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Activation of a Development-Specific Gene, *dofA*, by FruA, an Essential Transcription Factor for Development of *Myxococcus xanthus*

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FruA is an essential transcription factor for *Myxococcus xanthus* development. The expression of *tps* and *dofA* genes is *fruA* dependent. In this study, we show by gel shift and footprint assays with the C-terminal DNA-binding domain of FruA and by a *lacZ* fusion assay that FruA may directly activate *dofA* expression during development.

Myxococcus xanthus is a gram-negative soil bacterium that exhibits a communal lifestyle during vegetative growth and fruiting body development (3, 4). When nutrients are limited, *M. xanthus* cells form multicellular fruiting bodies filled with myxospores, which are dormant and resistant to various environmental stresses. The developmental process is achieved by a series of sophisticated intercellular signaling pathways that regulate the expression of a specific set of genes (11, 14).

FruA is a response regulator (RR) of the two-component system that is essential for aggregation, fruiting body formation, and sporulation during development (5, 13). FruA has been proposed to play a key role in the C-signal transduction system (16). C signal is a cell surface-associated protein encoded by the *csgA* gene and is essential for aggregation, fruiting body formation, and sporulation during development (6, 14). Analysis of the protein expression patterns in the wild-type, *fruA*::Tc, and *csgA731* strains during development indicated that developmental genes under the control of FruA can be classified into two groups: one that is C signal independent and one that is C signal dependent (7). The production of five proteins was found to be *fruA* dependent but C signal independent, and one protein was dependent on both *fruA* and C signal. Among them, protein S (the *tps* gene product) (9) and DofA, both of which are C signal independent, have been identified by sequence analyses. Protein S is a well-characterized spore coat protein which has structural similarity to βγ-crystallins (10). DofA does not show significant similarity to known proteins (7).

The DNA-binding domain of FruA binds specifically to the *dofA* promoter region. To examine whether FruA directly regulates the expression of *dofA* and *tps*, a gel shift assay was performed with the DNA fragments containing the *dofA* and *tps* promoter regions. The C-terminal DNA-binding domain, from Pro at position 152 to Leu at position 229, of FruA tagged with His_8 (FruA-DBD-His_8) was used in this study. FruA-DBD-His_8 was overproduced with the T7 promoter expression system and was purified with the use of Ni-nitrilotriacetic acid resin (QIAGEN) according to the manufacturer's instructions. Since the region from nucleotides (nt) −128 to −57 with respect to the transcription initiation site is sufficient for the developmental induction of *dofA* (8), for gel shift assays, DNA fragments containing the *dofA* promoter region from nt −150 to −32 were amplified by PCR with the chromosomal DNA of *M. xanthus* DZF1 and oligonucleotide primers −150T and −32B (Fig. 1). Forward primers −150T and −250T contain a HindIII site and reverse primers −32B and +10B contain a BamHI site for subsequent cloning. PCR products were digested with HindIII and BamHI and cloned into pBluescript SK− (Stratagene). DNA fragments for the probe were labeled with [α-^{32}P]dCTP by using the Klenow fragment of DNA polymerase I after digestion of the plasmid with HindIII and BamHI and isolating the fragments by polyacrylamide gel electrophoresis (PAGE). The probe containing the *tps* promoter region, from nt −250 to −40, required for the developmental expression of *tps* (2), was also prepared by using primers 5′TCAAGCTTGCCGGTACACCCACGAC3′ and 5′TCG GATCCTACAGTACCGTATCCGTC3′ for PCR amplification as described above.

When the gel shift assay was performed as described previously (19), FruA-DBD-His_8 was able to bind to the *dofA* promoter, and two types of complexes were observed (Fig. 2A). The addition of purified anti-FruA antibody to the reaction mixture resulted in the observation of the upshifted band, indicating that the detected complexes indeed consisted of the *dofA* promoter and FruA-DBD-His_8. On the other hand, FruA-DBD-His_8 was unable to bind to the *tps* promoter under the same conditions (Fig. 2B). Although the expression of *dofA* and *tps* was not observed in the *fruA*::Tc strain and both genes were *csgA* independent (7), FruA appears to directly activate *dofA* expression and to indirectly regulate *tps* expression. However, it is possible that the *tps* promoter tested in this study may have a lower-affinity site for FruA-DBD-His_8 or may require another factor for binding of FruA-DBD-His_8.

FruA-DBD-His_8 binds to two regions in the *dofA* promoter. To determine the sequences in the *dofA* promoter recognized by FruA-DBD-His_8, a footprint assay was performed by the DNase I method with a probe containing the promoter region from nt −250 to +10 (Fig. 1). The plasmid containing the region from nt −250 to +10 was constructed with primers −250T and +10B (Fig. 1) as described above. DNA fragments for the probe were prepared by digesting the plasmid with

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ACGCACGGCCCTGGCCAACGGCGGCTACGTCATCAACAACGGCAGCAGCT   -331
TCATCATGGCCATGGAGTTCACGCCCACCGGCGTGCAGGCCAGCGCCCTG   -281
CTGACCTACAGCGAGTCCAGCAACCCGGCCTCGCCGTACTACGCGGACCA   -231
                              -250T
GACGCGGCTGTTCTCCCAGAAGCAGTGGCGGCCCATCCTCTCTTCACCTCCG -181
AGGAAATCGCCGCCGCGCCGGCTGAGCAGATCACCCTCACCGGCGACTGA   -131
                              -150T
CGTCCCCGCCGGCCCCCGATTCGTCGGGGGCCGGACACCTCGCGGCCCGG    -81
                                                  -82
CAGGGCAACGTCGGGCCGCGGATGTCCGCCCTCCAGTCTGATTCCGTCTG    -31
     b     -67          -57     a     -42      -32B
CTCCAGGGACGCGCGCCAATGGGTGCGCAGACCGTCAGGCGCCGCCGTTC    +20
                              +1    +10B
GTTCCTGGCGAAGGGGCACCGGGATTTCCCGGAGGAATCACCGAGGAAGC    +70
AGCAGGGGGACATCATG                                     +87
            Met
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FIG. 1. The promoter region of the *dofA* gene. The transcription initiation site is indicated by a bold letter and +1. The translation initiation codon is indicated by bold letters and Met. The regions from nt −82 to −67 and from nt −57 to −42, protected from DNase I in the presence of FruA-DBD-His_8 in the footprint assay, are indicated with bold lines. A highly GC-rich inverted repeat sequence is indicated by arrows. The sequences of the primers used in PCR amplification, −250T, −150T, −32B, and +10B, are underlined.

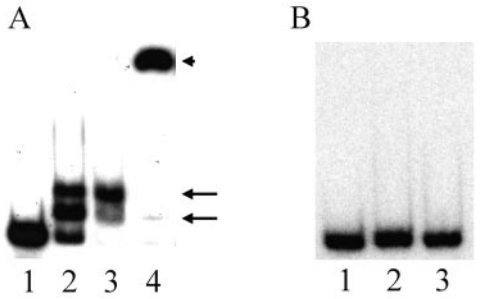

FIG. 2. Gel shift assay. (A) The *dofA* promoter. The probe containing the region from nt −150 to −32 was mixed with FruA-DBD-His_8, and the binding patterns were analyzed by PAGE. Lane 1, no FruA-DBD-His_8; lane 2, 0.5 ng/μl of FruA-DBD-His_8; lane 3, 1 ng/μl of FruA-DBD-His_8; lane 4, 1 ng/μl of FruA-DBD-His_8 and anti-FruA antibody. FruA-DBD-His_8/*dofA* promoter complexes are indicated by arrows. Anti-FruA antibody/FruA-DBD-His_8/*dofA* promoter complexes are indicated by an arrowhead. (B) The *tps* promoter. The probe contains the region from nt −250 to −41. Lane 1, no FruA-DBD-His_8; lane 2, 0.5 ng/μl of FruA-DBD-His_8; lane 3, 1 ng/μl of FruA-DBD-His_8.

HincII, located in the cloning vector, and BamHI and isolating the fragment by PAGE. The isolated DNA fragments were then labeled with [α-^{32}P]dCTP by using Klenow fragment of DNA polymerase I. Thus, only the top strand was labeled.

Two regions, from nt −57 to −42 (region a) and from nt −82 to −67 (region b), were found to be protected from DNase I in the presence of FruA-DBD-His_8 (Fig. 3). In addition, the sites at −87, −60, and −38 were found to be hypersensitive to DNase I. It appears that region a has a slightly higher affinity to FruA-DBD-His_8 than region b does, judging from the results of the footprint assay. Although apparent sequence similarity is not found in these regions, the sequence 5′AGGGC3′ from nt −79 to −75 in region b is found to be the complementary sequence of 5′GCCCT3′ from nt −53 to −49 in region a (Fig. 1). Therefore, it is possible that these sequences are critical for the binding of FruA-DBD-His_8 to the *dofA* promoter. Binding sites located in the reverse orientation are also found in some promoters regulated by the RR. Spo0A, for instance, regulates many genes which have binding sites in both orientations in their promoters (17).

To examine the roles of these regions on *dofA* expression in vivo, various regions of the *dofA* promoter were translationally fused to the *lacZ* gene and introduced into the phage Mx8 attachment gene *attB* in the *M. xanthus* chromosome (18). Since it has been shown that the *dofA* promoter region up to nt −128 is sufficient for the developmental regulation of *dofA* expression (8), the *dofA* promoter regions up to nt −128, −91, −62, and −41 were examined. The activity of β-galactosidase was measured at 20 h of development. The deletion of the highly GC-rich inverted repeat sequence (Fig. 1) (promoter −91) decreased β-galactosidase activity to 45% of that of the promoter up to nt −128 (promoter −128), and the deletion of region b (promoter −62) and region a (promoter −41) decreased its activity to 13% and 4%, respectively. These results agree well with the results previously described (8). Therefore, regions a and b were important for the developmental regulation of *dofA* expression. It is worth noting that the spacing between regions a and b was unimportant, since deletion of the region from nt −68 to −60 had no effect (8).

Implications. Since region a seems to have a slightly higher affinity to FruA-DBD-His_8 than region b as described above, FruA may bind in vivo first to region a and then to region b to activate the *dofA* gene. Furthermore, as the activity of the promoter containing only region a was less that half of that containing both regions a and b, it is likely that regions a and b synergistically activate the *dofA* gene. Moreover, because the deletion of the highly GC-rich inverted repeat sequence drastically affected the activity of the *dofA* promoter, some additional factor(s) may be required for the full activation of the *dofA* gene during development.

It will be important in the future to characterize the nature of the entire FruA protein, namely, the function of the N-terminal receiver domain of FruA, since the C-terminal domain of the RR does not always function in the same fashion as the entire RR. In the case of Spo0A, Spo0A functions as both an activator and a repressor, and it has been proposed that the N-terminal domain of Spo0A inhibits transcription activation unless it is phosphorylated (17). It is possible that FruA also functions as a repressor, since some proteins are not repressed in Δ*fruA* mutants during development (7).

The *fruA* gene is essential for development, and the *dofA* gene is not (8). Therefore, FruA appears to regulate other genes that are essential for development. It has been shown that FrzCD methylation and *devRS* expression are under the control of both FruA and C signal (5, 15). It is possible that FruA regulates the expression of *frzF*, which encodes the methyltransferase for FrzCD, although *frzF* is located in the *frz* operon containing *frzA*, *frzB*, *frzCD*, *frzE*, and *frzG* upstream of *frzF* (12). On the other hand, the *devRS* genes are part of the *dev* operon, which contains three genes upstream of the *devRS* genes (1). Thus, identification of the promoter regions of *frzF* and *devRS* is essential for understanding how FruA regulates FrzCD methylation and *devRS* expression.

We are now attempting to identify a consensus sequence of FruA binding sites by using randomized oligonucleotides as probes. Since sequencing of the *M. xanthus* genome has been

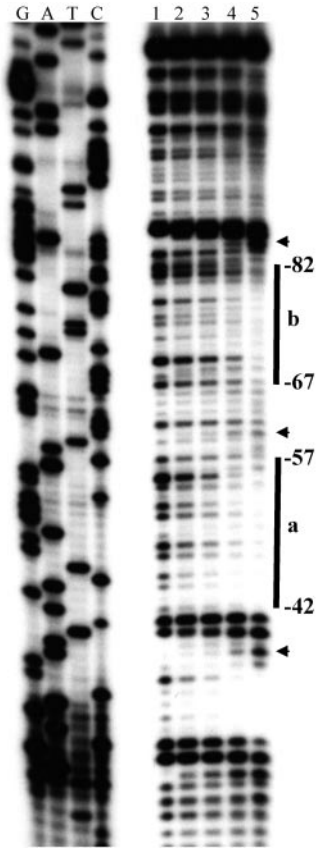

FIG. 3. Footprint assay. Lane 1, no FruA-DBD-His_8; lanes 2 to 5, 0.25, 0.5, 1, and 2 ng/μl of FruA-DBD-His_8, respectively. Lanes G, A, T, and C represent sequence ladders generated by a primer, 5′GAT CCGCCTGACGGTCTGCGCACCCA3′, which can hybridize to the 3′ end of the top strand of the probe. The sites that are hypersensitive to DNase I, at −87, −60, and −38, are indicated by arrowheads.

completed (http://www.ncbi.nlm.nih.gov), it may be possible to identify target genes of FruA by searching the genome for sequences homologous to FruA-binding sites. Identification of genes regulated by FruA is important for elucidation of the molecular mechanisms of the signal transduction pathways during development.

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