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Transcription from Two Promoters and Autoregulation Contribute to the Control of Expression of the *Salmonella typhimurium* Flagellar Regulatory Gene *flgM*

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The *flgM* gene product has been shown to be a negative regulator of flagellin transcription in *Salmonella typhimurium* (K. L. Gillen and K. T. Hughes, J. Bacteriol. 173:2301–2310, 6453–6459, 1991; K. Ohnishi, K. Kutsukake, H. Suzuki, and T. Iino, Mol. Microbiol. 6:3149–3157, 1992). Mud-*lac* fusions to the *flgM* gene were isolated and used to characterize the regulation of *flgM* gene expression. Transcription of the *flgM* gene was decreased more than 30-fold in strains with the flagellar master regulatory genes, *flhC* and *flhD*, deleted. A class 2 flagellar defect caused a slight increase of *flgM* gene transcription unless a wild-type copy of the *flgM* gene was present, in which case transcription was decreased threefold. A deletion in the gene for the alternative sigma factor σ^{28} (FliA) caused a fourfold decrease in *flgM* expression. Insertional inactivation of a gene upstream of the *flgM* gene (*flgA*) in a *fliA* mutant strain caused transcription of the *flgM* gene to be decreased to a basal level. Northern (RNA) blot analysis confirmed the presence of two transcripts through the *flgM* gene, one which initiates upstream of the *flgM* gene and a second which initiates upstream of the *flgA* gene.

Synthesis and assembly of functional flagella in *Salmonella typhimurium* and *Escherichia coli* require the input of more than 40 genes. Many of these gene products are actually known to be structural proteins, found in the completed flagellum. Other gene products are involved in gene regulation or in the assembly process or have unknown functions. (For a recent review, see reference 18.)

Regulation of flagellar gene expression has been studied, and a hierarchy of gene expression has been determined (13, 16). For *S. typhimurium*, genes have been grouped into three classes, with each subsequent class dependent upon earlier classes for expression (16). Class 1 consists of the *flhCD* genes (26). These genes are activated by the cyclic AMP-cyclic AMP receptor protein complex (15, 26, 33). They are required for expression of all flagellar genes, but the exact role of these master regulatory genes in promoting flagellar gene synthesis remains unclear.

Class 2 contains most of the known flagellar genes, including those encoding structural components of the hook and basal body of the flagellum. Known regulatory genes in this class are the *fliK* gene, which regulates hook length (30), and the *fliA* gene, which encodes an alternative sigma factor required for transcription of the class 3 genes (16, 21). In addition, the *flgA* gene has been reported to be required both for flagellar assembly (20) and for flagellar gene regulation (14). Expression of class 2 genes is required for class 3 gene expression to occur.

The genes for flagellin are grouped among the class 3 genes in the regulatory hierarchy. Other members of this class include the genes for hook-associated proteins as well as motility and chemotaxis genes. Regulatory genes among this class may include the *fliD* gene, which encodes hook-associated protein 2. When the *fliD* gene was insertionally inactivated, increased expression of the *fliC* flagellin gene occurred (16). The *fliD* gene is now known to be the first gene in an operon which includes flagellar genes *fliS* and *fliT*, which may be regulatory genes (12).

Recently we have reported (4) the discovery of a new regulatory gene, the *flgM* gene, which is responsible for negative regulation of flagellin gene transcription when one or more class 2 gene products are missing or defective. Preliminary characterization (3) showed that transcription from the *flgM* promoter was dependent on the presence of a functional copy of the gene for the class 3 sigma factor, σ^{28}, encoded by the *fliA* gene. This makes the *flgM* gene a class 3 gene. Subsequently, it was suggested (22) that the FlgM protein mediates its negative regulation of flagellin genes (and presumably of all class 3 operons) by interacting with and inactivating σ^{28}.

To further understand the role played by the *flgM* gene product in recognizing the presence of class 2 flagellar mutations and regulating flagellin transcription, we sought to determine how various flagellar mutations affect *flgM* gene expression. In this paper we report that *flgM* gene transcription is negatively affected by the presence of both a class 2 flagellar mutation and a wild-type copy of the *flgM* gene. Although a deletion of the *fliA* gene caused a reduction of *flgM* gene expression, this level was well above that seen for a strain with the master flagellar regulatory genes, *flhC* and *flhD*, deleted. We determined that the residual expression of the *flgM* gene in a Δ*fliA* strain was caused by read-through transcription from the adjacent *flgA* gene. These results suggest that the *flgM* gene is expressed from two promoters and may be classified as a member of both the class 2 and class 3 flagellar genes.

MATERIALS AND METHODS

Media and strains. The strains used in this study are shown in Table 1. Media and growth conditions were as described previously (4). Phage P22 *HT105/1 int-201* (24) was used for most transductions in *S. typhimurium*. P22 *int-201* was used for transduction of the MudP22-Q and MudP22-P elements.

Transposon insertions. Transcriptional and translational *lacZ* fusions to the *flgM* gene were obtained by localized

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TABLE 1. Strains

Strain	Genotype	Source or reference[a]
LT2	Wild type	J. Roth
KK2503	*fliC*::Tn*10*	SGSC
MY0645	*flhC*(Ts) (chemotactic at 30°C, nonchemotactic at 42°C)	R. Macnab
PY13518	F′114 (Ts) Lac$^+$ *zzf-20*::Tn*10 zzf-3551*::MudP/*leuA414 hsdSB* Fels	34
PY13757	F′114 (Ts) Lac$^+$ *zzf-20*::Tn*10 zzf-3553*::MudQ/*leuA414 hsdSB* Fels	34
SJW1511	*fla-1511*(ΔP_{flgAB}) vh2$^-$ *fljB*$^{c,n,x,\ off}$	S. Yamaguchi
SJW1529	Δ*flgA1529*vh2$^-$ *fljB*$^{c,n,x,\ off}$	S. Yamaguchi
TE1335	F′128 Pro$^+$ Lac$^+$ (P22 *HT105/1 int-201 sieA44*)/*trp* Δ*lacZ4* Str$^-$ Su$^-$	T. Elliott
TH714	*fljB5001*::MudJ	4
TH1380	F′128 *zzf-1066*::MudJ/Δ*proAB47 pyrB64*	
TH1886	*flgM5096*::Tn*10*dCm	4
TH1983	*flgM5096*::Tn*10*dCm *fla-2157*(Δ*flgG-L*) *fljB5001*::MudJ	3
TH1984	*fliA2087 fliC*::Tn*10 flgM5096*::Tn*10*dCm *fla2157* (Δ*flgG-L*) *fljB5001*::MudJ	3
TH2150	*fla-2157*(Δ*flgG-L*) *fljB5001*::MudJ	4
TH2230	*fla-2157*(Δ*flgG-L*)	4
TH2231	*fla-2039*(Δ*tar-flhD*)	4
TH2251	*fliA2087*	4
TH2507	*flgM5207*::MudJ	
TH2508	*flgM5208*::MudK	
TH2509	*DUP1113*[(*flgM5207*)*MudJ*(*purB1879*)]	
TH2510	*DUP1114*[(*flgM5208*)*MudK*(*purB1879*)]	
TH2511	*flgM5209*::Tn*10*dTc	
TH2512	*flgA5210*::Tn*10*dTc	
TH2513	*flgA5210*::Tn*10*dTc *flgM5207*::MudJ	
TH2514	*flgA5210*::Tn*10*dTc *flgM5207*::MudJ Δ*fliA2328*	
TH2515	Δ*flgA1529 flgM5207*::MudJ	
TH2516	*fla-1511* (ΔP_{flgAB}) *flgM5207*::MudJ	
TH2517	Δ*flgA1529 flgM5208*::MudK	
TH2518	*fla-1511* (ΔP_{flgAB}) *flgM5208*::MudK	
TH2519	*fla-2211* (Δ*fliE-K*) *fliC5002*::MudJ vh2$^-$ *fljB*$^{c,n,x,\ off}$	
TH2520	*pyrC691*::Tn*10 flhC*(Ts) vh2$^-$ *fljB*$^{c,n,x,\ off}$	
TH2521	Δ*fliA2328*	
TH2523	*fla-2157* (Δ*flgG-L*) Δ*fliA2328 fliC*::Tn*10 fljB5001*::MudJ	
TH2524	*fla-2157* (Δ*flgG-L*) Δ*fliA2328 fliC*::Tn*10 fljB5001*::MudJ *flgM5096*::Tn*10*dCm	
TH2525	Δ*fliA2328 fliC*::Tn*10 fljB5001*::MudJ	
TH2526	Δ*fliA2328 fliC*::Tn*10 fljB5001*::MudJ *flgM5096*::Tn*10*dCm	
TH2527	*fla-2157* (Δ*flgG-L*) *fliC*::Tn*10 fljB5001*::MudJ	
TH2528	*fla-2157* (Δ*flgG-L*) *fliC*::Tn*10 fljB5001*::MudJ *flgM5096*::Tn*10*dCm	
TH2529	*fliC*::Tn*10 fljB5001*::MudJ	
TH2530	*fliC*::Tn*10 fljB5001*::MudJ *flgM5096*::Tn*10*dCm	
TH2546	Δ*flgA1529 fljB5001*::MudJ	
TH2547	*fla-1511* (ΔP_{flgAB}) *fljB5001*::MudJ	
TH2548	Δ*fliA2328 fljB5001*::MudJ	
TH2549	*fla-2157* (Δ*flgG-L*) *flgA5210*::Tn*10*dTc *fljB5001*::MudJ	
TH2550	*fla-2157* (Δ*flgG-L*) *flgM5207*::MudJ	
TH2551	*DUP1113*{[*fla-2157* (Δ*flgG-L*) *flgM5207*]*MudJ*(*purB1879*)}	
TH2552	*fla-2039* (Δ*tar-flhD*) *flgM5207*::MudJ	
TH2553	*DUP1113* [(*flgM5207*)*MudJ*(*purB1879*)] *fla-2039* (Δ*tar-flhD*)	
TH2554	Δ*fliA2328 flgM5207*::MudJ	
TH2555	*DUP1113* [(*flgM5207*)*MudJ*(*purB1879*)] Δ*fliA2328*	
TH2556	*fla-2157* (Δ*flgG-L*) *flgM5208*::MudK	
TH2557	*DUP1114* {[*fla-2157* (Δ*flgG-L*) *flgM5208*]*MudK*(*purB1879*)}	
TH2558	*fla-2039* (Δ*tar-flhD*) *flgM5208*::MudK	
TH2559	*DUP1114* [(*flgM5208*)*MudK*(*purB1879*)] *fla-2039* (Δ*tar-flhD*)	
TH2560	Δ*fliA2328 flgM5208*::MudK	
TH2561	*DUP1114* [(*flgM5208*)*MudK*(*purB1879*)] Δ*fliA2328*	
TH2562	Δ*flgA1529 flgM5207*::MudJ Δ*fliA2328*	
TH2563	*fla-1511* (ΔP_{flgAB}) *flgM5207*::MudJ Δ*fliA2328*	
TH2564	Δ*flgA1529 flgM5208*::MudK Δ*fliA2328*	
TH2565	*fla-1511* (ΔP_{flgAB}) *flgM5208*::MudK Δ*fliA2328*	
TH2570	*flgC5215*::MudJ	
TT459	*pyrC691*::Tn*10*	J. Roth
TT8046	F′128 *zzf-1066*::MudA/Δ*proAB47 pyrB64*	J. Roth
TT9703	F′152 *zzf-1093*::MudB/*nadA56*	J. Roth
TT10213	*purB1879*::MudA (Lac$^+$)	J. Roth
TT10217	*purB1883*::MudA (Lac$^-$)	J. Roth
TT10288	*hisD9953*::MudJ *his9944*::MudI	11
TT10381	*hsiD1284*::MudK *hisA9944*::MudI	11
TT10423	F′ Pro$^+$ Lac$^+$ *zzf-1831*::Tn*10*dTc/Δ*proAB47*	J. Roth
TT11201	*pncX242*::MudJ	J. Roth
W1689	Δ*fliA2328* vh2$^-$ *fljB*$^{c,n,x,\ off}$	S. Yamaguchi

[a] Unless indicated otherwise, all strains were constructed during the course of this work. SGSC, *Salmonella* Genetic Stock Center.

mutagenesis with the defective MudJ and MudK transposons as described previously (11). P22 phage stocks grown on strains TT10288 and TT10381 were used to transduce LT2 to Km^r on minimal medium. The transductants were pooled, and P22 was grown on the pool. The resulting lysate was then used to transduce recipient strain TH2520 [*pyrC*::Tn*10* *flhC*(Ts)] to $pyrC^+$ by selecting for growth on minimal medium. Because the *pyrC* gene is located near the *flgM* flagellar gene, it was expected that this selection would bring in the linked *flgM*::Mud alleles by cotransduction. Transductant colonies were replica printed to minimal medium containing X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and kanamycin and incubated at 30 and 42°C.

Since expression of flagellar genes is known to be dependent upon the *flhCD* regulatory genes, it was expected that expression of *lacZ* from Mud insertions in flagellar genes would show temperature dependence in a *flhC*(Ts) mutant strain. Km^r colonies which were Lac^+ at 30°C but Lac^- at 42°C were retained as putative *flgM*::Mud insert strains. Some insert-containing strains were P22 phage resistant; the Mud markers were rescued from these strains by mating them with *E. coli* TE1335, which carries P22 on the F plasmid, and preparing a phage stock from the mixed culture. The phage stock was then used to transduce TH1886 (*flgM*::Tn*10*dCm) to Km^r. Mud inserts which showed 98 to 100% linkage to the *flgM5096*::Tn*10*dCm allele were sequenced to determine the location of the insert.

Tn*10*dTc (32) transposon insertions in the *flgM* gene were obtained by random mutagenesis of the *S. typhimurium* chromosome. Phage grown on strain TT10423 (F::Tn*10*dTc) was used to transduce TH2519 (Δ*fliE-K fliC*::MudJ) carrying Tn*10* transposase-producing plasmid pNK972 (32) to Tc^r on triphenyltetrazolium chloride (TTC)-lactose. Colonies which appeared to have a $FlgM^-$ phenotype (the ability to express *fliC* [Lac^+] despite flagellar defects) were saved as putative *flgM*::Tn*10*dTc insert strains. These inserts were checked for linkage to a *flgM*::Tn*10*dCm allele.

Sequencing. Strains carrying MudJ or MudK insertions were converted into strains containing MudP22-P or MudP22-Q insertions by using P22 *int-201* phage grown on PY13757 or PY13518 (34). DNA adjacent to the insertions was isolated by inducing the defective P22 contained within the MudP22 element with mitomycin (2 μg/ml; Sigma Chemical Co.) and extracting DNA from the resultant phage particles (34). Single-stranded DNA was prepared by using T7 gene 6 exonuclease (United States Biochemicals) (6), and the Mu-specific primers MuL (MudP) and MuR (MudQ) (34) were used to sequence from the point of insertion. Dideoxy sequencing (25) with commercial kits (Sequenase; United States Biochemicals) was performed according to the manufacturer's instructions. To sequence from the Tn*10*dTc inserts described above, strains which contained both the Tn*10*dTc transposon insertion and a *flgC*::MudP22-Q insertion were constructed. DNA obtained by inducing the MudP22 element in the *flgC* gene as described above was digested with *Xba*I, which cuts once in the Mud element and once in Tn*10*dTc. Following T7 gene 6 exonuclease treatment, the DNA sequence adjacent to the Tn*10*dTc element was obtained by using a primer specific for the Tn*10* element (5′ CCAAAATCATTAGGGGATTC [7]).

Plasmid construction. Plasmid pKG16 ($flgA^+$ Δ*flgM*) was constructed by digestion of the 6.5-kb plasmid pKG10 ($flgA^+$ $flgM^+$) (3) with *Pst*I and *Eco*NI. The 1.5-kb $flgM^+$ fragment was separated from the 5-kb $flgA^+$ fragment by gel electrophoresis, and the DNA was purified from the agarose by using GeneClean II (Bio 101). The 5-kb fragment containing the vector and the $flgA^+$ gene was treated with T4 DNA polymerase (Pharmacia) and deoxynucleoside triphosphates (Pharmacia) and religated by using T4 DNA ligase (New England BioLabs). Calcium chloride-competent *E. coli* DH5α cells were transformed (23) with the ligation mixture, and plasmid-containing cells were selected by plating on solid medium containing ampicillin. Potential clones were screened by restriction analysis.

Northern (RNA) blot. Total RNA was isolated from cells lysed in a sucrose–Triton X-100–lysozyme buffer by using guanidine thiocyanate followed by ultracentrifugation (23). The RNA-containing pellet was resuspended in RNase-free H_2O (sterile water for irrigation; Baxter Healthcare Corp.), quantitated by spectrophotometric methods, and stored in aliquots at −80°C.

Samples containing 10 μg of RNA in a salt buffer containing 50% formamide were electrophoresed through a 1% formaldehyde gel (17). The RNA was transferred to a GeneScreen-Plus membrane (New England Nuclear) according to the manufacturer's instructions. Hybridization and wash steps were carried out at 55°C in sodium phosphate-sodium dodecyl sulfate buffers (1).

A probe for the *flgM* transcript was made by digesting plasmid pKG12 (3) with *Eco*NI and *Ase*I to obtain a 197-bp internal portion of the *flgM* gene. Probes for the *flgA* and *flgN* transcripts were obtained similarly by *Sma*I-*Pvu*II digestion of pKG16 and *Bsm*I digestion of pKG10 to obtain 350- and 271-bp fragments, respectively. The DNA fragments were gel purified (23) and labeled by random priming (2) with [α-^{32}P]dATP (New England Nuclear), and unincorporated nucleotides were separated from labeled product by using a Sephadex G-50 spin column (23).

Primer extension and RNA slot blot. The *flgM* gene is located within a 6.5-kb chromosomal insert contained in the plasmid pMH71 (9). We previously isolated a derivative of this plasmid (3) in which the *flgM* gene was inactivated by Tn*1000*. Primer GD2 (28) is complementary to the Tn*1000* insert in the *flgM* gene and was radiolabeled by using T4 polynucleotide kinase (United States Biochemical) and [γ-^{32}P]ATP (New England Nuclear).

RNA was isolated from strains carrying plasmid pMH71::Tn*1000* as described above. Primer extension reactions were carried out with 50 μg of RNA, 100 ng of GD2, Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories), 5× buffer supplied by the manufacturer, and deoxynucleoside triphosphates for 30 min at 42°C. The reaction mixtures were loaded onto a polyacrylamide gel next to a sequencing ladder of pMH71::Tn*1000*, using GD2.

RNA prepared for the slot blot was isolated from strains carrying the mutations Δ*fliA*, Δ*flgG-L*, and *flgM*::Tn*10*dCm, in all combinations. In addition, these strains carried a *flgM*::Tn*1000*-inactivated allele on a plasmid which served as the target for a radiolabeled primer. The RNA was diluted to 20, 10, and 5 μg/ml, and aliquots of 300 μl from each dilution were transferred to a nitrocellulose membrane by using a Schleicher & Schuell Minifold II slot blotting apparatus according to the manufacturer's instructions. The labeled GD2 primer was used for probing for *flgM*-specific RNA in SSPE (0.18 M NaCl, 10 mM $NaPO_4$, 1 mM EDTA, pH 7.7)-Denhardt's solution at 37°C (23). The quantitative data shown were derived from analysis obtained with the help of the PhosphorImager laboratory at the Fred Hutchinson Cancer Research Center.

Merodiploid construction. Strains carrying a chromosomal duplication of the *flgM* gene were constructed by the method of Hughes and Roth (10) as follows. Phage P22 grown on TT8046 (F::MudA) or TT9703 (F::MudB) was used to convert

flgM::MudJ and *flgM*::MudK alleles to *flgM*::MudA and *flgM*::MudB alleles, respectively, by selection for Ap^r and screening for Km^s. Phage grown on the *flgM*::MudA strains was added to phage grown on strain TT10213 (*purB*::MudA [Lac^+]) or TT10217 (*purB*::MudA [Lac^-]). The phage mixture was used to transduce wild-type strain LT2 to ampicillin resistance. The MudA and MudB transposons are too large to be inherited by homologous recombination with a single transduced fragment. Inheritance requires recombination between two transduced fragments, each carrying part of the Mud element, that simultaneously infect the recipient cells. When P22 lysates grown on a *purB*::MudA and a *flgM*::MudA strain are mixed and used to transduce a recipient to MudA-encoded ampicillin resistance, both the parental *flgM*::MudA and *purB*::MudA recombinant types as well as hybrid recombinants between the different donors can be inherited. If the MudA inserts in the *flgM* and *purB* genes are in the same orientation on the chromosome, then inheritance of the hybrid recombinants by homologous recombination results in either a duplication or a deletion of the region of the chromosome between the Mud elements.

Cells receiving a *purB*::MudA fusion grew poorly because of the nature of the mutation. Hence, the larger colonies, which had either the *flgM*::MudA allele or the *flgM-purB* duplication MudA allele, were screened for the duplication phenotype. Cells carrying a duplication will at some frequency lose the Mud cassette (and lose ampicillin resistance) by homologous recombination in the absence of selection for ampicillin resistance. The duplication (*flgM-purB*) *flgM*::MudB alleles were obtained in the same way, using phage grown on strains carrying the *flgM*::MudB and *purB*::MudA alleles. Since the use of the Lac^+ *purB*::MudA allele but not the Lac^- allele resulted in a *flgM-purB* duplication, it can be determined that the *purB* gene is transcribed in the same direction as the *flgM* gene and has a counterclockwise orientation in the chromosome.

β-Galactosidase assay. Strains were assayed for β-galactosidase production by a standard protocol (19). Numbers given are averages of two assays done in triplicate, with high and low numbers discarded.

RESULTS

The *flgM* gene is dependent on the *fliA* gene for expression. We have shown that transcription of the *flgM* gene is dependent upon the FliA alternative sigma factor, because primer extension-mediated mapping of the transcriptional start site was greatly reduced in a strain carrying a point mutation in the *fliA* gene (3). It was not clear whether the transcription product observed was due to the leakiness of the *fliA* point mutant strain used or instead was the result of transcription due to the promoter being recognized by RNA polymerase carrying another alternative sigma factor. We repeated our experiments to determine if the *flgM* promoter is used in the absence of the *fliA* gene. The primer extensions were performed with RNA isolated from wild-type, *fliA* point mutant, and *fliA*-deleted strains. These results are shown in Fig. 1. As reported previously, primer extension using RNA isolated from the *fliA* point mutant showed some primer extension product (lane 1), but much less than that from a wild-type strain (lane 3). However, when RNA isolated from a strain which was completely missing the *fliA* gene was assayed (lane 2), the primer extension product which maps to the *flgM* promoter was completely lacking. Hence, no transcription from the *flgM* promoter occurs in the complete absence of the *fliA* gene; it is likely that the nature of the *fliA* point mutant is responsible for the observed transcription of the *flgM* gene in that background.

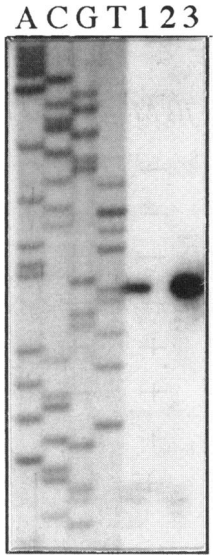

FIG. 1. Transcriptional mapping of the *flgM* gene by primer extension. Lanes A, C, G, and T, sequencing ladder. Primer extensions are shown in lanes 1 to 3. Lane 1, TH1984 (*fliA*) carrying pMH71 *flgM*::Tn*1000*; lane 2, TH2524 (Δ*fliA*) carrying pMH71::Tn*1000*; lane 3, TH2528 (*fliA*$^+$) carrying pMH71::Tn*1000*.

Multiple factors, including FlgM, contribute to *flgM* gene regulation. Because of the dependence of the *flgM* promoter on the *fliA* gene for expression, the *flgM* gene has been grouped with the flagellin genes among the class 3 flagellar genes. Since the expression of the class 3 flagellin genes is linked to class 2 gene expression, it seemed possible that the same would be true for expression of the *flgM* gene. We used two methods to determine the expression of the *flgM* gene in various flagellar mutant backgrounds. First, RNA isolated from flagellar mutant strains was probed for *flgM*-specific transcription. Second, strains carrying *lacZ* transcriptional and translational fusions to the *flgM* gene were constructed and assayed for β-galactosidase activity.

Several observations can be made from the transcription results shown in Fig. 2A and B. First, the *flgM* gene product was autoregulatory: when a wild-type copy of the gene is present, transcription is decreased (compare either Fig. 2A or B, rows 1 and 5 and rows 2 and 6). This is especially true in cells carrying the *flgM*$^+$ gene and a class 2 flagellar mutation (Fig. 2B; compare rows 6 and 2), in which transcription is decreased at least threefold. This autoregulation is expected for a protein whose function is the inactivation of the σ^{28} (FliA) sigma factor (22).

Second, as expected for a class 3 gene in the absence of the *flgM* gene, transcription of the *flgM* gene is high in strains with both class 2 genes and the *flgM* gene deleted (Fig. 2A and B, rows 2). However, the *flgM* gene was substantially transcribed in a class 2 flagellar mutant despite the presence of a wild-type copy of the *flgM* gene (Fig. 2A and B, rows 6). Further, in the absence of the *flgM* gene, transcription in a class 2 flagellar mutant strain is consistently, although not substantially, increased over that in a wild-type strain (Fig. 2A and B; compare rows 2 and 1). These results differ from the known regulation of class 3 flagellin gene expression (16), which is greatly decreased in the presence of class 2 flagellar mutations in a

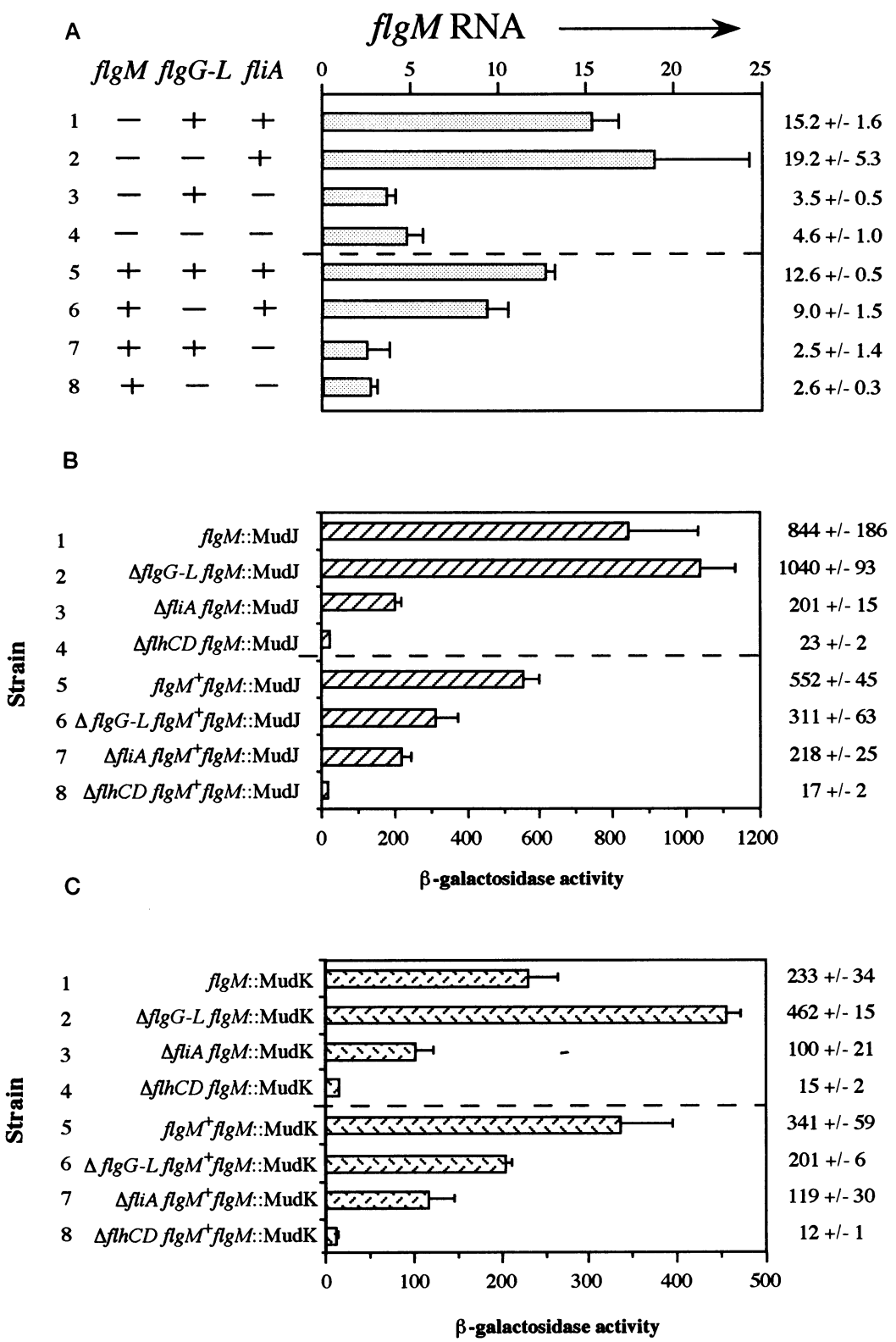

FIG. 2. Levels of *flgM* transcription and translation. (A) *flgM*-specific RNA detected by slot blot analysis. (B and C) β-Galactosidase activities of transcriptional and translational fusions, respectively, to the *flgM* gene in various mutant backgrounds. Genotypes of the strains assayed for β-galactosidase activity of the *flgM*::Mud fusions are shown on the left. Values (in Miller units) for the data are shown on the right. The *flgM*$^+$ allele in panels B and C originates from a tandem chromosomal duplication between the *flgM* and *purB* genes.

flgM$^+$ background, and indicate that the expression of the *flgM* gene is distinct from that of the flagellin genes.

Third, a strain with the *fliA* gene deleted has three- to fivefold-decreased *flgM* gene transcription (Fig. 2A, rows 3, 4, 7, and 8, and Fig. 2B, rows 3 and 7). However, the level of *flgM* transcription in a Δ*fliA* strain remains 10-fold higher than it is in a strain with the *flhCD* master regulatory genes deleted (Fig. 2B, rows 4 and 8). Only a basal level of transcription is observed in *flhCD* mutant strains. The level of *flgM* transcription in a *fliA* mutant strain observed in these experiments was not expected, since our primer extension results showed that the *fliA* gene was necessary for transcription from the *flgM* promoter. These data suggest that transcription in a Δ*fliA* strain originated elsewhere and will be further discussed in the next section.

Similar results were obtained for a translational *lacZ* fusion to the *flgM* gene (Fig. 2C). However, the presence of a class 2 flagellar mutation causes more than a slight increase in *flgM* gene expression. Expression increased about twofold in the absence of the *flgM* gene. However, similar to the case with the *flgM* transcriptional fusion, expression of the *flgM* gene in a flagellar mutant background is negatively regulated by the presence of a wild-type copy of the *flgM* gene.

Expression of the *flgM* gene in the absence of *fliA* is dependent on the promoter for the upstream *flgA* gene. Results from β-galactosidase assays and RNA slot blot analysis showed that transcription of the *flgM* gene occurred in the absence of the *fliA* gene; primer extension results preclude the possibility that transcription is occurring from the *flgM* promoter itself in the absence of the *fliA* gene. Hence, transcription of the *flgM* gene in the absence of the *fliA* gene must originate at a site other than the *flgM* promoter region. Several lines of evidence demonstrating the role of the upstream *flgA* gene in the transcription of the *flgM* gene will be described in this section. First, an insertion mutagenesis experiment resulted in the isolation of *flgA* transposon insertions which gave a FlgM phenotype for flagellin expression. Second, polar mutations in the *flgA* gene caused reduced expression of *flgM*::MudJ and *flgM*::MudK insertions as determined by β-galactosidase assays. Third, two *flgM*-specific transcripts were observed in a Northern blot analysis.

In a screen for additional insertions in the *flgM* gene, two classes of Tn*10*dTc inserts which were highly linked (98 to 100%) to the *flgM* gene were obtained. Both classes caused a FlgM mutant phenotype: they allowed expression of the flagellin-*lac* fusion despite the presence of a class 2 flagellar mutation (Δ*fliE-K*). This phenotype was seen as a white (Lac$^+$) colony color on TTC-lactose plates, although phenotypic differences were observed. Type I mutants retained the FlgM mutant phenotype indefinitely, remaining white (Lac$^+$) on TTC-lactose indicator plates after prolonged incubation. Colonies carrying the type II insertions gradually turned red (Lac$^-$) on indicator plates. It appeared as if there was a reduced level of *flgM* expression in the type II mutants which gradually built up to a level which could inhibit flagellin (and thus *lac*) transcription.

Wild-type cells carrying type II inserts were nonmotile or greatly reduced in motility when assayed on motility plates, whereas cells carrying the type I inserts were motile. Motility of the type II strains could be restored by complementation in *trans* with plasmid pKG16, which carries the *flgABCD* genes (but not the *flgM* gene). However, the regulatory defect in type II mutants was not restored by plasmid pKG16. The pKG16 plasmid did not complement the FlgM mutant phenotype: flagellar mutant (Δ*fliE-K*) strains carrying a flagellin-*lac* fusion and the type I or type II inserts continued to express flagellin despite the presence of the pKG16 plasmid. Both type I and type II mutants were complemented for their FlgM phenotype (i.e., they became Lac$^-$) by a plasmid carrying the *flgM* gene; however, the type II insertions were not complemented for motility. These complementation results suggest that the type II Tn*10*dTc insert strains have a defect in motility which is distinct from their defect in regulation of flagellin synthesis. Two of these inserts were sequenced; a type I insertion was located just upstream of the *flgM* gene, and a type II insertion was located in the *flgA* gene.

To determine the effects of mutations in the *flgA* gene on *flgM* gene expression, β-galactosidase assays of *flgM*::MudJ or *flgM*::MudK fusions were employed. In addition to the *flgA*::Tn*10*dTc allele described above, two strains with the *flgA* gene deleted were also used: one had the *flgAB* promoters deleted (ΔP_{flgA}), and the other was missing an internal portion of the *flgA* gene (Δ*flgA*). The effects of the different *flgA* alleles on *flgM* expression are shown in Fig. 3. Transcription of a *flgM*::Mud (-J or -K) fusion was increased by the presence of the internal *flgA* mutation (Fig. 3A or B, compare rows 2 and 1). These assays were carried out in the absence of a wild-type copy of the *flgM* gene and are similar to the Δ*flgG-L* deletion results shown in Fig. 2B, and C, rows 2. A strain with the *flgA* promoter deleted had decreased expression compared with that in the internal *flgA* deletion strain (Fig. 3A and B, rows 2 and 3). A strain carrying a transposon insertion in the *flgA* gene had an approximately 1.5-fold decrease in expression of a *flgM*::MudJ fusion compared with that in the wild type (Fig. 3A, rows 1 and 6). However, expression of the *flgM* gene is eliminated in a strain carrying both a mutation disrupting transcription of the *flgA* gene (ΔP_{flgA} or *flgA*::Tn*10*dTc) and a deletion in the *fliA* gene (Fig. 3A, rows 5 and 7, and Fig. 3B, row 5). A strain carrying the internal *flgA* mutation and a Δ*fliA* mutation shows essentially the same level of expression as the strain with Δ*fliA* alone (Fig. 3A, row 4, and Fig. 2B, row 3). Unlike the *flgA* transposon insertion and promoter deletion mutations, the internal *flgA* mutation does not affect *flgM* transcription and is apparently not polar on the *flgM* gene.

To confirm the role of the *flgA* promoter in *flgM* expression, transcripts through the *flgM* gene were examined. The results are shown in Fig. 4A and B. Two transcripts, approximately 1.6 kb (labeled I) and 0.8 kb (labeled II), were observed when RNA isolated from LT2 was hybridized with a probe internal to the *flgM* gene (Fig. 4A). The *flgM* transcripts from the chromosomal gene were present at very low levels. To amplify *flgM* transcripts, RNA was isolated from strains carrying either plasmid pMS531 or plasmid pMH71. Plasmid pMS531 (27) carries the *fliA* gene, encoding σ^{28}, which promotes transcription from the *flgM* promoter. Plasmid pMH71 (9) contains both the *flgA* and *flgM* genes. When RNA isolated from wild-type strain LT2 carrying the pMS531 plasmid was probed for *flgM* transcription (Fig. 4B, lane 1), the intensity of the smaller band (II) was increased. Only the smaller band can be seen in a strain missing the *flgA* promoter (Fig. 4B, lane 2). This strain (ΔP_{flgA}) is also carrying the pMS531 plasmid, so it is not clear why there is such a low level of transcription from the *flgM* promoter. When the RNA was probed with the *flgA* gene (Fig. 4C), transcript I was clearly seen in the LT2/pMS531 lane (Fig. 4C, lane 1). Although there appears to be some transcript I in the pMS531/ΔP_{flgA} lane (Fig. 4C, lane 2), this level was similar to the background level due to hybridization of the probe to rRNA. Transcript II can not be seen in any of the lanes in Fig. 4C. These data suggest that transcript II results from transcription from the *flgM* promoter (recognized by σ^{28} [FliA]) and that transcript I results from transcription from the *flgA* promoter. In all lanes, an intermediate-sized

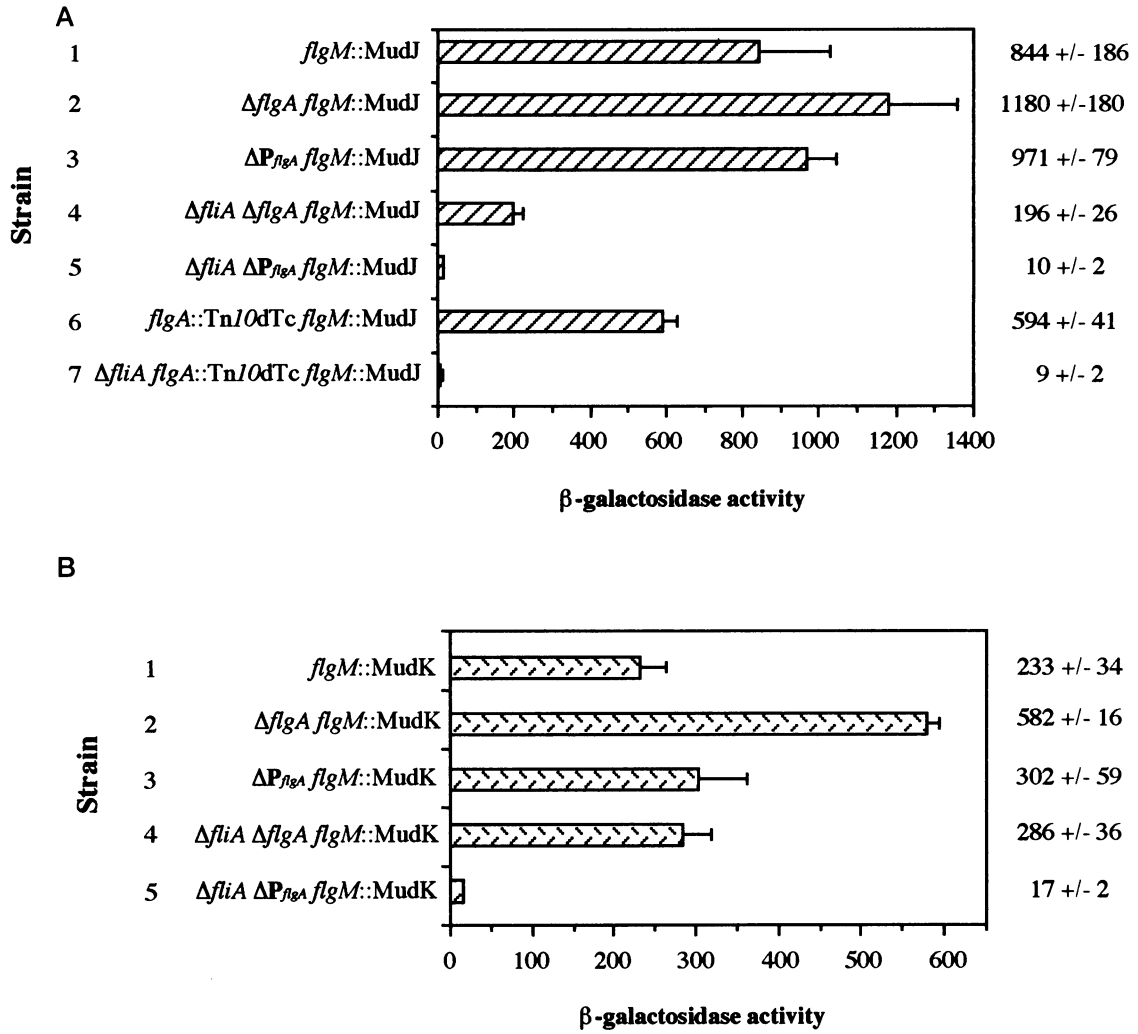

FIG. 3. Transcription and translation of the *flgM* gene in *flgA* mutant strains. Genotypes of the strains assayed for β-galactosidase activity of the *flgM*::Mud fusions are shown on the left. Values (in Miller units) for the data are shown on the right. A deletion of the *flgA* gene is shown as Δ*flgA*, while a deletion of the promoter for the *flgA* gene is shown as ΔP_{flgA}. There is no wild-type copy of the *flgM* gene in these assays.

band is seen; it may be due to a nonspecific binding of the probe, as it is recognized by both probes but does not disappear when the *flgA* promoter is deleted. Finally, RNA isolated from a strain carrying the pMH71 plasmid ($flgA^+M^+$) consists predominantly of transcript I (Fig. 4B, lane 3, and Fig. 4C, lane 3); this is probably due to autoregulation of the *flgM* promoter. These results are consistent with the hypothesis that transcription from the *flgA* promoter proceeds through the *flgM* gene and that the *flgM* gene is transcribed both from its own, σ^{28} (FliA)-dependent promoter and from the upstream *flgA* promoter.

The amount of flagellin transcription is dependent on *flgM* gene expression. The effects of the *flgA*::Tn*10*dTc insertion and the *flgA* deletions on flagellin transcription were assayed in a *fljB*::MudJ strain. The results shown in Fig. 5 show that the insertion and promoter-deletion *flgA* mutations, which affect *flgM* transcription, also allow wild-type levels of flagellin to be expressed. The *flgA* internal deletion (row 3) resulted in a shutoff of flagellin transcription, similar to the case with another class 2 flagellar mutation (Δ*flgG-L*, row 2). Both mutations have the same effect that a *fliA* mutation does (row

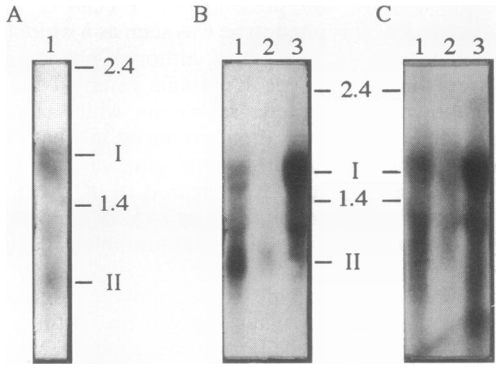

FIG. 4. Northern blot analysis of *flgM* and *flgA* gene transcription. (A) RNA from wild-type LT2 probed with the *flgM* gene. (B and C) RNA probed with the *flgM* and *flgA* genes, respectively. Lanes: 1, wild-type strain LT2 carrying $fliA^+$ plasmid pMS531; 2, strain SJW1511 (ΔP_{flgA}) carrying $fliA^+$ plasmid pMS531; 3, TH1983 carrying $flgM^+$ $flgA^+$ plasmid pMH71. Transcripts are indicated by I and II. Locations of the molecular size markers (1.4 and 2.4 kb) are shown.

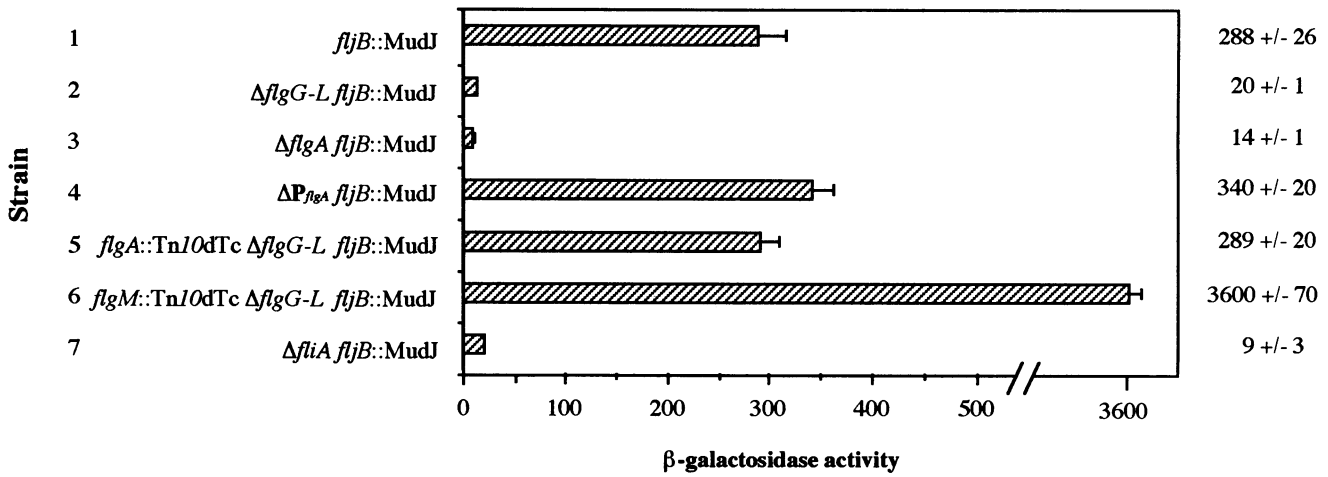

FIG. 5. Transcription of the flagellin *fljB* gene. Genotypes of the strains assayed for β-galactosidase activity of the *fljB*::MudJ fusion are shown on the left. Values (in Miller units) for the data are shown on the right.

7). However, the *flgA* promoter deletion allowed significant flagellin transcription (row 4), as did the *flgA*::Tn*10*dTc insertion (row 5). These two mutations behaved much like a weaker version of the *flgM*::Tn*10*dTc insertion (row 6); taken together with the above results, this suggests that these *flgA* mutations reduce the transcription of the *flgM* gene and that the apparent regulatory effect of a *flgA* mutation (14) may be due to its effect on *flgM* gene transcription. The internal *flgA* deletion mutation causes a loss of flagellin expression, as expected of a class 2 flagellar mutation in the presence of a functional *flgM* gene. In strains carrying a *flgM*::Tn*10*dTc allele in a flagellar mutant background, expression of flagellin is increased to at least three times the wild-type level. These data provide further evidence that the *flgM* gene product is the negative regulator of flagellin transcription and that the *flgA* gene does play a role in flagellin transcription by promoting transcription of the *flgM* gene.

DISCUSSION

It is known that flagellin transcription is repressed in strains carrying a mutation in any one of a number of flagellar genes (13, 16, 29). In an earlier mutant screen for a negative regulator of flagellin expression (4), an insertion in the *flgM* gene, as well as a number of insertions not linked to known flagellar genes, was obtained. The insertion in the *flgM* gene relieved repression of flagellin expression in all class 2 flagellar mutant backgrounds studied except a strain carrying a mutation in the gene for the class 3 sigma factor, *fliA*; thus, it was determined that the *flgM* gene product was a negative regulator of flagellin expression. In an effort to understand the role of the *flgM* gene product in flagellin regulation, we characterized the regulation of the *flgM* gene in several flagellar mutant backgrounds.

Complementary experiments assaying levels of *flgM* gene transcription by RNA slot blot analysis and β-galactosidase activity of *lacZ* fusions to the *flgM* gene resulted in three observations: (i) the *flgM* gene was autoregulated, especially in the presence of a class 2 flagellar mutation; (ii) transcription of the *flgM* gene was responsive to the presence of class 2 flagellar mutations (i.e., it decreased when a wild-type copy of the *flgM* gene was present and increased in the absence of the *flgM* gene); and (iii) the *flgM* gene was transcribed in the absence of the gene for alternative sigma factor σ^{28} (the *fliA* gene) at a level well above (10 times) that in a strain with the *flhCD* master regulatory genes deleted. Promoter mapping by primer extension showed that the *flgM* gene was not transcribed from its own promoter in the absence of the *fliA* gene. Hence, *flgM* gene transcription in the absence of the *fliA* gene must originate elsewhere.

Several lines of evidence suggested that transcription of the *flgM* gene in the absence of the *fliA* gene was dependent upon transcription through the upstream *flgA* gene. In a search for additional *flgM* insertion mutations, two classes of *flgM*-linked Tn*10*dTc insertion mutations were obtained. Type I mutations consisted of insertions within the *flgM* gene, as expected. The second type of insertion mutation appeared to cause a FlgM mutant phenotype by decreasing the levels of *flgM* transcription but not eliminating FlgM function entirely. One member of the type II insertions was sequenced and was located within the *flgA* gene. Analysis of transcription of the *flgM* gene in *flgA* and *fliA* mutant strains supported the hypothesis that the *flgM* gene is transcribed both from its own promoter and from the upstream *flgA* promoter; transcription of the *flgM-lacZ* fusion in a *fliA* mutant background was eliminated when the *flgA* promoter was deleted.

Northern blot analysis confirmed the presence of two transcripts through the *flgM* gene, one originating from the *flgM* promoter and the other from read-through transcription of *flgA* gene. This analysis also showed that the *flgM* gene on a multicopy plasmid was transcribed predominantly from the *flgA* promoter. Since we have shown that the FlgM protein negatively regulates its own transcription (presumably by inhibiting transcription from the *flgM* promoter by inactivating σ^{28}), it is likely that the high levels of the FlgM protein present in these cells greatly reduce transcription from this promoter.

Sequence analysis (5) showed that the *flgA* promoter (16) is oriented in the direction opposite to that of the *flgB* promoter (16) on the chromosome (Fig. 6). The two transcripts through the *flgM* gene were approximately 1.6 and 0.8 kb in length. Since the size of the *flgM* gene from the start of transcription to the stop of translation is only 348 bp, it appears likely that the transcription must proceed downstream for another 450 bases through an open reading frame of 420 bp which we have called *flgN* (3, 22).

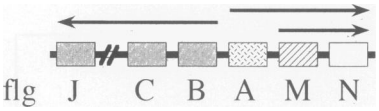

FIG. 6. Locations and orientations of the *flgM* and *flgA* genes on the chromosome. Genes *flgD*, *flgC*, *flgB*, *flgA*, and *flgM* and putative gene *flgN* are indicated by boxes. Directions of transcription are indicated by arrows.

For *E. coli*, Komeda and Suzuki (14, 31) had previously found that four of five *flgA* mutations allowed expression of flagellin in a flagellar (*fla*) *flgA* double mutant background. From these data it was proposed that the *flgA* gene encoded a bifunctional protein which was required for both flagellar assembly and repression of flagellin transcription in a *fla* mutant strain (14). Our results suggest that in *S. typhimurium* the FlgA protein is not bifunctional; rather, this protein has an exclusively structural role. The *flgA* gene, however, does participate in flagellin regulation, in that its promoter is partially responsible for the transcription of the downstream regulatory gene *flgM*.

The findings in this paper may shed some light on the role of *flgM* in a wild-type cell. The levels of flagellin in a *flgM* mutant are greatly increased compared with the wild type. When a plasmid carrying the *fliA* gene is placed in a *flgM* flagellin-*lac* strain, the cells form small colonies, presumably because of high levels of class 3 proteins and/or β-galactosidase (5). The motility of wild-type cells carrying a plasmid encoding the *flgM* gene is greatly reduced (5). One possible role of the *flgM* gene in the absence of flagellar mutations, then, may be in controlling the amount of flagellin produced in the cell.

These experiments show that the *flgM* gene is a member of both the class 2 and class 3 flagellar genes. This makes the *flgM* gene distinct from other class 3 genes, such as the flagellin genes, and in fact accounts for differences between *flgM* regulation and flagellin gene regulation: the flagellin genes are not transcribed in either a class 2 flagellar mutant background or a *fliA* mutant background, while the *flgM* gene is transcribed in both backgrounds, although at a lower level. It is likely that much of the *flgM* expression in a flagellar mutant background is due to transcription from the *flgA* promoter. The *flgM* gene is not the only class 3 gene which can be transcribed in a *fliA* mutant background. The hook-associated proteins, encoded by class 3 genes, have been purified from a *fliA* mutant strain (8).

The known activities of the *flgM* gene product suggest several possible rationales for its transcription from two promoters with distinct regulatory patterns. The FlgM protein interacts with and inactivates the FliA alternative sigma factor (22). The second promoter may be important in temporal regulation, so that the FlgM protein can be made concurrently with σ^{28} and thus can immediately be available to prevent transcription of the flagellin genes under appropriate conditions. Upon initial induction of the flagellar pathway, it may be important to keep the late genes repressed until hook-basal body structures are completed and substrates for flagellin polymerization are available. Another role may be in maintaining a balance between FlgM and FliA proteins in the cell. The levels of the two proteins are important for regulation of flagellin expression: negative regulation of flagellin transcription in a class 2 flagellar mutant background may be overcome either by inactivating the *flgM* gene (Fig. 4) or by overexpressing the *fliA* gene (5). In addition, expression of a flagellin-*lac* fusion in a wild-type cell is decreased when the *flgM* gene is overexpressed (5). Differential regulation of the *flgA* and *flgM* promoters in response to flagellar mutations or levels of flagellin synthesis would be a third possible reason for the existence of the second promoter. These possibilities await further study.

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