Multiple Control of Flagellum Biosynthesis in Escherichia coli: Role of H-NS Protein and the Cyclic AMP-Catabolite Activator Protein Complex in Transcription of the flhDC Master Operon

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Little is known about the molecular mechanism by which histone-like nucleoid-structuring (H-NS) protein and cyclic AMP-catabolite activator protein (CAP) complex control bacterial motility. In the present paper, we show that crp and hns mutants are nonmotile due to a complete lack of flagellin accumulation. This results from a reduced expression in vivo of fltA and fltC, which encode the specific flagellar sigma factor and flagellin, respectively. Overexpression of the flhDC master operon restored, at least in part, motility in crp and hns mutant strains, suggesting that this operon is the main target for both regulators. Binding of H-NS and CAP to the regulatory region of the master operon was demonstrated by gel retardation experiments, and their DNA binding sites were identified by DNase I footprinting assays. In vitro transcription experiments showed that CAP activates flhDC expression while H-NS represses it. In agreement with this observation, the activity of a transcriptional fusion carrying the flhDC promoter was decreased in the crp strain and increased in the hns mutant. In contrast, the activity of a transcriptional fusion encompassing the entire flhDC regulatory region extending to the ATG translational start codon was strongly reduced in both hns and crp mutants. These results suggest that the region downstream of the +1 transcriptional start site plays a crucial role in the positive control by H-NS of flagellum biosynthesis in vivo. Finally, the lack of complementation of the nonmotile phenotype in a crp mutant by activation-deficient CAP mutated proteins and characterization of cfs, a mutation resulting in a CAP-independent motility behavior, demonstrate that CAP activates flhDC transcription by binding to its promoter and interacting with RNA polymerase.

The structure and the function of the flagellum in Escherichia coli and Salmonella typhimurium have been extensively studied for many years. Its biosynthesis seems to play a crucial role in adaptation to various environmental conditions and is affected by numerous adverse conditions (51). Furthermore, motility has frequently been associated with virulence and/or inflammatory response in various microorganisms, such as Bordetella bronchiiseptica (1), Vibrio cholerae (19), and S. typhimurium (13). Finally, it has recently been shown that motility is critical for colonization and/or biofilm formation, e.g., in Vibrio fischeri (23) and in E. coli (45).

In E. coli, numerous mutations are known to alter motility, especially those affecting synthesis of bacterial membrane components, such as porins (27) and lipopolysaccharide (44). Moreover, several regulators have been shown to be involved in the control of swarming behavior in E. coli and S. typhimurium, in particular, the cyclic AMP (cAMP)-catabolite activator protein (CAP) complex (54, 61) and the histone-like nucleoid-structuring (H-NS) protein (7, 26). The former positively or negatively regulates a vast number of genes involved in various functions in E. coli (12). The latter controls expression of many genes regulated by environmental parameters, such as pH, temperature, and osmolarity (3). Mutations in crp or in hns, the structural genes of CAP and H-NS, respectively, result in a complete loss of motility in E. coli (7, 54). However, little is known about the molecular mechanism by which these regulatory proteins control flagellar gene expression.

In E. coli and S. typhimurium, flagellum biosynthesis requires expression of nearly 50 genes clustered at several places on the chromosome. Their transcription forms an ordered cascade in which the expression of one gene located at a given level requires the transcription of another one at higher level (35). At the top of the hierarchy, the flhD and flhC genes constitute the master operon which controls the expression of all other flagellar genes. In E. coli, the FlhD and FlhC proteins have been shown to act as positive regulators of the flagellar regulon (33). The flhD gene, one of those located at the second level of the cascade, encodes the flagellar sigma factor σ28. This protein is required for the expression of most genes located at the third level, e.g., fltC, the flagellin structural gene (35).

In the present study, we investigated the mechanism by which H-NS and the cAMP-CAP complex regulate flagellum biosynthesis. By gel retardation and footprinting experiments, we demonstrated the abilities of both regulators to bind to the flhDC regulatory region. Despite a similar complete loss of flagellin accumulation in hns and crp mutants, in vitro transcription assays showed that CAP activates expression of the master operon while H-NS represses it. Using transcriptional fusions, we demonstrated that the positive control by H-NS of flagellar gene expression in vivo requires the region downstream of the +1 transcriptional start site.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Strains carrying the hns-1001 (7) and

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RNA dilution buffer (water, 20 mM NaCl, and formaldehyde in the ratio 5:3:2) at 65°C for 15 min. The 20 mM SSC solution (3 M NaCl–0.3 M trisodium citrate adjusted to pH 7) was treated and 5 μg of total RNA in avian myeloblastosis virus reverse transcriptase reaction buffer (Boehringer Mannheim). Plasmid pDIA528 was constructed by cloning a similar DNA fragment into the pKK232-8 vector (Pharmacia) after PCR amplification with the oligonucleotides O1 (5'-CGGGATCCCTGCGCAA CATCC-3') and O2 (5'-CCCAAGCTTGGCAGAACACCATCC-3') and O3 (5'-CCCAAGCTTGGCAGAACACCATCC-3'). The resulting 435-nucleotide fragment was cloned into the plasmid pKK232-8 (Pharmacia). Plasmid pDIA545 was constructed by PCR amplification of the flhDC regulatory region with the primers O1 (5'-GGGAGATCCCTGCGCAA CATCC-3') and O3 (5'-CCCAAGCTTGGCAGAACACCATCC-3'). The resulting DNA fragment was cloned into the BamHI and HindIII sites of plasmid pKK232-8. Similarly, plasmid pDIA559 was constructed by PCR amplification of the flhDC promoter with the primers 5'-GGGAGATCCCTGCGCAA CATCC-3' and O3 (5'-CCCAAGCTTGGCAGAACACCATCC-3'). The resulting DNA fragment was cloned into the BamHI and HindIII sites of plasmid pKK232-8.

**Protein purification.** CAP, H-NS, and RNA polymerase were prepared as described previously (references 20, 60, and 24, respectively).

**Gel retardation experiments.** Plasmid pDIA525, containing the flhDC promoter region, was cleaved by EcoRI, HindIII, NdeI, and SphI. DNA fragments (100 ng) were incubated with H-NS for 15 min at room temperature in the reaction mixture previously described (22). Protein-DNA complexes were resolved on 3% MetaPhor agarose gel with Tris-borate-EDTA as the running buffer. Similar experimental conditions were used with CAP except that the reaction mixture and running buffer both contained 200 μM cAMP.

**Oligonucleotide labeling.** The oligonucleotides used in footprinting analysis and primer extension experiments were end labeled with phage T4 polynucleotide kinase and [γ-32P]ATP (3,000 Ci/mmol) according to standard procedures (48). The reaction was performed according to standard procedures (4), with some modifications. Ten picomoles of end-labeled O3 oligonucleotide complementary to the region including the translational start site of flhDC mRNA (see Fig. 2) was precipitated with ammonium acetate and ethanol at −20°C, washed with 70% ethanol, dried, and resuspended in 40 μl of dimethyl pyrocarbonate-treated 10 mM Tris–1 mM EDTA (pH 7.6) buffer to a concentration of 2.5 ng/μl. Five nanograms of primer was annealed with 10 ng of total RNA in avian myeloblastosis virus reverse transcriptase reaction buffer (Boehringer Mannheim) and 1 mM dithiothreitol in 60°C for 15 min. The reaction was kept going while the temperature slowly decreased to 30°C. RNAsIn (20 U) (Promega) was added, and the reaction was performed with 40 U

### TABLE 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype*</th>
<th>Reference or source</th>
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<tbody>
<tr>
<td>FB8</td>
<td>Wild type</td>
<td>10.1</td>
</tr>
<tr>
<td>MG1655</td>
<td>Wild type</td>
<td>29a</td>
</tr>
<tr>
<td>BE1815</td>
<td>pMG1655 cp::Sm</td>
<td>29a</td>
</tr>
<tr>
<td>BE1816</td>
<td>MG1655 hns-1001</td>
<td>This study</td>
</tr>
<tr>
<td>BE1817</td>
<td>MG1655 cp::Sm hns-1001</td>
<td>This study</td>
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<tr>
<td>PS2209</td>
<td>Wild type</td>
<td>7</td>
</tr>
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<td>PS2209 hns-1001</td>
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</tr>
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<td>BE1818</td>
<td>PS2209 cp::Sm</td>
<td>This study</td>
</tr>
<tr>
<td>BE1919</td>
<td>PS2209 hns-1001 cp::Sm</td>
<td>This study</td>
</tr>
<tr>
<td>ILV70-2</td>
<td>Δcfr efs</td>
<td>57</td>
</tr>
</tbody>
</table>

* nt, nucleotide.

**Probe labeling.** A 651-bp DNA probe corresponding to part of the flhDC coding region was generated by PCR amplification with oligonucleotides 5'-CATTA AACAGCCCTCTGC-3' and 5'-ATTGAAGCTGGTATTAATGACTTGTGCC-3' and the PCR DIG Probe synthesis kit (Boehringer Mannheim) according to the manufacturer's instructions.

**Quantitative analysis of mRNA.** RNA (500 ng) was denatured in 300 μl of RNA dilution buffer (water, 20× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], and formaldehyde in the ratio 5:3:2) at 65°C for 15 min. The 20× SSC solution (5 M NaCl–0.3 M trisodium citrate adjusted to pH 7) was treated with diethylpyrocarbonate. It was then applied to Hybond N+ nylon filters (Amersham) with a PR600 SlotBlot applicator ( Hoefer Scientific). The RNAs were covalently cross-linked to the membrane by UV cross-linking at 0.51 J/cm². DIG-labeled probe (20 μl) was hybridized to the immobilized RNA at 50°C for 16 h with DIG Easy Hyb buffer (Boehringer Mannheim). The membrane was washed two times with 2× SSC–0.1% sodium dodecyl sulfate at room temperature and then two times with 0.1× SSC–0.1% sodium dodecyl sulfate at 68°C. The labeled probe was visualized with the CSPD chemiluminescence detection system (Boehringer Mannheim) and Hyperfilm-MR X-ray film (Amersham). Bands were scanned with a JX-330 SHARP scanner and quantified with DDI software PDQuest based on a SUN computer system.

**DNA manipulations.** Plasmid pDIA25 was constructed by PCR amplification as previously described (6). Briefly, a DNA fragment containing the flhDC promoter region was generated with the primers O1 (5'-GAATTCCTGCGCA ACATCC-3') and O2 (5'-CCCAAGCTTGGCAGAACACCATCC-3') (see Fig. 2) and the overhang of total DNA from E. coli FB8. The 308-bp PCR fragment was purified with the High Pure PCR purification kit (Boehringer Mannheim) and cloned into the pSR plasmid, restricted by EcoRI and HindIII sites to remove the gal promoter located upstream of a λ op terminator. Plasmid pDIA528 was constructed by cloning a similar DNA fragment into the pKK232-8 vector (Pharmacia) after PCR amplification with the oligonucleotides O1 (5'-CGGGATCCCTGCGCAA CATCC-3') and O2 (5'-CCCAAGCTTGGCAGAACACCATCC-3') and O3 (5'-CCCAAGCTTGGCAGAACACCATCC-3'). The O1' primer differed from the O1 primer by the presence of a BamHI site instead of an EcoRI site. Plasmid pDIA545 was constructed by PCR amplification of the flhDC regulatory region with the primers O1 (5'-CGGGATCCCTGCGCAA CATCC-3') and O3 (5'-CCCAAGCTTGGCAGAACACCATCC-3'). The resulting 435-nucleotide fragment was cloned into the plasmid pKK232-8 (Pharmacia). Plasmid pDIA546 was constructed by cloning a 423-bp BamHI/NheI fragment containing the flhDC regulatory region of pDIA528 into plasmid pCDI0 (36). The fragment was purified from agarose gel with the JETsorb kit (GENOMED). Plasmid pDIA551 containing the promoter region and part of the coding sequence of the flhDC operon was constructed by PCR amplification of a 1,048-nucleotide fragment with primers O1 (5'-CGGGATCCCTGCGCAA CATCC-3') and O3 (5'-CCCAAGCTTGGCAGAACACCATCC-3'). The resulting DNA fragment was cloned into the BamHI and HindIII sites of plasmid pKK232-8. Similarly, plasmid pDIA559 was constructed by PCR amplification of the flhDC promoter with the primers 5'-GGGAGATCCCTGCGCAA CATCC-3' and O3 (5'-CCCAAGCTTGGCAGAACACCATCC-3'). The 276-bp fragment was cloned into the BamHI and HindIII sites of plasmid pKK232-8.
of avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim) at 42°C for 90 min. One microliter of 0.5 M EDTA (pH 8.0) and 1 μl of DNase-free pancreatic RNase (Boehringer Mannheim) were added, and the reaction was further incubated at 37°C for 30 min. The reaction mixture was precipitated with ammonium acetate and ethanol, washed with 70% ethanol, and resuspended in formamide loading buffer. As a reference, sequencing reactions were performed with the Thermosequenase radiolabeled terminator cycle sequencing kit from Amersham with the same primer used in primer extension experiments.

Footprinting analysis. DNase I footprinting experiments were performed as previously described (36) with some modifications. A 308-nucleotide fragment containing the fliC promoter region and a 435-nucleotide fragment containing the entire flhDC regulatory region as previously described (36) with the following modifications. Seven microliters of the reaction mixture containing H-NS at 334 nM and/or CAP at 50 nM was incubated at 30°C for 10 min with RNA polymerase at 120 nM final concentration. Then, 3.5 μl of a mixture containing nucleotide triphosphates and heparin was added to perform the polymerization at 30°C for 10 min. Quantitative data were obtained with a PhosphorImager (Molecular Dynamics).

RESULTS

The reduced expression of the flhDC master operon in crp and hns mutants results in a lack of flagellin biosynthesis. In E. coli, CAP- and H-NS-deficient strains are known to be completely nonmotile (7, 54). To evaluate the flagellin content in hns and crp mutants, total-protein extracts were analyzed by two-dimensional gel electrophoresis. In the wild-type strain, a protein was resolved as a single spot of pI 4.7 and an apparent mass of 53 kDa, in agreement with the theoretical values computed from the FliC amino acid sequence. This protein was undetectable in extracts of crp and/or hns mutants compared to extracts of the wild type (Fig. 1A). An internal fragment of the purified polypeptide was subjected to microsequencing. The amino acid sequence obtained matched the sequence of the fliC gene product (data not shown). This result provides evidence that the loss of motility associated with hns and crp mutations results from a complete lack of flagellin, in agreement with previous examination of such mutant strains by electron microscopy (7, 54).

To ascertain whether the lack of flagellin accumulation in crp and hns mutants resulted from a reduced expression of the fliC gene located at the third level in the flagellar-gene cascade, we performed a comparative analysis of flagellin-encoding mRNA in crp and/or hns strains. In contrast to the wild-type strain, fliC mRNA was not significantly different from the background level in the mutant strains (Fig. 1B), in agreement with the absence of flagellin observed in these mutants (Fig. 1A). The role of CAP and H-NS in the initiation of fliC transcription was investigated by measuring the activity of a cat-fliC transcriptional fusion in crp and/or hns strains. The strains were grown to mid-exponential phase, which corresponds to the highest level of flagellar-gene expression (46). More than a 10-fold decrease in fliC transcription was observed in crp and hns strains, compared to the level measured in the wild-type strain (Fig. 1C).

FliC synthesis requires the expression of fliA, encoding the specific flagellar sigma factor, located at the second level in the flagellar hierarchy. Therefore, to study the role of H-NS and CAP in flagellar-gene expression, we constructed transcriptional fusions between the cat gene and the fliA gene, encoding the specific flagellar sigma factor, as well as fliL and flhB, two genes also located at the second level in the ordered cascade and regulated by the FlhDC regulatory proteins (33). In comparison with the wild-type strain, more than a 10-fold decrease in cat activity was measured in the presence of hns and/or crp mutations (data not shown). This suggests that both regulators mainly control flagellar-gene expression by affecting flhDC lo-
TABLE 2. Motility of E. coli strains on semisolid agar plates

<table>
<thead>
<tr>
<th>Strain genotype</th>
<th>Plasmid (protein)</th>
<th>Motility*</th>
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<tr>
<td>Wild-type</td>
<td>None</td>
<td>30 ± 3</td>
</tr>
<tr>
<td>hns-1001</td>
<td>None</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>hns-1001</td>
<td>pPM61 (FlhD and FlhC)</td>
<td>28 ± 3</td>
</tr>
<tr>
<td>crp::Sm</td>
<td>None</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>crp::Sm</td>
<td>pPM61 (FlhD and FlhC)</td>
<td>8 ± 1</td>
</tr>
</tbody>
</table>

*Expressed as the diameter of the swarming ring (in millimeters) after 15 h at 30°C. The data are the mean values ± standard deviations of three independent experiments.

flhDC transcriptional control by H-NS and cAMP/CAP

Identification of CAP and H-NS binding sites in the flhDC promoter region. To determine the mechanism of regulation by the cAMP-CAP complex and H-NS, and their abilities to bind the flhDC promoter region, gel retardation experiments were performed with purified proteins. Plasmid pDIA525 carrying the flhDC promoter was digested by different restriction enzymes to generate various DNA fragments used as competitors for binding to both regulators. A preferential binding of H-NS to the flhDC promoter was observed when the concentration of H-NS reached 2 nM (Fig. 4). In addition, the 191-bp DNA fragment corresponding to the blu promoter was also retarded by H-NS, in agreement with previous results (6, 34, 62). Similarly, the 301-bp fragment corresponding to the flhDC promoter region was found to be specifically retarded in the presence of the cAMP-CAP complex (Fig. 4). Indeed, a 15 nM concentration of CAP protein was sufficient to promote a significant retardation in the electrophoretic mobility of this fragment. At a 50 nM concentration of CAP, a full retardation of the flhDC promoter region was observed. A competitive gel shift assay was also performed with a PCR-amplified DNA fragment encompassing the flhDC promoter (positions −213 to +14 with respect to the transcriptional start site) and the region extending downstream of the +1 transcriptional start site (position +14 with respect to the transcriptional start site to the ATG codon), respectively (Fig. 2). After amplification, this fragment was restricted by the DdeI restriction enzyme to generate two DNA fragments of 200 and 235 bp. The sole DNA fragment corresponding to the flhDC promoter (positions −213 to +14 with respect to the +1 site) was specifically shifted in the presence of CAP. The electrophoretic mobility of flhDC arises from the G residue located 198 nucleotides upstream from the ATG start codon (Fig. 2). Hexamers (−35 and −10) showing 50 and 67% similarity, respectively, with the σ54 consensus, were identified upstream from the transcriptional start site. Two of these boxes are separated by a 17-bp spacer (Fig. 2). The sizes of transcripts obtained by in vitro transcription experiments and primer extension experiments performed from these transcripts further confirmed the location of the +1 site (see below). Finally, the location of the transcriptional start site was in full agreement with that of a single-stranded region within the open complex. A characteristic permanganate reactivity with single-stranded thymine residues was indeed observed at positions −11 and −9 at the noncoding strand (data not shown).

**FIG. 2.** Regulatory region of the flhDC master operon. The nucleotides are numbered relative to the transcriptional start site (+1), indicated by a solid bent arrow. The unique CAP binding site consensus sequence is indicated by a box. Regions protected by H-NS are underlined with solid lines. Downstream of the +1 site, DNase footprinting assays were performed on the sole noncoding strand. The positions of the −10 and −35 sequences are indicated in italics. Nucleotide substitutions identified in the flhDC promoter of a cys strain are indicated by arrowheads. The ATG translation initiation codon identified by mass spectrometry and a putative ribosome binding site (RBS) are indicated in boldface and by a dotted line, respectively. Dash arrows numbered O1/O1 to O3 represent domains used in PCR amplification and/or +1 mapping. O1/O1′-to-O2 and O1′-to-O3 PCR fragments were used to construct plasmids pDIA525 and pDIA528 and plasmids pDIA545 and pDIA546, respectively.
this DNA-protein complex was further retarded in the presence of H-NS. This suggests that both regulators were able to bind together to the flhDC promoter region. Strikingly, in the presence of H-NS alone, a similar retardation was observed for both DNA fragments (data not shown), suggesting that H-NS could also bind to a region downstream of the +1 site. These results are in agreement with those obtained by footprinting experiments (see below).

To determine the precise location of CAP and H-NS binding sites in the flhDC regulatory region, DNase I footprint experiments were performed on the flhDC promoter (residues −213 to +78 with respect to the +1 transcriptional start site). The CAP-CAP complex protected a region between positions −56 and −83 from DNase I cleavage (Fig. 5A and B). This region contains a CAP binding site consensus sequence (positions −61 to −82) centered at position −71.5 with respect to the transcription start site. The same DNA fragment was used in footprinting experiments with H-NS. Unlike CAP, several binding sites were identified on the flhDC promoter, i.e., −178 to −170, −158 to −148, −139 to −130, −126 to −116, −110 to −85, −64 to −50, −40 to −27, −1 to +26, and +32 to +44 with respect to the +1 site (Fig. 5). The region extending from −64 to −50 was protected by H-NS alone and in part by CRP alone (Fig. 5C). In contrast, in the presence of both regulators, a new pattern was observed. Indeed, bands −58 and −59 remained visible while bands −54, −55, and −56 were no longer detected. This indicates that the two proteins together are able to bind the same DNA fragment, in agreement with what we observed in gel retardation experiments (data not shown). Such a modification in the protection pattern has been reported as a consequence of the simultaneous binding of RNA polymerase and cAMP-CAP to the lacUV5 promoter region (29). Moreover, the binding of CAP to its site did not alter H-NS binding to the sites identified in the flhDC regulatory region, as shown by H-NS footprinting observed downstream of the CAP binding site, i.e., −40 to −27, −1 to +26, and +32 to +44 (Fig. 5C). Finally, DNase footprint experiments performed on the region extending from the +1 site to the ATG start codon revealed that H-NS is able to bind to several sites, i.e., +75 to +84, +104 to +119, +134 to +144, and +162 to +174 (Fig. 2).

**The region downstream of the +1 transcriptional start site is required in activation by H-NS but not by CRP.** In vitro transcription experiments were performed with plasmid pDIA525 (Table 1) carrying the flhDC promoter region. A 169-bp transcript was observed (Fig. 6A), in agreement with the position of the transcriptional start site (Fig. 3). In the presence of 50 nM cAMP-CAP complex, in vitro transcription of the master operon was increased more than threefold. In contrast, the presence of 334 nM H-NS in the reaction mixture resulted in a sevenfold decrease in flhDC transcription. When H-NS and CAP were both present, the in vitro transcriptional level was close to that observed in the absence of both regulators (Fig. 6A). Similar results were obtained with plasmid pDIA545 synthesizing a 271-bp transcript encompassing the whole flhDC regulatory region extending to the ATG start codon (Fig. 6B).

To investigate the discrepancy between the repressor effect of H-NS in vitro (Fig. 6) and its activator effect in vivo (Fig. 1), we constructed two transcriptional fusions by using the cat reporter gene. The flhDC promoter (residues −213 to +78 with respect to the +1 transcriptional start site) and the whole regulatory region (residues −213 to +205) were cloned into pKK232-8, giving pDIA528 and pDIA545, respectively. While a fivefold decrease in enzymatic activity was observed from plasmid pDIA528 in the crp mutant (Table 3), more than a twofold increase in cat activity was measured in the hns strain, suggesting that H-NS represses the activity of the flhDC promoter in vivo. Moreover, the cat activity was restored to a level close to that in the wild type in an hns crp double mutant. These results are in agreement with those obtained in vitro (Fig. 6A). In contrast, up to a 100-fold decrease in cat activity was measured from the fusion carried by plasmid pDIA545 in a crp mutant and in an hns crp double mutant. More importantly, a threefold decrease in activity was observed in the hns strain, suggesting that the in vivo flhDC activation by H-NS requires the entire regulatory region encompassing both the flhDC promoter and the region extending downstream of the +1 transcriptional start site to the ATG translational codon. Similar results were obtained with a flhDC-lacZ transcriptional fusion located on the chromosome (data not shown).

**Activation by cAMP-CAP complex of the flhDC master operon results from a direct interaction with RNA polymerase.** Promoter mutations, in particular, those in the lac operon, are known to release transcription from the requirement for cAMP and CAP. Mutant strains, known as cfs (constitutive flagellar synthesis), synthesize flagella in a CAP-independent
manner (54). To determine whether this phenotype was associated with a mutation located in the flhDC promoter region, we examined its nucleotide sequence in a cfs mutant strain (57). We observed two nucleotide substitutions, i.e., an A→C transversion close to the CAP binding site and a C→T transition in the −10 box. This substitution increases the homology of the −10 region with the Pribnow box consensus sequence (Fig. 2). All attempts to isolate similar mutants showing an H-NS-independent swarming in hns strains were unsuccessful (unpublished results).

In most cases, the cAMP-CAP complex activates transcription by recruiting RNA polymerase through the activating region I (residues 156 to 162) (12). We wanted to confirm that the CAP-dependent activation of the flhDC operon resulted from a similar mechanism. Therefore, we complemented the loss of motility in a crp mutant by wild-type and two mutated CAP proteins carrying amino acid substitution T158A or H159L in activating region I (15). In contrast to the wild-type CAP, both mutated proteins were unable to complement the nonmotile phenotype in the crp mutant (Table 4). Taken together, these results suggest that the cAMP-CAP complex positively regulates expression of the flagellar master operon by interaction with RNA polymerase.

**DISCUSSION**

One of the most striking features of microorganisms is their ability to grow under a wide range of osmolarity, temperature, or nutrient availability conditions. To survive under detrimental conditions, bacteria have acquired the ability to adapt their
structures and physiologies rapidly. These mechanisms are based on the existence of multiple regulatory networks in which genes are regulated in a coordinate manner in response to environmental stimuli. Motility and chemotaxis are processes known to be affected by numerous factors, e.g., salts and temperature (32). This suggests that flagellar biosynthesis is a complex process involving multiple controls on gene expression.

In the present study, we attempted to determine more precisely the role of H-NS and cAMP-CAP on flagellar gene expression and, in particular, on the flhDC master operon. We showed that mutations affecting hns or crp resulted in up to a 100-fold decrease in flhDC-cat expression (Table 3). Although the decrease in cat activity was less in the hns mutant than in the crp strain, it is still sufficient to explain the complete lack of motility associated with hns mutation in E. coli (7, 14, 55). First, the absence of flagellin synthesis in hns and crp mutants resulted from a similar strongly reduced expression in flIC, the flagellin structural gene (Fig. 1), and in fliA, encoding the flagellar sigma factor (data not shown). These data are in agreement with a previous electron microscopy observation showing that E. coli mutant strains deficient in H-NS or CAP lack flagella (7, 54). Second, it has been previously demonstrated that mutations affecting the level of either the heat shock protein DnaK (52) or the initiating factor of chromosomal DNA replication, DnaA (40), or mutations resulting in a modification of the membrane phospholipid content (50), as well as some adverse conditions such as temperature (51), can lead to a strong reduction in fliA and/or fliC transcription despite a moderate reduction in flhDC expression. Third, a high level of flhDC expression from a multicopy plasmid was able to overcome the moderate repression of the master operon in the hns strain (Table 3), resulting in a restoration of motility in this mutant (Table 2). Although, it has been shown that H-NS can interact with FlgG, a protein of the flagellar motor (14, 37), our results provide evidence that the altered swarming behavior in such an hns mutant mainly results from the control by H-NS of flagellum synthesis rather than of its functioning.

To investigate the molecular mechanism by which H-NS protein and the cAMP-CAP complex control the flhDC operon, we performed gel shift assays and DNase I footprinting experiments. These experiments demonstrated that both proteins are able to bind to the regulatory region of the master operon in vitro. A unique CAP binding site was identified centered at position −71.5 upstream from the +1 transcription start site (Fig. 5). Its location relative to the +1 site and its strong homology with the CAP binding site consensus sequence suggest that the binding of the cAMP-CAP complex to this site leads to flhDC activation (12, 53). On the other hand, several H-NS binding sites were identified in the flhDC regulatory region (Fig. 5). Such multiple binding sites have been previously observed with H-NS on the promoter region of different E. coli genes, such as hns (17), proU (34), lac (47), mmb (56), and virF (16).

H-NS was shown to positively affect the synthesis of flagella (Fig. 1), although it has usually been described as a general repressor of transcription (59). Therefore, it was of interest to know whether the positive effect of H-NS on flhDC expression was a direct consequence of its binding to the promoter region of the master operon. In vitro transcription experiments performed with H-NS showed that this regulator represses in vitro expression of the master operon (Fig. 6). In contrast, we demonstrated that CAP is able to promote flhDC activation (Fig. 6). Such a positive regulatory role is further supported by characterization of a cfs mutation. The nucleotide substitutions observed in the promoter region of the cfs mutant, known to synthesize flagella in a CAP-independent manner (Fig. 2), are somewhat reminiscent of the mutations in the lacUV5 promoter, known to restore promoter activity in the absence of the cAMP-CAP complex. Moreover, CAP proteins carrying mutations in activating region I, a region involved in interactions between CAP and the C-terminal part of the RNA polymerase α subunit, were unable to complement the loss of motility in crp mutant strains (Table 4). This provides evidence that the positive control of CAP results from a direct interaction between the cAMP-CAP complex and RNA polymerase.

![FIG. 6. In vitro transcription assay by RNA polymerase from flhDC promoter.](https://example.com/figure6)

TABLE 2. Motility of E. coli strains expressing wild-type or mutated CAP protein on semisolid agar plates

<table>
<thead>
<tr>
<th>Strain genotype</th>
<th>Plasmid (protein)</th>
<th>Motility*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>None</td>
<td>30 ± 3</td>
</tr>
<tr>
<td>crp::Sm</td>
<td>None</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>crp::Sm pDCRP</td>
<td>(CAP)</td>
<td>21 ± 2</td>
</tr>
<tr>
<td>crp::Sm pDCRP-T158A</td>
<td>(mutated CAP)</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>crp::Sm pDCRP-H159L</td>
<td>(mutated CAP)</td>
<td>3 ± 1</td>
</tr>
</tbody>
</table>

*Expressed as the diameter of the swarming ring (in millimeters) after 15 h at 30°C. The data are the mean values ± standard deviations of three independent experiments.

![TABLE 3. Effect of hns and/or crp mutations on flhDC-cat transcriptional fusion activity](https://example.com/table3)
Recently, interactions between H-NS and various regulators have been demonstrated. For example, H-NS is known to interfere with σ^7 and Lrp in transcription of the osmC gene (8) and with FNR and CAP in the regulation of cai and fix operons (11). In some cases, the role of the activator, e.g., CAP, in the regulation of bgl and pap operons (18, 41) and σ^7 in the regulation of csgBA (2) has been thought to relieve, at least in part, the repression mediated by H-NS. Using in vivo (Table 3) and in vitro (Fig. 6) assays, we demonstrated that CAP relieves the repression mediated by H-NS on the activity of the sole flhDC promoter region. However, this mechanism is not sufficient to explain the complex regulation affecting the flhDC master operon, as suggested by our previous observation that css mutants synthesizing flagella in a CAMP-CAP-independent manner remain nonflagellate in an hns background (7). First, gel retardation and footprinting experiments demonstrated that H-NS and CAP are able to bind together to the flhDC promoter fragment, without altering significantly the binding of the other regulator (Fig. 5). Moreover, an hns crp double mutant was completely deficient in flagellin accumulation and in flIC mRNA synthesis (Fig. 1), in agreement with the lack of motility recently observed in a similar mutant in S. typhimurium (30). Finally, H-NS exerted a positive control in vivo of the full-length flhDC regulatory region extending to the translational ATG start codon (Table 3).

During the last decade, the regulation of the proU operon has been extensively studied, in particular, with regard to H-NS (21). However, it has recently been demonstrated that the mechanism of proU repression by H-NS cannot be explained solely by the binding of the regulator to the promoter region (28). Similarly, our data provide evidence that the in vitro binding of H-NS to the flhDC regulatory region (Fig. 6) is not sufficient to explain its clear positive control observed in vivo on flagellar-gene expression (Fig. 1 and Table 3). One hypothesis would be that H-NS acts indirectly on flhDC by regulating the synthesis of, or by interacting with another protein required for full expression of, the master operon. The observation that the positive control by CAP was also modulated by the region extending from the +1 transcriptional start site to the ATG translational codon (Table 3) further supports the involvement of an ancillary factor in the regulation of flhDC expression in vivo. In any case, an understanding of the mechanism by which H-NS affects expression of the flagellar master operon, the first example of positive control studied so far at the molecular level, will require further investigation.

The function of the flagellum-chemotaxis regulon seems to play an important role in adaptation to stressful environmental conditions. In this respect, the master operon flhDC constitutes a good example of stress-responsive genes. The regulation of its expression requires a complex network involving several regulators. In addition to CAP and H-NS, Hu, Fis, and/or Lrp have been suggested to affect the flagellum-chemotaxis regulon in E. coli (42), S. typhimurium (43), and Proteus mirabilis (25). Such a multiple control of motility in enterobacteria could be the basis of a fine tuning of flagellar-gene expression in response to environmental challenges.

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