The FlhD/FlhC Complex, a Transcriptional Activator of the *Escherichia coli* Flagellar Class II Operons

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The *Escherichia coli* *flhD* operon encodes two genes, *flhD* and *flhC*. Both gene products were overproduced and purified. The purified proteins formed a complex consisting of two FlhD and two FlhC molecules. Mobility shift assays showed that the FlhD/FlhC complex had a DNA-binding activity and bound to the upstream regions of *flaA*, *flhB*, and *flaL* operons (class II), which are under direct control of the *flhD* operon. DNase I footprinting analyses of FlhD/FlhC binding to the three class II promoter regions revealed protection of a 48-bp region of the *flaA* operon between positions –41 to –88, a 50-bp region of the *flhB* operon between positions –28 to –77, and a 48-bp region of the *flaL* operon between positions –29 to –76. In vitro transcription experiments demonstrated that the FlhD/FlhC complex is a transcriptional activator required for the transcription of the three class II operons examined in vitro.

The *Escherichia coli* flagellar regulon consists of at least 14 operons which encode more than 40 genes. Studies of the regulation of the flagellar regulon showed that the genes in the regulon constituted their own transcriptional hierarchy and allowed for coordinate expression (13) (Fig. 1). At the top of the hierarchy is the *flhD* master operon, which is composed of the *flhD* and *flhC* genes. Expression of this operon is required for the expression of all of the remaining operons. Class II consists of operons which are under direct control of the master operon. The genes contained in these operons encode a flagellum-specific sigma factor (σ^{28} or FlhA), which is required for the transcription of class III operons, and many structural components assembled in the early and middle stages of flagellar synthesis as well as some proteins of unknown functions. Class IIIa operons are under the dual control of FlhD/FlhC and FlhA, whereas class IIIb operons are under the control of FlhA (for reviews, see references 18 and 19).

Through genetic techniques, it is known that FlhD and FlhC are the master regulatory proteins of the flagellar transcriptional regulon. However, little is known about the molecular mechanism by which gene expression in the regulon is controlled. It was proposed that FlhD and FlhC may function together as a σ^{28} homolog in *E. coli* (2, 10). This proposal was based on the observation of a *Bacillus subtilis* σ^{28} promoter consensus sequence similarity, found in the upstream region of seven *E. coli* flagellar operons which lacked the promoter consensus sequence recognized by σ^{70} (9), and on the observation that both *flhD* and *flhC* gene products functioned as *trans*-acting positive regulators of the flagellar regulon. Consistent with this idea, an amino acid sequence similarity between σ^{28} of *B. subtilis* and both FlhD and FlhC has been reported (10). Later, a 28-kDa flagellum-specific sigma factor was isolated (2). However, the sigma factor activity was shown to reside in the class II FlhA protein, not in FlhD or FlhC (23).

Further analysis at the DNA sequence level indicated that a derivative of the flagellum-specific consensus sequence TT(A/

T)GCCGATAACG was present in those operons at class II in the transcriptional hierarchy. Because those operons were under direct control of FlhD and FlhC, it was suggested that the 13-base consensus might be the target site recognized by FlhD and FlhC (4). However, deletion analysis showed that the class II consensus sequence was not required for FlhD- and FlhC-regulated expression of the *flhB* operon (5a).

In this report, we demonstrate that the proteins encoded by the *flhD* master operon are DNA-binding proteins. They bind to the upstream regions of the class II operons. We show that FlhD and FlhC form heterotetramers and activate class II operon transcription in vitro. The FlhD protein cannot bind to the promoter regions of the class II operons and cannot initiate transcription from those operons in the absence of FlhC. We propose that FlhD/FlhC complex formation is required for transcriptional regulation in the flagellar regulon.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *E. coli* strains and plasmids used in this study are listed in Table 1.

Plasmids pXL25, pXL26, and pXL27 were constructed as follows. Single-stranded synthetic oligonucleotides (Table 2) were designed to create a 5' *Nde*I site or a 3' *Hind*III site flanking the coding regions and were used for subcloning. The template used for amplification was pMC39, in which the *flhD* operon was previously cloned (12). Pairs of primers used for the PCR synthesis of the *flhD*, *flhC*, and *flhD-flhC* open reading frames were 5'FLHD and 3'FLHD; 5'FLHC and 3'FLHC; and 5'FLHD and 3'FLHC. Vent DNA polymerase (New England Biolabs) was used for primer extension reactions. The PCR products treated with *Nde*I and *Hind*III were inserted into pT7-7 that had been digested with *Nde*I and *Hind*III to generate plasmids designated pXL25, pXL26, and pXL27.

Overproduction and purification of FlhD and FlhC proteins. Overproduction was achieved by using a two-plasmid pT7 system (28). MC1000 *flhD::kan* cells harboring plasmids pXL25, pXL26, or pXL27 and pGp1-2^{cmi} were grown overnight at 30°C in LB medium supplemented with penicillin (100 µg/ml), kanamycin (50 µg/ml), and chloramphenicol (25 µg/ml). The cultures were diluted 100-fold into a medium of the same composition and grown at 30°C. At a cell density of 40

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FIG. 1. Transcriptional hierarchy of operons in the flagellar regulon as proposed by Macnab (18, 19). The order of transcription in the regulon is presented. Activation of the *flhD* operon is under catabolite repression, regulated by a cyclic AMP/catabolite gene activator protein (c-AMP/CAP) complex (27). Arrows with solid or dashed arrows between classes indicate activation or inhibition, respectively.

Klett units, the cultures were shifted to 42°C for 30 min and then shifted back to 30°C. After 2 h, the cells were harvested by centrifugation.

For purification of FlhD and FlhC, the cells were disrupted by sonication in 20 mM Tris (pH 7.9) and then centrifuged at $31,000 \times g$ for 30 min. The supernatant was loaded onto a heparin cartridge of a Bio-Rad Econo system (Bio-Rad Laboratories), and the proteins were eluted with a linear gradient of NaCl from 0 to 0.75 M. Fractions containing FlhD and FlhC were then pooled and passed through a Sephacryl S-200 (Pharmacia) column equilibrated with 20 mM Tris (pH 7.9) and 0.5 M NaCl. The molecular weight of the FlhD/FlhC complex was determined by using lysozyme (14,000), carbonic anhydrase (29,000), ovalbumin (45,000), bovine serum albumin (BSA; 68,000), and alcohol dehydrogenase (150,000) as protein standards.

The FlhD protein was purified by first passing the FlhD lysate through a DEAE-blue column. Next, the FlhD-containing flowthrough was precipitated with 40% $\text{NH}_4(\text{SO}_4)_2$, redissolved, and loaded onto a Sephadex G-50 column. The FlhD-containing fractions were collected and concentrated with $\text{NH}_4(\text{SO}_4)_2$. The buffer used throughout the purification process was 20 mM Tris (pH 7.9). All protein purity was deter-

TABLE 1. *E. coli* strains and plasmids used in this study

Strain or plasmid	Relevant genotype or comments	Reference or source
<i>E. coli</i> strains		
MC1000 <i>flhD</i> ::Tn10	<i>flhD</i> ::Tn10	5
YK4131	<i>flhD</i>	15
YK4136	<i>flhC</i>	16
Plasmids		
pMC39	pTTQ18- <i>flhD</i> - <i>flhC</i>	12
pXL25	pT7-7- <i>flhD</i>	This study
pXL26	pT7-7- <i>flhC</i>	This study
pXL27	pT7-7- <i>flhD</i> - <i>flhC</i>	This study
pT7-7	T7 promoter	28
pGp1-2 ^{cm} l	T7 RNAP	25
pIK905	pUC118- <i>fliA</i>	I. Kawagishi
pMC250	pKK232-8- <i>flhB</i> promoter	12
pJM4	pBR322- <i>fliL</i>	20
pMG1	pMK2004- <i>flhB</i>	12

TABLE 2. Primers used in this study^a

Name	Sequence ^b (5'→3')
5'FLHD	5'-GGGGGGCATATGCATACCTCCGAGTTGCTG
3'FLHD	5'-GGGGGGAAGCTTTGATCAGGCCCTTTCTTGGC
5'FLHC	5'-GGGGGGCATATGAGTGAAAAAGCATTGTT
3'FLHC	5'-GGGGGGAAGCTTCAGTTAAACAGCCTGTACTCT
5'fliAFP	5'-CGGGATCCATCCGCCAACATAAA
3'fliAFP	5'-CCTTCAGCGGTATAGAGT
3'fliA	5'-CTTCGTGACGCACCA
5FLHBFP	5'-CCGGAATTCTGATAAGGCGATGAC
5FLHBFP	5'-GATCCGTCGGTGTGGTA
MBR21	5'-ATGCCCGACAGTCGACGGGCC
5'fliLFP	5'-AGCTTTAGTGGTCAG
3'fliL	5'-TAACCTGCGCTGGCACA

^a All primers were purchased from Operon Technologies Inc., Alameda, Calif.

^b Boldface letters signify sequences in operons.

mined by densitometric scanning of Coomassie blue-stained sodium dodecyl sulfate (SDS)-polyacrylamide gels.

Labeling of DNA. The radioactive DNA templates used in this study were prepared as follows. For the *fliA* promoter, two primers, 5'fliAFP and 3'fliAFP (Table 2), were used to synthesize by PCR a 215-bp fragment whose 5' and 3' termini extended to -163 and +43, respectively, relative to the transcriptional +1 position of the *fliA* gene. Primer 5'fliAFP created a *Bam*HI site. The PCR product was labeled by T4 polynucleotide kinase by using [γ -³²P]ATP (Amersham). *fliA* footprinting of the template and nontemplate strands was carried out by digesting the labeled fragment with *Eco*RI, which cut at position +21, and *Bam*HI, respectively. Further purification of the desired fragments was unnecessary since the very short fragments ran off the bottom of the footprinting gels.

For the *flhB* footprinting of the nontemplate strand, a 3'-end-labeled DNA template was obtained by digestion of plasmid pMC250 with *Bam*HI, which cut within site about 240 bp upstream of *flhB*, filling in with [α -³²P]dGTP by using T7 sequenase (U.S. Biochemical Corp.), and subsequent digestion with *Pvu*II, which gave rise to a 385-bp fragment. Further purification of the desired fragment was achieved by electrophoresis on a 1.5% agarose gel followed by electroelution of the fragment. The labeled template strand probe was generated by labeling the 334-bp PCR fragment (see discussion of in vitro transcription assay below) at the 5' end by using T4 polynucleotide kinase and [γ -³²P]ATP and digestion with *Mbo*II, which cut at position +101. The labeled 234-bp fragment (-134 to +101) was recovered by electroelution.

The DNA template for *fliL* footprinting of the noncoding strand was prepared by digestion of pJM4 with *Hind*III, fill-in labeling of the linearized plasmid with T7 Sequenase and [α -³²P]dATP, and further digestion with *Bam*HI, which cut at position +75. The desired 3'-end-labeled 308-bp fragment was purified by electroelution after gel fractionation. The 358-bp DNA fragment (see discussion of in vitro transcription assay below) was used for the *fliL* template strand footprinting. The PCR product was end labeled with [γ -³²P]ATP and then digested with *Mbo*I, which cut at position +66. The 5'-end-labeled fragment was isolated as described as above.

Mobility shift assay. The binding reaction mixture (20 μ l) contained 10 mM Tris (pH 7.9), 100 mM KCl, 5 mM EDTA, 1 mM dithiothreitol, 5% glycerol, 10 ng of poly(dI-dC), 2.5 pM ³²P-labeled DNA, and 5 μ g of lysate as indicated. After 20 min at 30°C, the samples were loaded onto 4% polyacrylamide gels, and the bands were visualized by autoradiography.

DNase I footprinting assay. The footprinting reaction mixture (200 μ l) contained 10 mM Tris (pH 7.9), 100 mM KCl, 5 mM EDTA, 1 mM dithiothreitol, 5% glycerol, 200 ng of

FIG. 2. Overproduction and purification of the FlhD and FlhC proteins, analyzed on SDS-15% polyacrylamide gels and visualized by Coomassie brilliant blue staining. Positions of M_r markers and their sizes are shown at the left in kilodaltons. (A) Total protein pattern of uninduced (lanes 1, 3, and 5) and induced (lanes 2, 4, and 6) cultures carrying the information for the proteins indicated. Arrows indicate the induced proteins. (B) Purification and complex formation. Lanes: 1, total proteins; 2, clear lysate; 3, pooled material from the heparin cartridge; 4 to 16, equal volumes of fractions 22 to 34 taken from the Sephacryl S-200 column. The position of BSA is indicated.

poly(dI-dC), 100 nM ^{32}P -labeled DNA, and 1 μg of proteins as indicated. After 20 min of incubation at 30°C, 20 μl of DNase I (60 ng for samples without protein added and 240 ng for samples with protein) in DNase I dilution buffer (10 mM Tris [pH 7.9], 100 mM MgCl_2 , 100 mM CaCl_2) was added, the samples were incubated at room temperature for 30 s, and then 60 μl of saturated ammonium acetate was added to stop the cleavage reaction. The DNA was then phenol-chloroform extracted and ethanol precipitated. The digested DNA samples were resuspended in loading buffer (47% formamide, 10 mM EDTA, 0.05% bromophenol blue, 5% xylene cyanol) and subjected to electrophoresis in a 6% polyacrylamide-7 M urea gel, which was dried and examined by autoradiography. As size markers for the DNase I footprints, Sanger sequencing ladders (26) were generated, using plasmid pIK905 and primer 5'fliAFP or 3'fliAFP for the *fliA* promoter, plasmid-primer pair pMG1-5FLHBFP or pMC250-5FLHBFP for the *flhB* promoter, and plasmid pJM4 and primer 5'FLILFP for the *flhL* promoter. The primers used in sequencing match the beginning of the fragments used for the footprint analysis.

In vitro transcription assay. All transcription templates were PCR products. For the *fliA* operon, two primers, 5'fliAFP and 3'fliA (Table 2), were used to synthesize a 272-bp fragment. The PCR product was then treated with *Hin*PI to generate a 248-bp DNA template which carried the *fliA* promoter-regulatory region and the first 100 bp into the coding region of *fliA*. For the *flhB* operon, 5FLHBFP and MBR21 (Table 2) were used to synthesize a 334-bp fragment which gave a 201-nucleotide transcript. For the *flhL* operon, 5'FLILFP and 3'fliL (Table 2) were used to synthesize a 358-bp fragment which gave a 126-nucleotide transcript. Transcription runoff reactions were carried out at 37°C in a total volume of 25 μl . The transcription buffer contained 10 mM Tris (pH 8.0), 50 mM KCl, 5 mM MgCl_2 , 1 mM CaCl_2 , 100 μg of BSA per ml, 100 mM potassium glutamate, 0.1 mM EDTA, and 1 mM dithiothreitol. ATP, GTP, and CTP were each

present at 100 μM ; unlabeled UTP was present at 10 μM , and 10 μCi of [α - ^{32}P]UTP was used per reaction. For each reaction, 1.5 μg of heparin was added. Template DNA was present at 3 nM, and 1 U of *E. coli* RNA polymerase (RNAP) core or holoenzyme (Epicentre Technologies) and 30 U of RNase inhibitor (U.S. Biochemical) were used per reaction. One microgram of FlhD/FlhC or FlhD was included as indicated.

In a typical single-cycle transcription assay, reaction mixtures (19 μl) containing the buffer, template, RNase inhibitor, and proteins as indicated were incubated at 37°C for 15 min. Then 1 μl of RNAP (1 U) was added. After incubation at 37°C for 10 min to allow the formation of open complexes, a 5- μl mixture of deoxynucleoside triphosphates, [α - ^{32}P]UTP, and heparin was added, and the mixtures were incubated at 37°C for an additional 15 min to allow the formation of full-length transcripts. The reactions were stopped by extraction with phenol-chloroform (1:1) once and chloroform three times. The samples were denatured in denaturing-loading buffer (7 M urea, 0.1 M Na_2EDTA , 5% [vol/vol] glycerol, 0.05% [wt/vol] bromophenol blue, 0.05% xylene cyanol) at 70°C for 5 min and subjected to electrophoresis on 8 M urea-acrylamide gels followed by autoradiography.

RESULTS

Overproduction and purification of FlhD and FlhC. Figure 2A shows the overproduction of FlhD and FlhC. The overproduced proteins have molecular masses of 22 kDa (FlhC) and 13 kDa (FlhD), which are consistent with earlier studies (1, 4) and the predicted M_r from DNA sequencing data. In the overexpression of proteins from plasmid pXL27, which contained both *flhD* and *flhC*, the amount of FlhD synthesized was much greater than that of FlhC (Fig. 2A, lane 2). In this plasmid, the synthesis of FlhC was dependent on its own ribosome-binding

FIG. 3. Mobility shift analysis of clear lysates performed on the *flhA* promoter (−163 to +43). Lanes: 1, free probe; 2 to 5, clear lysates (5 μ g of each) from MC1000 *flhD::kan/pGp1-2^{cm}/pXL27*, uninduced (lane 2) and induced (lane 3), and from MC1000 *flhD::kan/pGp1-2^{cm}/pXL25*, uninduced (lane 4) and induced (lane 5).

site in the operon, whereas the synthesis of FlhD was dependent on the strong ribosome-binding site of pT7-7.

FlhD and FlhC were purified from lysates of cells carrying pXL27 (see Materials and Methods). During protein purification, we found that both FlhD and FlhC bound to a heparin cartridge and coeluted from the column at 0.5 M NaCl. At this step, FlhD and FlhC were about 90% pure, as determined by densitometric scanning of a Coomassie blue-stained SDS-polyacrylamide gel (data not shown). Unlike in the unpurified lysate, FlhD and FlhC were isolated from the heparin column at a constant ratio of 1 to 2 (FlhD to FlhC), as judged from Coomassie blue staining. This finding suggested that a complex of a 1-to-1 molar ratio may be formed. The proteins were further purified, and the complex formation was confirmed by passing the heparin eluates through a Sephacryl S-200 column. As shown in Fig. 2B (lanes 6 to 16), the proteins were about 96% pure. The proteins were coeluted from this column at the same volume as BSA. The 68-kDa size of the complex and the apparent molar ratio of 1:1 suggested that FlhD and FlhC form an oligomeric complex of two FlhD and two FlhC molecules in solution.

When applying the lysate containing overproduced FlhD to the heparin column, we found that no FlhD bound. The FlhD protein was purified to over 97% purity (see Materials and Methods). When we overexpressed proteins from the construction which contained only *flhC* (pXL26), however, inclusion bodies were formed, and no soluble FlhC protein could be detected. Therefore, FlhC was soluble only when complexed with FlhD. Attempts to purify the insoluble protein did not succeed and were not further pursued.

The DNA-binding activity of FlhD and FlhC. To investigate how FlhD and FlhC proteins regulate the transcription of the class II operons, a mobility shift assay was first used (Fig. 3). The lysate containing both overproduced FlhD and FlhC proteins showed a DNA-binding activity (lane 3), but the FlhD lysate did not (lane 5). Figure 3 shows the result when activity was tested on the *flhA* promoter. Similar results were obtained with the *flhB* and *flhL* promoters (data not shown).

The specific binding sites of FlhD and FlhC proteins were identified by DNase I footprinting assays on the three class II promoters, using purified proteins. DNase I footprinting was carried out on both strands of the DNA fragments. Figure 4A (lanes 1 to 8) shows that a 48-bp region of the *flhA* promoter was protected. This region extended from −41 to −88 relative to the *flhA* transcriptional start site. In lanes 9 to 16, a 50-bp region of the template strand and a 49-bp region of the nontemplate strand of the *flhB* promoter were protected. For the template strand, however, protection of the region from

−28 to −77 was interrupted at position −68. A 48-bp region of the template strand and a 49-bp region of the nontemplate strand of the *flhL* promoter were protected; these regions extended from −29 to −76 and −31 to −79, respectively, relative to the *flhL* transcriptional start site (lanes 17 to 24). At various positions, DNase I-hypersensitive sites were observed in the presence of FlhD/FlhC. An overview of the FlhD/FlhC-mediated contacts on the promoter regions of three class II genes is given in Fig. 4B.

Transcriptional regulation of the class II operons *in vitro*.

We examined the ability of purified FlhD/FlhC and FlhD to activate the initiation of transcription in a defined transcription system. In Fig. 5 (lanes 1 to 4), we present the results of transcription assays done with the *flhA* promoter. A 248-bp DNA fragment which extends from −147 to +100 was used as a template for measuring transcription from the *flhA* promoter. In the presence of the σ^{70} holoenzyme alone, there was no transcript synthesized (lane 1). When FlhD/FlhC was added, a 100-bp transcript was synthesized (lane 3). As expected, FlhD/FlhC could not initiate transcription in the absence of σ^{70} (lane 2). In the absence of FlhC, FlhD did not stimulate transcription in the system that we used (lane 4). The same results were obtained when we used the *flhB* and *flhL* promoters (lanes 5 to 8 and 9 to 12, respectively). The FlhD/FlhC complex as well as σ^{70} and core RNAP are required for initiation of transcription at these class II promoters *in vitro*. In control experiments, it was demonstrated that the σ^{70} holoenzyme did not have detectable σ^{28} activity (data not shown). These data demonstrate that the master positive regulatory proteins, FlhD and FlhC, function together as a transcriptional activator but not as a two-component sigma factor in activation of transcription from these class II operons.

DISCUSSION

Genetic analysis of flagellar gene expression in *E. coli* suggests that both *flhD* and *flhC* gene products are required as *trans*-acting positive regulators of transcription (13, 17). Positive regulators can be either DNA-binding proteins or alternative sigma factors, and these classes of positive regulators cannot be distinguished in genetic experiments (24). On the basis of some sequence similarity to known sigma factors, it has been proposed that FlhD and FlhC may act together as an alternative sigma factor for RNAP (2, 4, 8, 10, 11, 18, 19). The overproduction and purification of the FlhD and FlhC proteins allows us to understand the properties and biological role of those two positive transcriptional regulators. From data collected on three available class II promoters, we conclude that the FlhD/FlhC complex activates transcription of flagellar class II genes by binding to the upstream control regions of those operons.

Previous attempts to express FlhD and FlhC at high levels were not successful (1, 4), partly because of lack of strong ribosome-binding sequences in the *flhD* operon and lack of convenient restriction sites for subcloning. Here, we used the PCR technique to engineer and amplify the *flhD*, *flhC*, and *flhD-flhC* coding regions and then subclone each of them into pT7-7. This approach created translational fusions that utilized the efficient phage ribosome-binding site in the vector pT7-7 without altering any of the sequences between the ribosome-binding sequence and the start codons. The resultant plasmids, pXL25, pXL26, and pXL27, were found to complement YK4131, YK4136, and MC1000 *flhD:Tn10*, respectively, as assayed on swarm plates (data not shown).

The DNA-binding activity was first detected in the lysate which contained both overproduced FlhD and FlhC (Fig. 3,

FIG. 4. Analysis of FlhD/FlhC binding to three class II promoters by DNase I footprinting. (A) DNase I cleavages of DNA fragments in the absence (lanes 3, 7, 11, 15, 19, and 23) or presence (lanes 4, 8, 12, 16, 20, and 24) of FlhD/FlhC. In lane pairs 3-4, 11-12, and 19-20, the labeled DNA fragments represent the template (t) strands, whereas in lane pairs 7-8, 15-16, and 23-24, the nontemplate (nt) strands were labeled. Regions protected by FlhD/FlhC are indicated by brackets. Products of the dideoxynucleotide sequencing reactions were also electrophoresed adjacent to the footprinting reactions (lanes 1 [T], 2 [C], 5 [T], 6 [A], 9 [C], 10 [G], 13 [A], 14 [T], 17 [T], 18 [C], 21 [A], and 22 [T]). (B) DNA sequences of the upstream regions of three class II genes. The sequence data are from references 17a and 23 (*fliA*), 12 (*flhB*), and 16 (*fliL*). +1 indicates the transcriptional start site. Bases hypersensitive to DNase I digestion are indicated with asterisks. The sequences underlined indicate the protection regions.

lane 3) but not in the lysate which contained only overproduced FlhD (Fig. 3, lane 5) or FlhC (data not shown). Since overproduction of FlhC leads to the formation of insoluble inclusion bodies, we could not test whether FlhC has DNA-

binding activity. During purification of FlhD/FlhC, mobility shift assays were used to monitor activity. Footprinting studies assayed on purified FlhD/FlhC (Fig. 4) and the crude lysate (data not shown) revealed the same protection pattern, dem-

FIG. 5. Activation of transcription from three class II promoters by purified proteins. The composition of each reaction mixture is given above the corresponding lane. E, core RNAP; $E\sigma^{70}$, core RNAP saturated with σ^{70} ; D, *flhD* product; D/C, FlhD/FlhC complex. Positions of RNA M_r markers and their sizes in nucleotides (nt) are indicated at the left. Arrows indicate transcripts initiated at the following promoters in the presence (+) or absence (-) of the indicated proteins: *flhA* (lanes 1 to 4), *flhB* (lanes 5 to 8), and *flhL* (lanes 9 to 12).

onstrating that FlhD/FlhC is, in fact, responsible for the activity initially characterized in the lysate. The results presented here demonstrate that the *flhD* master operon encodes two proteins that contribute to a DNA-binding activity.

Arnosti and Chamberlin showed that RNAP containing σ^{28} can transcribe in vitro the *fliL* operon of *E. coli* (2); we do not know the significance of the data, since the transcription in vivo of this operon does not require the *fliA* gene (13, 17). However, DNA sequence analyses of the promoter regions of five class II operons for which sequences are available reveal that a σ^{28} consensus overlaps the -10 region in the *fliA*, *fliL*, *fliE*, and *fliF* promoters and the -120 region in the *flhB* promoter. The existence of σ^{28} consensus in class II promoters suggests a role of σ^{28} in regulation of the class II promoters. It is possible that class II genes are initially activated by FlhD/FlhC and then their expressions are further regulated by σ^{28} . Our data showed that initiation of transcription of the class II promoters in vitro requires σ^{70} and FlhD/FlhC in addition to core RNAP. This finding is consistent with genetic data showing that transcription of the class II operons requires the gene products of the *flhD* operon (13). The σ^{70} holoenzyme used for the in vitro transcription of class II promoters lacked σ^{28} activity, as tested on the class III *tar* promoter and by Western blotting (immunoblotting) using anti- σ^{28} serum (unpublished data). This finding demonstrates that the transcription observed in Fig. 5 results from σ^{70} but not σ^{28} . Although our data have shown that transcription of the class II promoters can be initiated by σ^{70} holoenzyme and FlhD/FlhC in vitro, they do not exclude the possibility that those class II operons are transcribed by σ^{38} holoenzyme in vivo, since σ^{38} is the central regulator of gene expression in the stationary phase, and some *E. coli* promoters can be recognized in vitro by both σ^{70} and σ^{38} holoenzymes (29).

Very little is known about the mechanism by which FlhD and FlhC influence transcription initiation by RNAP. The process

of promoter recognition involves polymerase binding to the recognition elements at -35 and -10. Sequence analysis of upstream regions of the three genes revealed that the -35 elements are far from consensus, suggesting that activators are involved in optimization of the promoter function. The results of our experiments demonstrate that FlhD and FlhC form a complex, bind DNA, and act together with σ^{70} holoenzyme in vitro. On the basis of DNase I footprinting data, the binding sites overlap the promoter -35 region in *flhB* and *fliL* and are near position -40 in the case of *fliA*. It is most likely that activation involves a direct contact between the bound FlhD/FlhC and RNAP. The DNase I-hypersensitive sites are about 10 bp apart, which suggests that FlhD/FlhC bind to one side of the DNA double helix and may bend DNA upon binding. Comparison of the protected regions of the three class II genes does not reveal any compelling conserved elements. Sequence analysis of FlhD and FlhC also does not reveal any likely helix-turn-helix motifs (7). At present, we do not know which protein contacts DNA or whether both do. DNase I footprinting data showed that the length of DNA bound by FlhD/FlhC is about 50 bp (Fig. 4B). FlhD/FlhC was judged to be a heterotetramer consisting of two subunits each of FlhD and FlhC with an aggregate molecular mass of 68 kDa. The protected sites do not have any apparent twofold symmetry, which suggests that only one heterodimer is bound to the site.

In eukaryotic systems, there are many examples of transcriptional regulation mediated by the activities of a pair of proteins via heterotypic dimerization. The potential advantage of heterodimerization is diversity and specificity in transcriptional regulation. In contrast, such heterotypic complexes are relatively rare in prokaryotic systems (22). The results of our studies show that complex formation plays an important part in the regulation of expression of class II operons. Mobility shift and in vitro transcriptional data reveal that FlhD by itself cannot bind to the upstream regions of class II promoters and fails to activate transcription from them. Direct binding of FlhC to the control regions of class II genes has not been tested because we have been unable to solubilize FlhC from inclusion bodies. We propose that the interaction of FlhD and FlhC results in a change which affects the FlhD/FlhC complex's DNA-binding activities or effector functions or both. Taken together, FlhD and FlhC activate flagellar gene transcription by interacting as heterocomplexes with the DNA-regulatory element known as the activator-binding site. Elucidating the structure of the FlhD and FlhC functional domains will be important in understanding the mechanism of transcriptional activation and will be the focus of our future studies.

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