This novel mechanism of *fmgA* combinatorial control was predicted to be conserved because similar *cis*-regulatory sequences have been found upstream of other developmentally regulated *M. xanthus* promoters (6, 31, 52, 59–61, 67).

The promoter region of a putative operon (named here *fmgBC* for FruA- and MrpC2-regulated genes B and C) at the Ω4499 locus in the *M. xanthus* chromosome has *cis*-regulatory sequences similar to those bound by MrpC2 in the *fmgA* promoter region. The *fmgBC* operon was identified by an insertion of the transposon Tn*5 lac* into *fmgC* (25). FmgB and FmgC are similar to reductase and oxidase components, respectively, of bacterial cytochrome P-450 systems, which typically are involved in catabolism or anabolism of unusual compounds (6). *M. xanthus* DNA upstream of *fmgBC* was cloned, a putative transcriptional start site was mapped, and the region from positions −100 to +50 was shown to encompass the promoter (6, 67). Expression from the *fmgBC* promoter was reduced in a *csgA* mutant but was restored upon codevelopment of the *csgA* mutant with wild-type cells, which supply C-signal, demonstrating that promoter activity is partially dependent on C-signaling (6, 24). Mutational analysis identified critical *cis*-regulatory sequences at positions −71 to −45 upstream of the promoter (67). This region contains two C boxes (consensus CAYYCCY; Y means C or T) and two 5-bp elements (consensus GAACA) (Fig. 1), which are sequence motifs found in the promoter regions of several developmentally regulated genes (6, 31, 52, 59–61, 67). In the *fmgA* promoter region, between positions −63 and −46, a 5-bp element is located 6 bp upstream of a C box, and this region is bound by MrpC2, while FruA binds cooperatively to a site located slightly upstream (35).

Here, we report that MrpC2 and FruA bind to sequences between positions −71 and −45 upstream of the *fmgBC* promoter, but the arrangement of binding sites is the reverse of that found in the *fmgA* promoter region. Nevertheless, the association of MrpC and/or MrpC2 with the *fmgBC* promoter region in vivo required FruA. Furthermore, there appeared to be cooperative binding of MrpC2 and FruA to *fmgBC* promoter region DNA in vitro. Our results demonstrate combinatorial control by MrpC2 and FruA at a second promoter and reveal surprising flexibility in the arrangement of the binding sites.

MATERIALS AND METHODS

Bacterial strains and plasmids. Strains and plasmids used in the present study are listed in Table 1.

Growth and development. *Escherichia coli* BL21(DE3) containing plasmids were grown at 37°C in Luria-Bertani (LB) medium (49) containing 200 μg of ampicillin per ml. *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 (14) or on CTT agar (1.5%) plates. When required, 40 μg of kanamycin sulfate per ml was added. Fruiting body development was performed on 1.5% TPM agar plates (10 mM Tris-HCl [pH 8.0], 1 mM KH_2PO_4-K_2HPO_4, 8 mM $MgSO_4$, [final pH 7.6]) as described previously (25).

Construction of *M. xanthus* strains and determination of *lacZ* expression during development. Strains containing pREG1727 or its derivatives integrated at the Mx8 phage attachment site, *attB*, were constructed by electroporation (18) of *M. xanthus*, and transformants were selected on CTT agar plates containing kanamycin sulfate. Transformants were screened on TPM agar plates containing 40 μg of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside)/ml in order to avoid rare transformants with unusual developmental *lacZ* expression (60). Three transformants were chosen for further analysis, and the β-galactosidase activity was measured as described previously (25).

Preparation of DNA fragments. DNA fragments spanning the *fmgBC* promoter region from positions −104 to −29 were generated by PCR using wild-type or mutant plasmid (Table 1) as the template and the oligonucleotide primers 5′-GCGCGAGGAGATTGCGTTCATAC-3′ (for −104) and 5′-GAGGAA TGGGCCGGAAGTTC-3′ (for −29). For the 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% polyacrylamide gel electrophoresis (49).

EMSAs. EMSAs were performed as described previously (68), except that binding reaction mixtures were incubated at 25°C for 15 min.

DNA-affinity chromatography. An *fmgBC* DNA fragment (positions −104 to −29) was synthesized by PCR with a 5′-biotin label at position −104, bound to streptavidin beads, and DNA-affinity chromatography was performed with the AS fraction as described previously (62).

Preparation of His_{10}-MrpC2, FruA-His_6, and FruA-DBD-His_8. Recombinant proteins were expressed in *E. coli* and purified as described previously (35, 39, 68).

ChIP. *M. xanthus* strains MSM1727.DZF1, MSM4499.DZF1, and MSM4499.FA were used for chromatin immunoprecipitation (ChIP) as described previously (68). The primers used for PCR of the *fmgBC* promoter region integrated ectopically were 5′-CTGCCAGGAATTGGGGATC-3′ (the upstream primer in

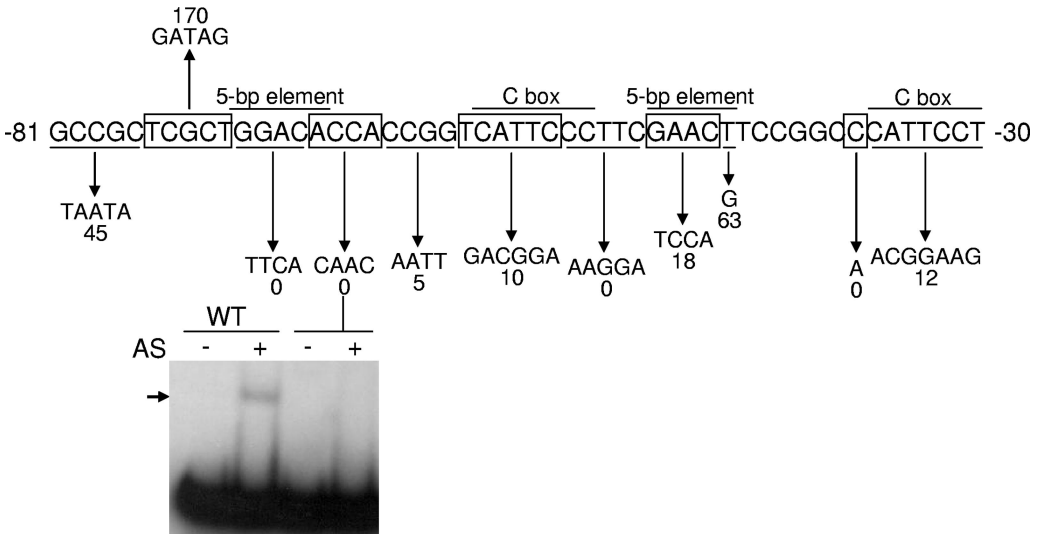

FIG. 1. Effects of mutations on *fmgBC* promoter activity in vivo and on DNA binding in vitro. The top part of the figure shows a summary of mutational effects on developmental *fmgB-lacZ* expression (67). The wild-type *fmgBC* upstream sequence is alternately boxed or underlined to indicate changed sequences, which are shown below the downward arrows. The number beneath each mutant sequence indicates the maximum β-galactosidase activity during development, expressed as a percentage of the maximum activity observed for the wild-type promoter. The bottom part shows EMSAs performed with ^{32}P-labeled *fmgBC* DNA (12 nM) spanning from positions −104 to −29 and proteins in the AS fraction (0.7 μg/μl). The arrow indicates the shifted complex produced by incubating the wild-type (WT) DNA fragment with the AS fraction. No complex was observed with a DNA fragment bearing the indicated mutation at positions −67 to −64.

the vector) and 5′-CGGATCCAGCGGGTGAGGTCGACGACG-3′ (the downstream primer with its 5′ end at position +50 of *fmgBC*). The primers used for PCR of the vector alone integrated ectopically were the same upstream primer as described above and 5′-CGGGCCATCCGCCAGTGG-3′ (downstream primer in the vector). The primers used for PCR of the *rpoC* coding region were described previously (68).

RESULTS

An insertion in *fmgC* reduces spore formation. *M. xanthus* strain DK4499 contains Tn*5 lac* Ω4499 inserted in *fmgC*, which was predicted previously to encode an oxidase of a cytochrome P-450 system (6). *fmgC* corresponds to MXAN4127 in the annotation of the genomic sequence (9). Only 59 bp upstream of *fmgC* is *fmgB* (MXAN4126), which was predicted previously to code for a reductase likely to function in the same P-450 system as FmgC, although the substrate and products of the system are unknown (6). The short distance between *fmgB* and *fmgC*, and the finding that their products are likely components of a P-450 system, suggested that the two genes might be cotranscribed. In agreement, 5′-deletion analysis and mapping of an mRNA 5′ end located a promoter upstream of *fmgB* capable of driving expression of *lacZ* during development similar to that observed for DK4499 containing Tn*5 lac* Ω4499 (6). The gene upstream of *fmgB* is in the opposite orientation (9). The gene downstream of *fmgC* is in the same orientation but is separated from the end of *fmgC* by an intergenic region of at least 243 bp and is predicted to encode a transposase, so it is unlikely to be cotranscribed with the putative *fmgBC* operon.

M. xanthus DK4499 bearing Tn*5 lac* Ω4499 aggregated normally under conditions that induce development, but the number of heat- and sonication-resistant spores that were able to germinate and form a colony was sixfold lower than observed for wild-type DK1622. The reduced sporulation of DK4499 is likely due to loss of FmgC, although we cannot rule out an effect of the Tn*5 lac* insertion on expression of *fmgB* (e.g., due to altered mRNA stability) or a gene downstream of *fmgC* (i.e., if transcription from the *fmgBC* promoter normally reads through a downstream gene). In any case, our results suggest that transcription from the *fmgBC* promoter is important for sporulation.

MrpC2 binds to a key *cis*-regulatory sequence in the *fmgBC* promoter region. Mutational analysis of the *fmgBC* promoter region was performed previously (67) and showed that sequences upstream of the promoter are important for its activity (Fig. 1). These regulatory sequences include two 5-bp elements and two C boxes, which are found in the promoter regions of several developmentally regulated genes (6, 31, 52, 59–61, 67). To identify putative transcription factors, we performed EMSAs with a DNA fragment from the *fmgBC* promoter region and partially purified DNA-binding proteins (AS fraction) from *M. xanthus* cells that had undergone 12 h of development, since *fmgBC* is expressed at this time (25). A single shifted complex was observed with a DNA fragment spanning from positions −104 to −29, but no complex was observed when the DNA fragment contained a mutation in the sequence from positions −67 to −64 (Fig. 1). Since this mutation was shown previously to eliminate *fmgBC* promoter activity in vivo (67), these results showed that a protein in the AS fraction binds to a crucial *cis*-regulatory sequence upstream of the *fmgBC* promoter.

To purify the putative activator protein from the AS fraction, DNA affinity chromatography was performed with the *fmgBC* DNA fragment (−104 to −29). The major species after purification was ~30 kDa in size (Fig. 2A). The affinity-purified protein (APP) generated a shifted complex of similar mobility, as observed with the AS fraction when the *fmgBC* DNA fragment with the wild-type sequence was used

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
E. coli		
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	35
SMFruAhis	BL21(DE3) containing pET11km/FruA-His$_6$	35
EDYFruA	BL21(DE3) containing pET11a/FDBD-H$_8$	68
M. xanthus		
DK1622	Wild type	17
DK4499	Tn*5 lac* Ω4499	25
MSM1727.DZF1	*sglA1 attB*::pREG1727	This study
MDY1727.FA	*sglA1 fruA*::TnV Ω786 *attB*::pREG1727	68
MSM4499.DZF1	*sglA1 attB*::pDY52	This study
MSM4499.FA	*sglA1 fruA*::TnV Ω786 *attB*::pDY52	This study
Plasmids		
pET11a/FDBD-H$_8$	pET11a with a gene encoding FruA-DBD-His$_8$ under the control of a T7 RNA polymerase promoter	57
pET16b/His$_{10}$-MrpC2	pET16b with a gene encoding His$_{10}$-MrpC2 under the control of a T7 RNA polymerase promoter	39
pET11km/FruA-His$_6$	pET11km with 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
pDY52	pREG1727 with 150-bp XhoI-BamHI fragment from pDY51	67
pDY100	pGEM7Zf with *fmgBC* DNA from positions −218 to +50	67
pDY133	pDY100 with a GCCGC-to-TAATA mutation at positions −81 to −77	67
pDY129	pDY100 with a GGAC-to-TTCA mutation at positions −71 to −68	67
pDY127	pDY100 with a ACCA-to-CAAC mutation at positions −67 to −64	67
pDY125	pDY100 with a CCGG-to-AATT mutation at positions −63 to −60	67
pDY49	pDY100 with a TCATTC-to-GACGGA mutation at positions −59 to −54	67
pDY121	pDY100 with a CCTTC-to-AAGGA mutation at positions −53 to −49	67
pDY47	pDY100 with a GAAC-to-TCCA mutation at positions −48 to −45	67
pDY117	pDY100 with a C-to-A mutation at position −37	67
pDY45	pDY100 with a CATTCCT-to-ACGGAAG mutation at positions −36 to 30	67

in EMSAs, and no complex was observed with the APP and the mutant (−67 to −64) *fmgBC* promoter region (Fig. 2B). It appeared that APP contained the putative activator protein from the AS fraction.

To identify the putative activator protein, the APP was subjected to mass spectrometry analysis after protease digestion. The peptide sequences primarily matched MrpC, a protein that is ~30 kDa in size, which is consistent with the size of the major species in the APP (Fig. 2A). MrpC is similar to CRP family transcription factors and is essential for *M. xanthus* development (54). MrpC2, a shortened form of MrpC that lacks the 25 N-terminal residues, is produced during development, and was identified in an AS fraction previously by DNA-affinity chromatography with the *fruA* promoter region (58). We infer that MrpC2 in the AS fraction and in the APP is responsible for the shifted complex we observed with *fmgBC* promoter region DNA.

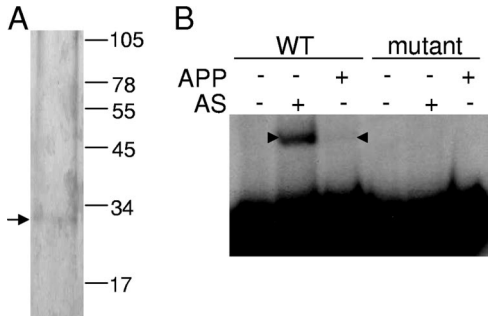

FIG. 2. DNA-affinity purification of protein that binds to the *fmgBC* promoter region. (A) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of protein purified from the AS fraction using *fmgBC* DNA (positions −104 to −29). The arrow indicates the major species in the APP after staining with silver. The numbers indicate the migration positions of molecular mass (in kilodaltons) standards. (B) EMSAs with ^{32}P-labeled *fmgBC* DNA (12 nM) spanning from positions −104 to −29 and proteins in the AS fraction or the APP. Arrowheads indicate the shifted complexes produced with the wild-type (WT) DNA fragment. No complex was observed with a DNA fragment bearing the ACCA to CAAC mutation at positions −67 to −64 (mutant).

To confirm that MrpC2 binds to the *fmgBC* promoter region fragment, N-terminally His-tagged MrpC2 (His$_{10}$-MrpC2) was expressed in *E. coli* and purified. His$_{10}$-MrpC2 displayed a pattern of binding to wild-type and mutant *fmgBC* DNA fragments similar to that of the AS fraction (Fig. 3). The mutation from positions −67 to −64 that resulted in the loss of shifted complex formation with the AS fraction (Fig. 1) and the APP (Fig. 2B) also caused the loss of shifted complex formation with His$_{10}$-MrpC2 (Fig. 3). This mutation includes 1 bp of a 5-bp element (Fig. 1); however, an adjacent mutation at positions −71 to −68, which changes the remaining 4 bp of the 5-bp element, did not markedly impair formation of shifted complexes with the AS fraction or with His$_{10}$-MrpC2 (Fig. 3). Likewise, none of the other mutations between positions −63 and −30 markedly impaired complex formation. The slower

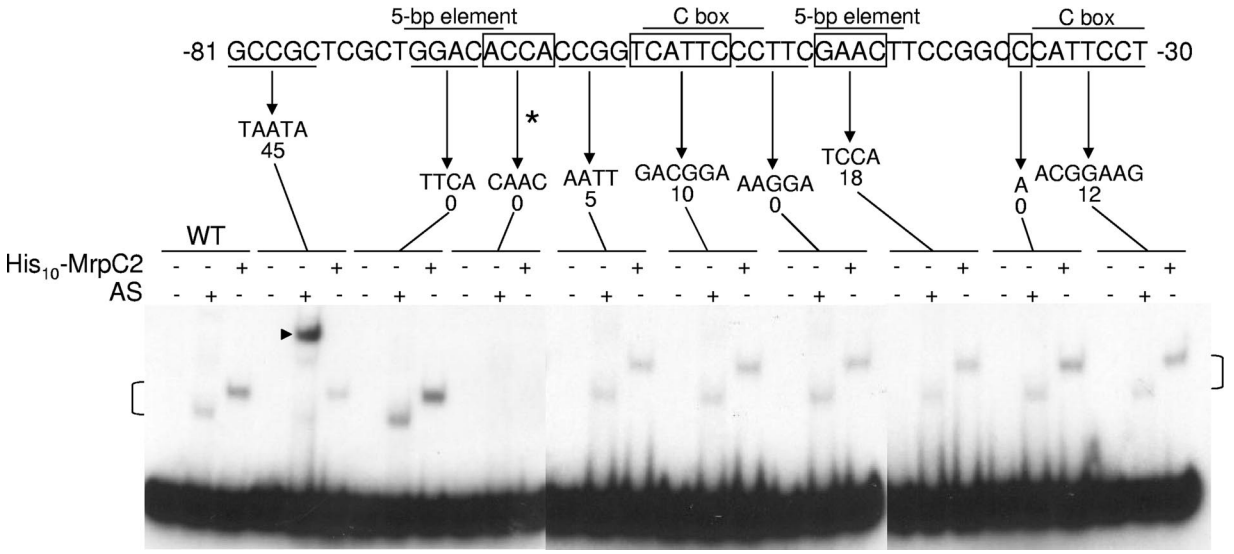

FIG. 3. Comparison of purified His_{10}-MrpC2 and the AS fraction for binding to the *fmgBC* promoter region. EMSAs with ^{32}P-labeled *fmgBC* DNA (2 nM) spanning from positions −104 to −29, wild-type (WT), or mutant as indicated and His_{10}-MrpC2 (1 μM) or the AS fraction (0.7 μg/μl). An asterisk indicates the mutation from positions −67 to −64 that impairs shifted complex formation. Brackets indicate the shifted complexes produced by the AS fraction and His_{10}-MrpC2 upon addition to wild-type *fmgBC* DNA and to most of the mutant DNA fragments. The mutation from positions −81 to −77 causes a novel shifted complex to form with the AS fraction (arrowhead). The image is a composite from three experiments, and in each experiment the wild-type *fmgBC* DNA served as a control, and the signal intensity of the shifted complexes was comparable to that shown. The results shown are representative of results observed in at least two experiments.

migration of the complex produced by His_{10}-MrpC2, compared to the complex produced by the AS fraction, is presumably due to the 10 His residues plus 8 additional residues present in the His_{10}-MrpC2 fusion protein. The mutation from positions −81 to −77 resulted in diminished formation of the complex that we believe contains MrpC2, by the AS fraction, and the appearance of a novel shifted complex. The novel complex appears to be due to an unknown protein in the AS fraction that is capable of binding to this mutant *fmgBC* DNA fragment, since purified His_{10}-MrpC2 did not show this effect. Rather, His_{10}-MrpC2 formed a complex that migrated at the expected position, suggesting that formation of the novel complex by the AS fraction might account for its diminished ability to form the complex that we believe contains MrpC2. We conclude that MrpC2 binds to an important *cis*-regulatory sequence at positions −67 to −64 in the *fmgBC* promoter region.

Since MrpC2 is similar to CRP family transcription factors, and cyclic nucleotides affect DNA binding by some family members (22), we examined His_{10}-MrpC2 binding to the *fmgBC* promoter region (−104 to −29) in the presence of different nucleotides. At concentrations designed to reflect physiological conditions, no effect of cyclic AMP (4 to 8 μM), cyclic GMP (20 to 40 nM), cyclic di-GMP (1 to 10 μM), ppGpp (50 to 400 μM), nucleoside triphosphates (400 μM), or deoxynucleoside triphosphates (200 μM) was observed (data not shown).

MrpC and/or MrpC2 associates with the *fmgBC* promoter region in vivo and this depends on FruA. ChIP assays were performed with polyclonal antibodies to MrpC, which also recognize MrpC2 (39), to determine whether MrpC and/or MrpC2 associate with the *fmgBC* promoter region during development. *M. xanthus* cells with the *fmgBC* promoter region (−100 to +50) integrated ectopically at a phage attachment site via site-specific recombination were collected after 12 or 18 h of development and subjected to ChIP with antibodies to MrpC or, as a control, immunoglobulin G (IgG). DNA recovered after ChIP was analyzed by PCR with primers designed to amplify the ectopic copy of the *fmgBC* promoter region. The PCR analysis showed that the *fmgBC* promoter region was enriched by ChIP with the anti-MrpC antibodies relative to the IgG control at 12 h (Fig. 4, lanes 5 and 6, top panel) and 18 h (Fig. 4, lanes 17 and 18, top panel) into development. PCR analysis with primers designed to amplify the *rpoC* coding region showed no enrichment of this region by ChIP with anti-MrpC antibodies relative to control antibodies at 18 h (Fig. 4, lanes 17 and 18, bottom panel), as reported previously (35), but at 12 h we unexpectedly yet reproducibly observed enrichment of the *rpoC* coding region by ChIP with anti-MrpC antibodies relative to control antibodies (Fig. 4, lanes 5 and 6, bottom panel). These results indicate that MrpC and/or MrpC2 is present in the vicinity of the *rpoC* coding region at 12 h into development, but not at 18 h, and that MrpC and/or MrpC2 is associated with the *fmgBC* promoter region at both times.

Recently, regulation of the *fmgA* gene was shown to be under combinatorial control by MrpC2 and FruA (35). Since the expression of *fmgA* occurs with similar timing during development as *fmgBC* (25) and the expression of both genes depends partially on C-signaling (4, 6, 24), to which FruA has been proposed to respond (5), we hypothesized that *fmgBC* is also under direct control by FruA. In the case of *fmgA*, association of MrpC and/or MrpC2 with the promoter region in vivo, as measured by ChIP-PCR analysis, was dependent on FruA (35). We carried out a similar analysis for *fmgBC* by performing ChIP-PCR analysis of a *fruA* mutant with the *fmgBC* promoter region (−100 to +50) integrated ectopically

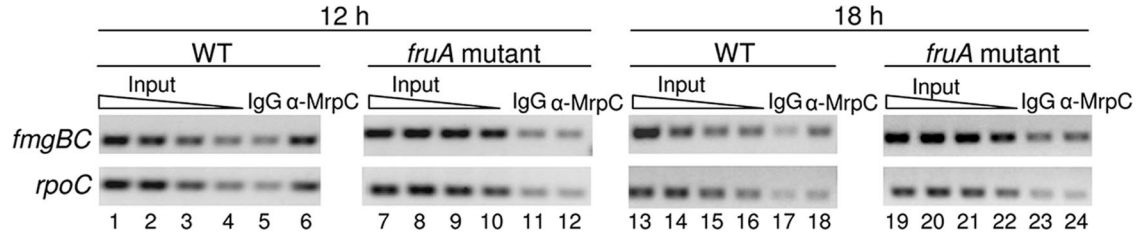

FIG. 4. Association of MrpC and/or MrpC2 with the *fmgBC* promoter region during development of wild-type and *fruA* mutant cells. ChIP analysis of *M. xanthus* with the *fmgBC* promoter region (−100 to +50) integrated ectopically in otherwise wild-type (WT) or *fruA* mutant backgrounds. At 12 and 18 h into development, cells were treated with formaldehyde and lysed, and cross-linked chromatin was immunoprecipitated with anti-MrpC antibodies or IgG as a control. DNA was amplified with appropriate primers for the *fmgBC* promoter region at the ectopic chromosomal site or with appropriate primers for the *rpoC* coding region as a control. A twofold dilution series of input DNA purified from 0.25, 0.125, 0.0625, or 0.03125% of the total cellular extract prior to immunoprecipitation was used as a template in parallel PCRs to show that the PCR conditions were in the linear range of amplification for each primer set.

as described above. In contrast to the wild-type strain, no enrichment of the *fmgBC* promoter region was observed with anti-MrpC antibodies relative to control antibodies at 12 h or 18 h into development (Fig. 4, lanes 11, 12, 23, and 24 [top panel]). Likewise, no enrichment of the *rpoC* coding region was observed with anti-MrpC antibodies relative to control antibodies (Fig. 4, lanes 11, 12, 23, and 24 [bottom panel]). We conclude that FruA is necessary for the association of MrpC and/or MrpC2 with the *fmgBC* promoter region during development and for the association of MrpC and/or MrpC2 with the *rpoC* coding region at 12 h into development.

FruA associates with the *fmgBC* promoter region in vivo and governs expression. If FruA plays a direct role in recruitment of MrpC and/or MrpC2 to the *fmgBC* promoter region, as observed previously for *fmgA* (35), it should be possible to detect FruA at the *fmgBC* promoter region by ChIP with antibodies against FruA. To test this expectation, ChIP was performed on the wild-type strain with the *fmgBC* promoter region (−100 to +50) integrated ectopically. At 12 h into development, enrichment of the *fmgBC* promoter region was observed with anti-FruA antibodies compared to control preimmune serum (Fig. 5, lanes 11 and 12). No enrichment was observed for a strain with vector lacking the *fmgBC* promoter region integrated ectopically (Fig. 5, lanes 5 and 6). We conclude that FruA associates with the *fmgBC* promoter region in vivo, which is consistent with the notion that it directly recruits MrpC and/or MrpC2.

If FruA plays a key role in regulation of *fmgBC*, expression of *fmgBC* is predicted to be impaired in a *fruA* mutant, as observed previously for *fmgA* (68). To test this prediction, *fruA* mutant and wild-type *M. xanthus* cells were transformed with a plasmid containing the −100 to +50 region of the *fmgBC* promoter transcriptionally fused to the *E. coli lacZ* gene. The plasmid integrates into the *M. xanthus* genome ectopically via site-specific recombination at a phage attachment site. As negative controls, strains bearing the vector with promoterless *lacZ* were also constructed. β-Galactosidase specific activity was measured in cell extracts at different times during development. The activity of each negative control strain was subtracted from that of the corresponding promoter-containing strain. The *fruA* mutation abolished developmental *lacZ* expression from the *fmgBC* promoter region (Fig. 6). This demonstrates that FruA governs *fmgBC* expression and, together with our other data, strongly suggests that FruA binds to the *fmgBC* promoter region and recruits MrpC and/or MrpC2, activating transcription.

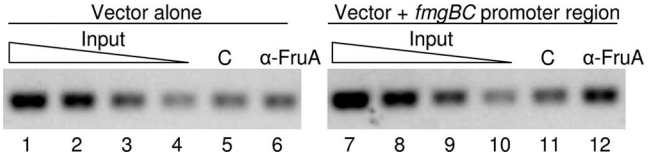

FIG. 5. Association of FruA with the *fmgBC* promoter region in vivo. ChIP analysis of *M. xanthus* with the vector alone or with the *fmgBC* promoter region (−100 to +50) integrated ectopically. At 12 h into development, cells were treated with formaldehyde and lysed, and cross-linked chromatin was immunoprecipitated with anti-FruA antibodies or preimmune serum as a control (lane C). A twofold dilution series of input DNA purified from 0.25, 0.125, 0.0625, or 0.03125% of the total cellular extract prior to immunoprecipitation was used as a template in parallel PCRs to show that the PCR conditions were in the linear range of amplification.

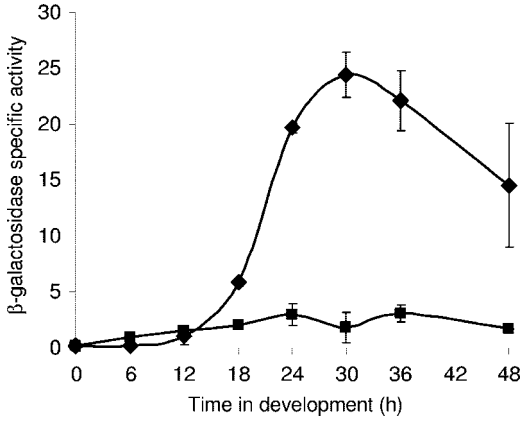

FIG. 6. Developmental expression from *fmgB-lacZ*. The *fmgBC* promoter region from positions −100 to +50 was fused to *lacZ*, and the β-galactosidase specific activity was measured during the development of *M. xanthus* wild-type (◆) and *fruA* mutant (■) cells. In each background, the activity from the vector with no promoter was measured as a negative control. Points show the average of three transformants, after subtracting the average of three transformants with the promoterless vector. The units of activity are nanomoles of *o*-nitrophenyl phosphate per minute per milligram of protein. Error bars depict one standard deviation of the data.

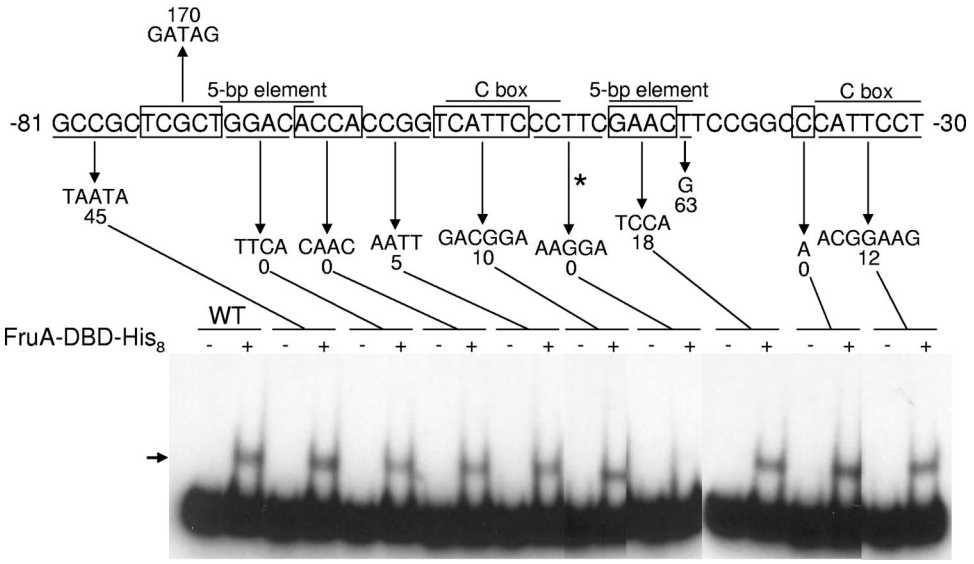

FIG. 7. Effects of mutations on binding of FruA-DBD-His_8 to *fmgBC* promoter region DNA. EMSAs with ^{32}P-labeled *fmgBC* DNA (2 nM) spanning from positions −104 to −29, wild type (WT), or mutant as indicated and FruA-DBD-His_8 (14 μM). A horizontal arrow indicates the shifted complex produced with wild-type DNA. An asterisk indicates the mutation from positions −53 to −49 that impairs shifted complex formation. The image is a composite from three experiments, and intervening lanes were removed from one of the images. In each experiment, the wild-type *fmgBC* DNA served as a control, and the signal intensity of the shifted complex was comparable to that shown.

The FruA DNA-binding domain binds to a key *cis*-regulatory sequence in the *fmgBC* promoter region. To determine whether FruA binds to the *fmgBC* promoter region, the C-terminally His-tagged FruA DNA-binding domain (FruA-DBD-His_8) was overexpressed in *E. coli*, purified, and used in EMSAs. FruA-DBD-His_8 generated a single shifted complex with a DNA fragment spanning from positions −104 to −29 of the *fmgBC* promoter region (Fig. 7). EMSAs with mutant probes localized the binding to positions −53 to −49, since a mutation in this region abolished the FruA-DBD-His_8 binding. This region was shown previously to be critical for *fmgBC* promoter activity (67). It includes part of a C box and lies immediately upstream of a 5-bp element.

Enhanced complex formation in the presence of FruA-His_6 andHis_{10}-MrpC2. The combination of FruA and MrpC2 led to enhanced formation of shifted complexes with *fmgA* promoter region DNA, due to cooperative binding of the two proteins to adjacent (possibly overlapping) sites (35). Both sites were required for the enhancement of shifted complex formation, as was full-length FruA (i.e., FruA-DBD-His_8 was insufficient), suggesting that the N-terminal domain of FruA might directly interact with MrpC2 (35). To test whether similar effects could be observed with *fmgBC* promoter region DNA, FruA-His_6 was overexpressed in *E. coli* and purified. As observed previously with *fmgA* promoter region DNA (35), the *fmgBC* promoter region (−104 to −29) was bound weakly by FruA-His_6 in EMSAs (Fig. 8A, lane 3), but the combination of FruA-His_6 and His_{10}-MrpC2 resulted in the formation of an abundant lower complex (LC) and a faint upper complex (UC) when analyzed on 5% polyacrylamide gels (Fig. 8A, lane 4). Migration of the LC was similar to that of complexes formed by either protein alone, suggesting that the LC is composed of DNA bound by His_{10}-MrpC2 or FruA-His_6. The slower migration of the UC was suggestive of DNA bound by both proteins simultaneously. When analyzed on 8% polyacrylamide gels, more UC was observed when both proteins were added to the *fmgBC* DNA fragment (Fig. 8B, lane 4), and more complex was observed when only FruA-His_6 was added (Fig. 8B, lane 3). The 8% polyacrylamide gel seems to stabilize FruA-His_6 binding to DNA under the conditions of the EMSAs, as observed previously with *fmgA* promoter region DNA (35).

To determine whether the binding of both proteins to DNA is required for the observed enhancement of complex formation, EMSAs were performed with mutant DNA fragments. A mutation at positions −67 to −64 that abolished His_{10}-MrpC2 binding (Fig. 3) also abolished enhancement of complex formation by the combination of proteins on 5% polyacrylamide gels; the UC was undetectable, and the faint LC was comparable in intensity to that formed by FruA-His_6 alone (Fig. 8A, lanes 7 and 8). On 8% polyacrylamide gels, a similar result was observed, except that a small amount of UC was detected (Fig. 8B, lane 8), perhaps indicating that FruA-His_6 facilitates weak binding of His_{10}-MrpC2 to the mutant site. Similarly, a mutation at positions −53 to −49 that abolished detectable binding of FruA-DBD-His_8 (Fig. 7) or FruA-His_6 (Fig. 8A, lane 11) resulted in no detectable UC on 5% polyacrylamide gels, and LC of an intensity comparable to that formed by His_{10}-MrpC2 alone (Fig. 8A, lanes 10 and 12). On 8% polyacrylamide gels, there was slight enhancement of LC and a small amount of UC (Fig. 8B, lanes 10 and 12). The small amount of UC might indicate that His_{10}-MrpC2 facilitates weak binding of FruA-His_6 to the mutant site. The slight enhancement of LC might result from the initial binding of both proteins, followed by dissociation of FruA-His_6. In any case, much less of the shifted complexes is observed with the DNA fragment containing the mutation at positions −53 to −49 (Fig. 8B, lane 12) than with the wild-type fragment (Fig. 8B, lane 4). MrpC2 and FruA appear to bind cooperatively to the *fmgBC* promoter region, as

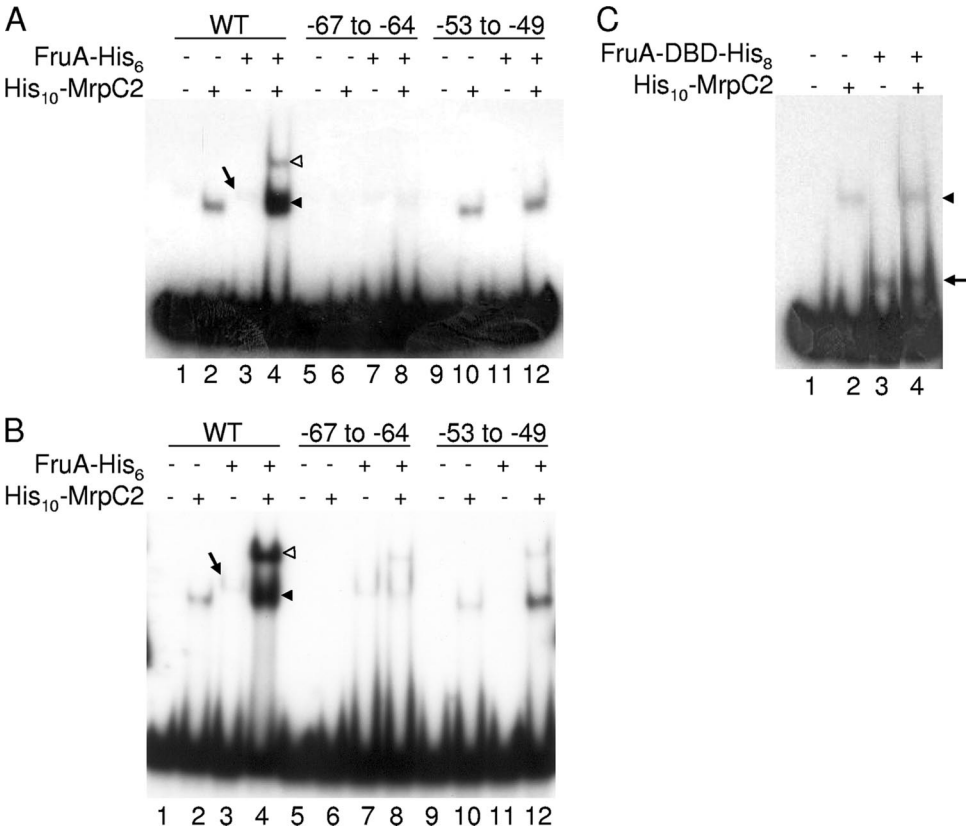

FIG. 8. EMSAs with MrpC2 and full-length FruA or just the DNA-binding domain of FruA. (A) Shifted complex formation with His_{10}-MrpC2 and full-length FruA-His_6 and the effect of mutations. EMSAs with ^{32}P-labeled *fmgBC* DNA (2 nM) spanning from positions −104 to −29, wild-type (WT), or mutant as indicated and no protein, 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, electrophoresed on a 5% polyacrylamide gel. A slanted arrow indicates the faint shifted complex produced by FruA-His_6 alone. The unfilled and filled arrowheads indicate the UCs and LCs, respectively, produced by the combination of proteins. (B) Same as in panel A except electrophoresed on an 8% polyacrylamide gel. (C) Shifted complex formation with His_{10}-MrpC2 and FruA-DBD-His_8. EMSAs with ^{32}P-labeled *fmgBC* DNA (2 nM) spanning from positions −104 to −29 and no protein, His_{10}-MrpC2 (1 μM), FruA-DBD-His_8 (14 μM), or both His_{10}-MrpC2 (1 μM) and FruA-DBD-His_8 (14 μM) as indicated. The arrowhead indicates the complex produced by His_{10}-MrpC2, and the arrow indicates the complex produced by FruA-DBD-His_8. Intervening lanes were removed from the image.

seen previously for the *fmgA* promoter region, although the arrangement of binding sites relative to the promoter is different. FruA binds upstream of MrpC2 in the *fmgA* promoter region (35), whereas FruA binds downstream of MrpC2 in the *fmgBC* promoter region (Fig. 3 and 7).

Despite the different arrangement of binding sites, we found that the *fmgA* and *fmgBC* promoter regions share the characteristic that FruA-DBD-His_8 is insufficient to enhance complex formation in combination with His_{10}-MrpC2 (Fig. 8C, lane 4). The complexes formed by the combination of proteins were similar to the complexes formed by His_{10}-MrpC2 or FruA-DBD-His_8 alone (Fig. 8C, lanes 2 and 3). We propose that the N-terminal regulatory domain of FruA interacts with MrpC2 at the *fmgBC* promoter region, mediating cooperative binding of the two transcription factors and subjecting *fmgBC* expression to combinatorial control similar to that observed for *fmgA*.

DISCUSSION

Our results demonstrate that MrpC2 and FruA bind to key *cis*-regulatory sequences upstream of the *fmgBC* promoter, placing it under similar combinatorial control as observed previously for *fmgA* (35). Surprisingly, the arrangement of binding sites for MrpC2 and FruA is different in the two promoter regions. FruA binds downstream of MrpC2 in the *fmgBC* promoter region (Fig. 3 and 7), whereas FruA binds upstream of MrpC2 in the *fmgA* promoter region (35). In both cases, FruA is required for promoter activity and for recruitment of MrpC and/or MrpC2 to the promoter region in vivo. In vitro, FruA and MrpC2 appear to bind cooperatively to both promoter regions, and this depends on the N-terminal regulatory domain of the FruA response regulator. Preliminary results, described below, indicate that cooperative binding by FruA and MrpC2 is a common mechanism of gene regulation during *M. xanthus* development. This mechanism is proposed to allow integration of positional information via short-range C-signaling with starvation signaling and cell death, controlling spatiotemporal gene expression and determining cell fate.

Combinatorial control of C-signal-dependent genes involving cooperative binding of FruA and MrpC2 appears to be a common mechanism of gene regulation during *M. xanthus* development. In addition to *fmgA* and *fmgBC*, the promoter region of the *dev* operon appears to utilize this mech-

anism. MrpC2 binds to a region that includes a 5-bp element and two C box-like sequences, and the addition of FruA greatly enhances complex formation in EMSAs (S. Mittal, P. Viswanathan, and L. Kroos, unpublished data). Expression of the *dev* operon is confined to fruiting bodies (16, 47) and has been proposed to be a crucial step in commitment of cells to differentiate into spores (23, 35). The gene identified by Tn*5 lac* Ω4403 encodes a putative serine protease whose role in development is unknown, but whose expression depends absolutely on C-signaling (7, 24). The promoter region contains two 5-bp elements in inverted orientation that are bound by MrpC2, and FruA appears to bind cooperatively (J. Lee, S. Mittal, and L. Kroos, unpublished data). Therefore, at least four promoter regions appear to be bound cooperatively by MrpC2 and FruA, since the combination of proteins greatly enhances formation of shifted complexes in EMSAs, and this was shown to correlate with cooperative binding at the *fmgA* promoter region by DNase I footprinting (35). Moreover, enhancement of shifted complex formation was shown to require the binding sites for both MrpC2 and FruA at both the *fmgA* (35) and the *fmgBC* (Fig. 8) promoter regions.

Although the combination of MrpC2 and FruA produces a strikingly similar enhancement of shifted complex formation in EMSAs with *fmgA* or *fmgBC* promoter region DNA, the arrangement of the MrpC2 and FruA binding sites is different in the two promoter regions. In the *fmgA* promoter region, mutations from positions −86 to −77 impaired the binding of FruA-DBD-His_8 (68) and mutations from positions −76 to −46 affected the binding of His_{10}-MrpC2 (35). In addition, DNA upstream of position −76 was found to be required for His_{10}-MrpC2 binding, suggesting that the MrpC2- and FruA-binding sites might partially overlap, with the two proteins presumably interacting with opposite faces of the DNA in the region of overlap (35). In contrast, FruA-DBD-His_8 and His_{10}-MrpC2 binding to the *fmgBC* promoter region was impaired only by mutations from positions −53 to −49 and from positions −67 to −64, respectively (Fig. 3 and 7). Adjacent mutations did not impair the binding of either protein, although these mutations had previously been shown to reduce promoter activity (67), suggesting that sequences important for binding in vivo might be missed under the in vitro conditions of the EMSAs. Alternatively, other transcription factors might bind to the adjacent sequences. In any case, FruA binds downstream of MrpC2 in the *fmgBC* promoter region, whereas FruA binds upstream of MrpC2 in the *fmgA* promoter region.

The different arrangement of FruA and MrpC2 binding sites in the *fmgA* and *fmgBC* promoter regions suggests a somewhat different mechanism of transcriptional activation from the two promoters. As noted previously, in the *fmgA* promoter region, the two proteins occupy a location typical for class I activators (35), which contact the C-terminal domain of the α subunits of RNA polymerase (1). In the *fmgBC* promoter region, FruA and MrpC2 occupy a similar location, but their positions relative to the promoter are reversed, so presumably a different contact(s) with the C-terminal domain of the α subunits of RNA polymerase would be involved in the activation of transcription. Two activators can contact the C-terminal domain of the α subunits of RNA polymerase at the same promoter, based on studies of both synthetic (28, 55) and natural promoters (2).

Despite the different arrangement of FruA and MrpC2 binding sites with respect to the *fmgA* and *fmgBC* promoters, the two proteins might interact with each other similarly at the two promoter regions. Our results show that the N-terminal regulatory domain of FruA is required for enhancement of shifted complex formation in combination with MrpC2 at both promoter regions (35) (Fig. 8C). This domain is similar to receiver domains of response regulators that are phosphorylated by histidine protein kinases (5, 42); however, it lacks two aspartate residues that are highly conserved in receiver domains and normally play an important role in phosphorylation of a third aspartate residue (5, 63). Moreover, several lines of evidence suggest that FruA might function without phosphorylation (35). Here, we showed that recombinant (presumably unphosphorylated) FruA-His_6 greatly enhances formation of shifted complexes in combination with His_{10}-MrpC2 at the *fmgBC* promoter region, and the receiver domain of FruA is required for enhancement (Fig. 8). Therefore, the unphosphorylated receiver domain of FruA might interact directly with MrpC2 to mediate cooperative DNA binding. Receiver domains that cannot or need not be phosphorylated have been described in bacterial DNA-binding proteins (11, 44, 50) and in proteins that regulate circadian rhythms in bacteria (36, 65) and plants (53). These proteins are sometimes called pseudo-response regulators. Whether FruA is a pseudo-response regulator (i.e., whether its receiver domain is phosphorylated in vivo) remains an open question but, to our knowledge, cooperative binding of a response regulator-like protein and an independent transcription factor (MrpC2) is a novel mechanism of gene regulation (35).

Consistent with the idea that FruA and MrpC2 interact similarly with each other at the *fmgA* and *fmgBC* promoter regions, the combination of proteins produces a strikingly similar enhancement of shifted complex formation in EMSAs with DNA from either promoter region (35) (Fig. 8). In both cases, the percentage of polyacrylamide in gels used in the EMSAs influenced the shifted complexes that were observed, with 8% gels (compared to 5% gels) facilitating the detection of FruA binding and the detection of UC that presumably represents FruA and MrpC2 bound to DNA. We infer that the two proteins bind cooperatively to DNA in solution, as demonstrated by DNase I footprinting in the case of *fmgA* (35), but FruA binding is less stable than MrpC2 binding, especially when analyzed on 5% gels, so LC is predominantly MrpC2 bound to DNA. Since their invention, it has been known that the gel matrix can influence the stability of protein-DNA complexes during EMSAs (8).

Another observation consistent with the idea that FruA and MrpC2 might interact similarly with each other at the *fmgA* and *fmgBC* promoter regions is that sequences matching the consensus binding site for FruA are in the opposite orientation in the two promoters. The consensus sequence for binding of FruA-DBD-His_8 is GGGC/TA/G(N_{4-6})C/TGGG (62). The sequence GGGTG(N_5)TGGG from positions −81 to −68 in the *fmgA* promoter region matches the consensus perfectly, and some mutations in this sequence impair FruA-DBD-His_8 binding in vitro (68). In the *fmgBC* promoter region, in the opposite orientation, the sequence GGGAA(N_4)CGGT from positions −52 to −64 matches the consensus except at two positions, and the mutation at positions −53 to −49 that impaired FruA-

DBD-His_8 binding in vitro overlaps this sequence (Fig. 7). MrpC is dimeric, and one type of site to which MrpC and MrpC2 bind is palindromic, with a consensus sequence of GTGTC(N_8)GACAC (39). Presumably, a dimer of MrpC or MrpC2 bound to such a palindromic site could present the same surface to FruA bound upstream or downstream. In the *fmgA* promoter region, the sequence GAGCG(N_8)CACAT from positions −67 to −50 is the best match to the consensus between positions −76 and −46, where mutations affected His_{10}-MrpC2 binding (35). In the *fmgBC* promoter region, the sequence ACGCC(N_8)GACAC from positions −83 to −66 matches half the consensus perfectly, and the mutation at positions −67 to −64 that impaired His_{10}-MrpC2 binding in vitro overlaps this sequence (Fig. 3). We hypothesize that the N-terminal domain of FruA can interact directly with dimeric MrpC2 to permit cooperative DNA binding, whether FruA binds upstream of MrpC2 (as at the *fmgA* promoter region) or whether FruA binds to a site in the opposite orientation downstream of MrpC2 (as at the *fmgBC* promoter region). This flexibility in the arrangement of FruA and MrpC2 at different promoters would presumably result in a different contact(s) with RNA polymerase and different levels of transcriptional activation.

Our finding that the response regulator-like FruA and the transcription factor/antitoxin MrpC bind cooperatively in different arrangements in the promoter regions of C-signal-dependent genes has important implications for *M. xanthus* development. Since MrpC2 appears to activate *fruA* transcription (58), combinatorial regulation of target genes by MrpC2 and FruA constitutes a coherent feed-forward loop, which is a motif found commonly in regulatory networks since it has beneficial characteristics (33, 34). One characteristic is that the expression of target genes is delayed until both transcription factors reach a sufficient concentration. Full expression of partially C-signal-dependent target genes such as *fmgBC* and the *dev* operon, which are important for sporulation, may be delayed until cell alignment in the nascent fruiting body causes a high level of C-signaling, which could affect FruA and/or MrpC2. Since a mutant defective in C-signaling accumulates FruA normally during development, it has been proposed that one or more histidine protein kinases alter the activity of FruA via phosphorylation in response to C-signaling (5, 15, 58). However, if FruA is not phosphorylated, perhaps C-signaling affects the concentration of MrpC2 and/or its precursor, MrpC. The accumulation of MrpC and MrpC2 is inhibited by the STPK cascade that leads to phosphorylation of MrpC during growth (39). Starvation triggers accumulation of both forms of the protein by counteracting the STPK cascade (39); however, the EspA signal transduction pathway appears to delay their accumulation in response to an unknown signal (13). Therefore, the concentrations of MrpC and MrpC2 appear to be linked to starvation and perhaps other developmental signals via several pathways. Only if starvation persists and the other putative signals, including C-signal, are received, would the MrpC2 concentration rise to a threshold that permits full expression of target genes in combination with FruA, committing the cell to form a spore. In its role as an antitoxin, binding of MrpC to the MazF toxin would prevent programmed cell death in cells destined to form spores (37). In cells destined to undergo programmed cell death, binding of MrpC to the *mazF* promoter region would activate transcription, leading to increased MazF. According to this model, MrpC is a key determinant of cell fate, and determining whether MrpC2 binds to MazF and/or the *mazF* promoter region is an important goal. Also, determining whether the effects of FruA on target gene expression depend solely on the strength of its binding sites and their position relative to MrpC2 binding sites, or whether FruA integrates additional signal inputs, is an important question for future studies. Signal-responsive auxiliary regulatory proteins have been shown to interact with the response regulator RcsB (32) and the pseudo-response regulator AmiR (43), so perhaps FruA interacts with other partners in addition to binding cooperatively to DNA with MrpC2.

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