INFECTION AND IMMUNITY, Apr. 2001, p. 2502–2511 Vol. 69, No. 4
0019-9567/01/$04.00+0 DOI: 10.1128/IAI.69.4.2502–2511.2001

Transcriptional Regulation of Divergent Capsule Biosynthesis and Transport Operon Promoters in Serogroup B *Neisseria meningitidis*

YIH-LING TZENG,[1] JOHN S. SWARTLEY,[2]† YOON K. MILLER,[1] RACHEL E. NISBET,[1] LI-JUN LIU,[1] JAY H. AHN,[1] AND DAVID S. STEPHENS[1,2,3]*

Departments of Medicine[1] and Microbiology and Immunology,[2] Emory University School of Medicine, and Department of Veterans Affairs Medical Center,[3] Atlanta, Georgia

Received 18 October 2000/Returned for modification 2 January 2001/Accepted 12 January 2001

The clinically important serogroups B, C, Y, and W-135 of *Neisseria meningitidis* produce sialic acid capsules that are critical in pathogenesis. In each of these serogroups, the capsule transport (*ctrABCD*) and capsule biosynthesis (*synABCD*) operons are divergently transcribed from putative promoters located in a 134-bp intergenic region (J. S. Swartley, J. H. Ahn, L. J. Liu, C. M. Kahler, and D. S. Stephens, J. Bacteriol. 178:4052–4059, 1996). In this study we further assessed the role of the intergenic sequence in the transcriptional regulation of the sialic acid capsules of *N. meningitidis*. Insertional mutagenesis or deletions of the 134-bp sequence in the serogroup B meningococcal strain NMB resulted in a marked reduction or elimination of *ctrABCD* and *synABCD* transcription, with a concomitant loss of encapsulation. Chromosomal transcriptional *lacZ-ermC* reporter fusions of *syn* and *ctr* promoters were constructed through allelic exchange. Using these constructs, both operons were found to be constitutively transcribed in meningococci, the biosynthesis operon about fourfold higher than the transport operon. Both promoters showed increased activity during stationary-phase growth. In addition to the promoters, a 70-bp 5′ untranslated region (UTR) upstream of *synA* was found to have a direct repeat and an inverted repeat that overlapped three putative integration host factor binding sites. Mutation of this 70-bp UTR and of the direct repeat upregulated both *syn* and *ctr* transcription. Regulation through the *synA* UTR was absent in a K1 *Escherichia coli* strain that produces identical capsular polysaccharide, implicating species-specific regulation. Meningococcal sialic acid capsule expression is initiated by divergent promoters in a 134-bp intergenic region, is repressed at the transcriptional level by the 5′ UTR of *synA*, is increased during stationary-phase growth, and shows species-specific regulation. Transcriptional regulation is another important control point for sialic capsule expression in *N. meningitidis*.

Capsular polysaccharide is a major virulence factor of *Neisseria meningitidis*. Of the 12 different meningococcal capsular polysaccharides so far defined, 5 (serogroups A, B, C, Y, and W-135) are most often associated with invasive disease. With the exception of serogroup A, these capsules are polymers of or contain sialic acid. Capsular polysaccharides protect the meningococcus from a variety of cellular and humoral host immune defenses, including phagocytosis, opsonization, and complement-mediated killing (16, 17), and allow survival during invasive meningococcal disease. In addition, the (α2→8)-linked polysialic acid capsule of serogroup B meningococci is a poor immunogen due to structural identity with surface antigens of human tissues such as the neural cell adhesion molecule N-CAM (34). Capsules also have an important role in meningococcal transmission by facilitating loss of meningococci from human mucosal surfaces and protecting the organism from environmental stress. During other events in human pathogenesis (e.g., nasopharyngeal colonization and attachment to epithelial and endothelial cells), meningococci that have downregulated or switched off capsule are selected (27, 35–37).

* Corresponding author. Mailing address: Division of Infectious Diseases, Emory University School of Medicine, 69 Butler St., SE, Atlanta, GA 30303. Phone: (404) 728-7688. Fax: (404) 329-2210. E-mail: dstep01@emory.edu.

† Present address: Office of Cooperative Research, Yale University, New Haven, CT 06520.

The genetic basis for the control of expression of meningococcal capsule has been partially elucidated. Frosch et al. (9) identified a 24-kb *cps* gene complex that encodes factors necessary for the expression of serogroup B capsule when cloned into *Escherichia coli*. We also defined this region in *N. meningitidis* by Tn*916* mutagenesis (28, 30, 33). Subsequent work has identified the genetic basis for the different meningococcal capsular polysaccharides (5, 32) and shown that regions A and C of the *cps* complex are critically involved in expression of the serogroups A, B, C, Y, and W-135 capsules (30, 32). For the sialic acid capsule-producing meningococci, region A consists of four polycistronic capsule biosynthetic genes, *synABCD*, while region C contains *ctrABCD*, responsible for capsule transport across the inner and outer membranes (Fig. 1A). We have shown that *synABCD* and *ctrABCD* are operons separated by a 134-bp intergenic region that contains putative promoters that initiate divergent transcription from adjacent start sites (Fig. 1B). Examination of the nucleotide sequences surrounding the transcriptional start sites (30) of both operons showed that the *synA* promoter had identity with the σ^{70} class of constitutive promoters, while *ctrA* was preceded by a perfect −10 extended promoter (19). Transcriptional activity of the putative promoters was confirmed when they were cloned in front of a *lacZ* reporter in *E. coli*, with the *synA* promoter exhibiting higher activity (30).

In this study we further investigated the hypothesis that the

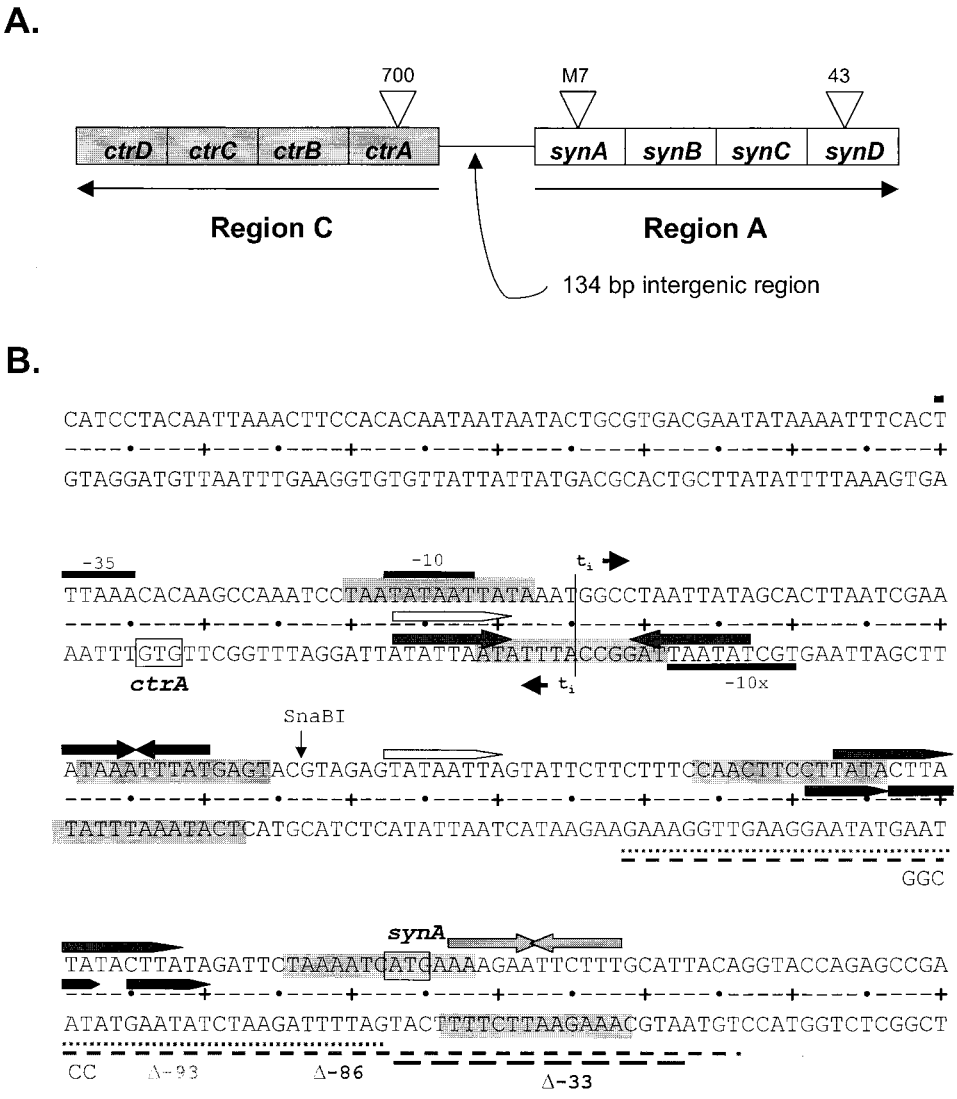

FIG. 1. (A) Schematic diagram of region A (biosynthesis operon, *synA*/*B*/*C*/*D*) and region C (capsule transport operon, *ctrA*/*B*/*C*/*D*) of the capsule locus in *N. meningitidis* with the locations of Tn*916* mutations (700, M7, and 43) noted (30). Arrows indicate the directions of transcription and the start codons of *synA* and *ctrA* separated by a 134-bp intergenic region. (B) Nucleotide sequence of the intergenic region from −95 to +145 of *synA* is shown. The −10 and −35 elements of the *synA* promoter are indicated above the sequence, while the extended −10 (−10x) sequence of *ctrA* is indicated below the sequence. The start codons for *synA* (ATG) and *ctrA* (GTG) are boxed. A vertical line marks the adjacent transcription initiation sites. The deletions of this region in mutants S(C)A286(Δ−86), S(C)A293(Δ−93), and S(C)A233(Δ−33) are indicated by broken lines underneath the sequence, and the base substitutions (GGCCC) in mutant S(C)A247 are specified below the sequence. Solid arrows above the sequence show the locations of inverted (IR) and direct (DR) repeats. Putative IHF binding sites are shaded in gray.

intergenic region separating the biosynthetic and capsule transport operons is critical for transcriptional regulation of serogroup B meningococcal capsule expression. Deletion, insertion, and site-directed mutagenesis of the intergenic region was performed, and transcriptional reporter gene fusions were constructed in order to define the role of the region in transcriptional regulation of meningococcal capsule expression.

MATERIALS AND METHODS

Strains and growth conditions. Strains used in this study are listed in Table 1. Meningococcal strains were grown on GC base agar (Difco Laboratories) supplemented with glucose and iron at 37°C with 3.5% carbon dioxide. Liquid cultures were vigorously aerated in GC broth with the same supplements and 4.3% sodium bicarbonate at 37°C. β-Galactosidase expression in reporter constructs was assayed using the Miller method (21). Meningococcal transformants containing the *lacZ-ermC* constructs were selected and maintained in the presence of erythromycin (3 μg/ml) (Sigma). *E. coli* strains were grown in Luria-Bertani (LB) broth (Bethesda Research Laboratories) at 37°C with appropriate antibiotic selection (erythromycin, 300 μg/ml; spectinomycin, 100 μg/ml; kanamycin, 50 μg/ml; and ampicillin, 100 μg/ml).

Transformation. Meningococci were transformed with DNA by the technique of Janik et al. (15). *E. coli* transformation was performed using the chemical transformation method described by Chung and Miller (4) or electroporation with a GenePulser (Bio-Rad).

DNA constructs. To create mutations in the 134-bp intergenic region, a 900-bp PCR product of primers LJ4 and JS44 (Table 2), containing the intergenic region and the 5′ ends of both the *ctrA* and *synA* genes, was cloned into pCR2.1 (Invitrogen) or pGEM-T (Promega), yielding pTINT and pGINT, respectively. An Ω-spectinomycin cassette with strong transcriptional and translational terminators on either side was obtained from pHP45Ω (23). A *Sma*I fragment of Ω

TABLE 1. Strains used in this study

Strain	Relevant characteristics[a]	Reference or source
E. coli		
DH5α	Cloning host	25
CAB1	K1 *E. coli* clinical isolate	Laboratory collection
N. meningitidis		
NMB	B:2B:P1.2,5:L2 (CDC8201085), parent strain for all following constructs	28
43	*synD*::Tn*916*, class I insertion	30
M7	*synA*::Tn*916*, class II insertion	33
700	*ctrA*::Tn*916*, class II insertion	30
ΩINT1	Ω(Sp^r) cassette inserted into *Sna*BI site within intergenic region	This study
ΔΩINT1	Same as ΩINT1 but with sequence between JS75 and JS74 primers deleted	This study
ΩINT2	Ω(Sp^r) cassette inserted into *Eco*RI site 10 bp downstream of *synA* ATG start codon	This study
ΔΩINT2	Same as ΩINT2 but with sequence between JS94 and JS93 primers deleted	This study
SA1	*lacZ-erm* cassette inserted into *Sna*BI site within intergenic region	This study
SA2	*lacZ-erm* cassette inserted into *Sna*BI site created within *synA* 339 bp from TTS	This study
CA2	*lacZ-ermC* cassette inserted into *Ssp*I site within *ctrA*, 285 bp from TTS	This study
SA286	SA2 strain with 70 bp between primers JS56 and JS86 deleted, removing both DR and IR	This study
CA286	CA2 strain with same deletion as SA286	This study
SA293	SA2 strain with 46 bp between primers JS56 and JS93 deleted, removing only DR	This study
CA293	CA2 strain with same deletion as SA293	This study
SA233	SA2 strain with 20 bp downstream of *synA* ATG start codon deleted, removing only IR	This study
CA233	CA2 strain with same deletion as SA233	This study
SA247	SSA2 strain with 5 bp (TTATA) mutated to CCGGG within DR	This study
CA247	CA2 strain with the same mutation as SA247	This study
988	*lacZ-ermC* cassette inserted into chromosomal locus 120A1	This study
SA295	*syn* promoter fragment containing 95 bp upstream of TTS fused to *lacZ-ermC* cassette and integrated into 120A1 locus	This study

[a] TTS, transcriptional start site; DR, direct repeat; IR, inverted repeat.

was inserted into the *Sna*BI site of pTINT and the *Eco*RI-Ω fragment was inserted into the *Eco*RI site of pGINT to generate pΩINT1 and pΩINT2, respectively. These plasmids were used to transform *N. meningitidis* strain NMB, and the spectinomycin-resistant transformants NMBΩINT1 and NMBΩINT2, respectively, were isolated. To create the NMBΔΩINT1 mutant, a ligation of the LJ4-JS75 and JS74-JS44 PCR products was used as a template and reamplified with external primers LJ4 and JS44. The resulting PCR product of the expected size was cloned into pCR2.1, and the Ω cassette was subsequently inserted into the *Sna*BI site to give pΔΩINT1. pΔΩINT1 was then used to transform strain NMB as described above. Analogously, the NMBΔΩINT2 mutant was generated by the LJ4-JS94 and JS93-JS73 pairs and cloned into pGEM, followed by the insertion of the Ω cassette into the *Eco*RI site. The mutations were verified by PCR amplification, Southern hybridization, and sequencing analysis.

To create the *ctrA*::*lacZ* (CA2) and *synA*::*lacZ* (SA1) reporter constructs, a PCR product of JS99-JS56 primers containing a unique *Ssp*I site within *ctrA* gene and a PCR product of LJ4-JS73 primers with a unique *Sna*BI site downstream of the *synA* transcriptional start site was cloned into pCR2.1. The appropriate transformants were digested with *Ssp*I or *Sna*BI and ligated with the *Bam*HI-blunted *lacZ-ermC* cassette, which contains a transcriptional terminator downstream of *ermC*, from pAErmC′G (22, 39). The constructs were transformed into *E. coli* strain DH5α, and transformants were selected on LB agar containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and erythromycin. The colonies were further tested for correct orientation of the *lacZ* gene relative to the transcription. The *lacZ-ermC* cassette was then integrated into wild-type meningococcal strain NMB via transformation and allelic exchange, with selection for erythromycin-resistant NMB transformants.

TABLE 2. Primers used in this study

Primer	Sequence
JS41	GGCCACTTAATTCTAATGCTTTG
JS44	GCTTGTTCATTTGCTACCAAGTGG
JS56	GAATACTAATTATACTCTACGTACTC
JS73	GAGAGATAAACGTAATGGTATTGC
JS74	GAGTACGTAGAGTATAATTAGTATTC
JS75	GTGTGGAAGTTTAATTGTAGGATG
JS86	GGTACCAGAGCCGACTTCGG
JS93	ATGAAAAGAATTCTTTGCATTACAGG
JS94	GTGTTTAAACTGAAATTTTATATTCGTC
LJ4	CCACCACCAAACAARACTGCCG
RN3	CAATACCATTACGTATATCTCTCG
RN7	CCAGCCGAAGCATAACCATCG
RN8	GAGAGATATACGTAATGGTATTGCC
YT01	CGGCTCTGGTACCTGTAGATTTTAGAATCT
YT03	CGATTGGGACGATATGACGG
YT05	GCCTGAGTGCCATCGCGCAT
YT29	TTATTTGTCGTCGTCGTCTTTGTAGTCATTAGTTAAATTATTAATACTGTTCGCGCC
YT30	GAGTAATTAAGAAGCTTGAGCAACTTCCTG
YT33	AGATTCTAAAATCTACAGGTACCAGAGCCG
YT47	GAATCTATAAGTACCCGGGTATAAGGAAGTTGG

A second *synA*::*lacZ* fusion (SA2) was generated downstream of the 20-bp inverted repeat. A unique *Sna*BI site was created by means of the PCR-ligation mutagenesis method of Ali and Steinkasserer (1). First, two halves of the *synA*-specific PCR products were amplified using special internal primers (JS74-RN8 and RN3-JS41) designed to introduce a new *Sna*BI restriction site into the *synA* sequence. These products were purified and mixed together (60 ng of each) to serve as the template for a secondary PCR amplification using nested primers (JS86-JS44). This product was ligated into pGEM and then transformed into *E. coli*. Colonies with the appropriate size insert were selected and digested with *Sna*BI to determine which insert had the new restriction site. Next, a blunt copy of the *lacZ-ermC* cassette was inserted into this new *Sna*BI site. This construct was transformed into NMB as described above.

Specific deletions of the intergenic region were created using similar PCR-ligation mutagenesis procedures. The sequence between primers JS56 and JS86 was removed in strains SA286 and CA286. The sequence between primers JS56 and JS93 was deleted in strains SA293 and CA293. Mutagenic primers YT01 and YT33 were used for removing the 20-bp palindromic sequence in strains SA233 and CA233, while the primer YT47, which contains a 5-base substitution to disrupt the direct repeat, was used for generating mutants SA247 and CA247.

Two promoter fragments, one between RN7 and JS73 and the other between LJ8 and JS56, were obtained by PCR and cloned into pBlue-Topo vector (Invitrogen) to generate transcriptional *lacZ* reporters. The promoter and *lacZ* fusions were released by *Spe*I-*Xba*I digestion and subcloned into pHP45 (*Sma*I) to give pYT140S and pYT141S. The resulting plasmids, which have the same promoter fragment as SA2 and SA1, were transformed into *E. coli* K1 strain CAB1. An ~5-kb PCR product that contains the *synA* promoter *lacZ-erm* fusion was amplified by *Taq* polymerase (Perkin-Elmer) and *Taq* Extender (Stratagene) from strain SA2 using primers LJ6 and JS44. This PCR fragment was cloned into an integration plasmid, which contained ~1 kb of intergenic meningococcal sequence. Incorporation of the PCR product within the *Hin*cII site of this sequence allowed homologous recombination into a chromosomal location that is distant from the *cps* locus. The double-crossover recombination into this locus and the intactness of the *cps* locus were confirmed by PCR.

RNA isolation and slot blots. Total RNA was purified according to the published procedure (30). RNA samples were denatured in 500 μl of cold denaturing buffer (10 mM NaOH, 1 mM EDTA) and immediately transferred to a Zeta-Probe GT membrane with a PR648 slot blot filtration manifold (Hoefer Scientific). The wells were rinsed once with 500 μl of denaturing buffer. The blotted membrane was rinsed in 2X SSC (1x SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS) before prehybridization for 5 min at 65°C in P buffer, composed of 0.5 M $NaHPO_4$ (pH 7.2), 1 mM EDTA, and 7% SDS. The membrane was hybridized with denatured DNA probe in P buffer overnight at 65°C. After hybridization, the membrane was washed twice in E buffer (40 mM $NaHPO_4$ [pH 7.2], 1 mM EDTA) containing 5% SDS and then twice in E buffer with 1% SDS, all at 65°C. The membrane was then subjected to autoradiography. The probe for *synA* transcription was PCR amplified from chromosomal DNA using JS44 and JS86 and labeled by random-primed labeling (Boehringer Mannheim) with [^{32}P]dATP (NEN DuPont).

Colony immunoblots. Colony immunoblots were performed essentially as described by Swartley et al. (30). Wild-type strain NMB served as the positive control for encapsulation, while the capsule-negative mutant strain M7 (28) was the negative control. The primary anti-serogroup B capsular monoclonal antibody 2-2-B was generously supplied by Wendell Zollinger (Walter Reed Army Institute of Research). The secondary antibody was goat anti-mouse immunoglobulin M (IgM)-IgG-alkaline phosphatase conjugate (Jackson Immunochemicals). The membranes were developed with NBT-BCIP (nitroblue tetrazolium–5-bromo-4-chloro-3-indolylphosphate).

Whole-cell ELISA. Serogroup B capsule-specific monoclonal antibody 2-2-B was employed in the whole-cell enzyme-linked immunosorbent assay (ELISA) following the published protocol (31) with minor modifications; 100 μl of a 1:3 dilution of the cell suspension (optical density at 650 nm [OD_{650}] = 0.1) was added to the microtiter plates. Incubation was done at 37°C instead of 33°C.

Statistical analysis. Student's *t* test with a two-tailed hypothesis was used to determine the significant difference ($P \leq 0.05$) between two variables in these studies.

RESULTS

Insertion and deletion mutagenesis confirmed that the *ctrA-synA* intergenic region is required for production and expression of serogroup B meningococcal capsular polysaccharide. A series of controlled deletion and Ω-spectinomycin cassette insertions in the 134-bp *ctrA-synA* intergenic region were created as described in Materials and Methods and are shown schematically in Fig. 2A. The production and expression of capsule were quantified by whole-cell ELISAs using monoclonal antibody 2-2-B, and the results are shown in Fig. 2B. The mutations within the intergenic region eliminated or dramatically reduced capsular polysaccharide expression ($P < 0.006$ for each mutant), indicating the essential role of this region in controlling the production and transport of capsule.

Transcription of *ctr* and *syn* operons is initiated from the intergenic region. The capsule-specific ELISA results suggested that capsule production is initiated within the intergenic region through transcriptional activation of the *syn* and *ctr* operons. To further confirm this hypothesis, changes in *synA* transcriptional level were examined by RNA slot blots. As shown in Fig. 3, the mutations within the 134-bp intergenic region reduced *synA* RNA expression. However, the *synA* mRNA signal from the *ctrA* mutant 700 (*ctrA*::Tn*916*), in which the transposon was at the bp 285 location of *ctrA* (−285 bp relative to the transcriptional start site of *synA*), yielded a *synA* mRNA signal similar to that of the wild-type strain. This result indicated that the 285-bp sequence upstream of the *synA* transcriptional start site was sufficient to control *synA* expression and the inactivation of *ctrA* had no significant influence on *synA* transcription.

***syn* promoter is constitutively more active than *ctr* promoter, and transcription from both promoters is growth phase dependent.** To further investigate the relative strength and possible mechanisms of regulation of the *syn* and *ctr* promoters, a *lacZ-ermC* cassette was inserted downstream of both promoters, creating transcriptional reporter strains. Two *syn* reporter strains were constructed, one with *lacZ* inserted in the *synA* coding region (SA2), and the other with *lacZ* in the 5′ untranslated region (UTR) of *synA* (SA1) (Fig. 2A). The *ctr* promoter strain (CA2) contained *lacZ* in the coding region. Overnight cultures of the meningococcal reporter strains were diluted into fresh GC broth and grown with aeration at 37°C, and the expression of β-galactosidase activity was monitored (Fig. 4A). The *ctr* promoter reporter strain (CA2) produced low levels of activity that increased moderately (~2-fold) when entering the stationary phase. The SA2 *syn* construct gave an expression pattern similar to that of CA2, but was about fourfold higher ($P < 0.05$). The SA1 *syn* construct consistently yielded ~2-fold higher activity than that of the SA2 reporter strain throughout growth ($P < 0.05$). These results suggested that the sequence between the insertion sites of SA1 and SA2 had a negative or downregulating role in *syn* transcription, yet the growth phase-dependent regulation was not located in this region, since SA1 still produced an ~2-fold increase in the stationary phase.

Determination of minimal promoter region required for *syn* transcription. As described above, a Tn*916* insertion (*ctrA* mutant 700) at bp −285 of the *synA* transcription start site did not alter the *synA* mRNA level in RNA slot blot experiments (Fig. 3). This result was also confirmed by the transcriptional reporter assays. As shown in Fig. 4B, the SA2 *syn* reporter produced similar activity in either the wild-type or *ctrA*::Ω background, indicating that no *cis* element was present beyond −285 bp. To confirm these results, a *lacZ* transcriptional fusion construct of *synA* was also generated at a distant heterologous chromosomal locus, site 120A1, which has no intrinsic pro-

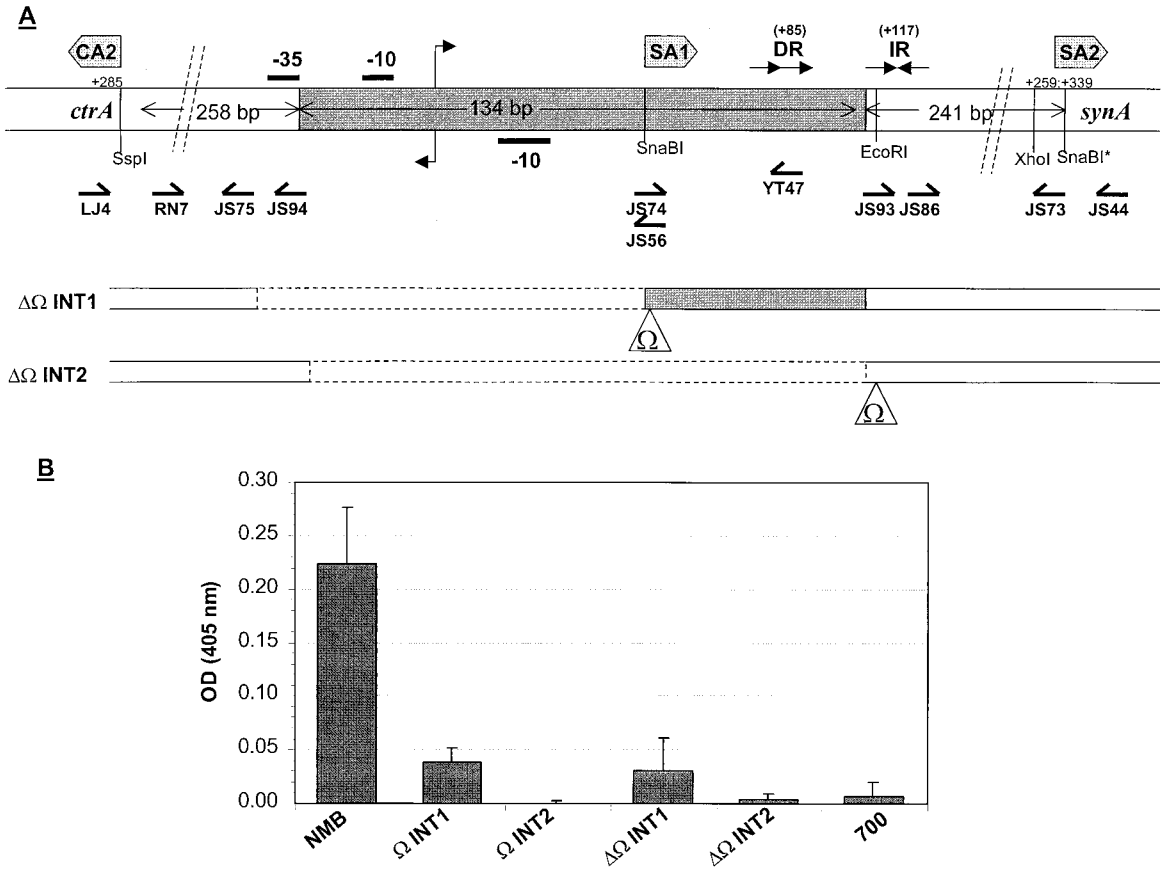

FIG. 2. (A) Schematic diagram of the capsule biosynthesis and transport operon intergenic region. The locations of various primers (half-arrows) used in this study are noted below the diagram. The shaded bar is the 134-bp intergenic region between the two start codons of *synA* and *ctrA*. Base numbering is from the corresponding transcriptional start sites, which are indicated by bent arrows. The repeats examined in mutagenesis studies are also shown as arrows. The Ω cassette was inserted into the *SnaB*I site within the 5′ UTR of *synA* in the two INT1 constructs, whereas the two INT2 constructs were created with the Ω cassette inserted at the start of the coding sequence. In addition, the sequence between primers JS75 and JS74 was deleted in the ΔΩINT1 strain, while the sequence between JS94 and JS93 was removed in the ΔΩINT2 construct. (B) Capsule whole-cell ELISA of wild-type strain NMB and meningococcal mutants ΩINT1, ΩINT2, ΔΩINT1, and ΔΩINT2. An unencapsulated *synA*::Tn*916* mutant (28) was used as the negative control. A *ctrA*::Tn*916* mutant, 700 (30), is included for comparison. Results are shown as the average values from three independent experiments.

moter activity. This construct, SA295, has an intact capsule locus and contains at the distant site the *lacZ* reporter and the −95 bp upstream sequence of the *synA* transcriptional start site. The SA295 construct had ~80% of the transcriptional activity of the SA2 strain. These results suggested that most of the required *cis* transcriptional determinants for the *syn* operon resided in the 95 bp upstream of the *synA* transcriptional start site.

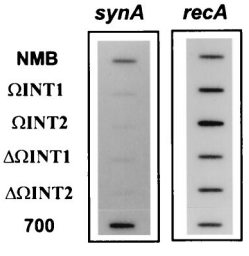

FIG. 3. RNA slot blots of *synA* transcription. Total-cell RNA was purified from parental strain NMB and mutants ΩINT1, ΩINT2, ΔΩINT1, and ΔΩINT2, as described in Materials and Methods. The mRNA level of *recA* was also measured and used as a control for RNA loading.

***syn* and *ctr* operons do not appear to be influenced by product inhibition or feedback regulation.** Many biosynthesis pathways utilize product inhibition or feedback regulation to control overall transcription and product synthesis. For example, the expression of capsule transport proteins could be regulated by the biosynthesis operon substrates (e.g., expression of the *ctrABCD* transport operon may be influenced by the amount of sialic acid or capsule polymer synthesized by the *synABCD* gene products). To test whether feedback regulation influenced the meningococcal capsule region, *ctr* transcriptional fusions were generated in backgrounds with biosynthesis operon mutations, *synA*::Tn*916*, *synC*::Ω or *synD*::Tn*916*, and the corresponding transcriptional activity was compared to that in constructs without these mutations. No significant difference in transcriptional activities was noted in these backgrounds (data not shown). Similarly, disruption of *ctrA* with insertion of an Ω cassette did not affect *syn* operon expression (Fig. 4B). In

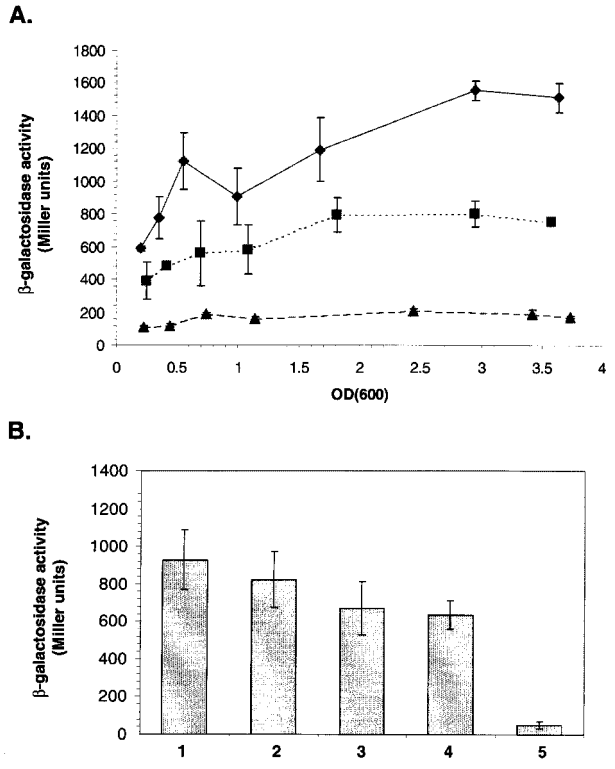

FIG. 4. (A) Expression of a *lacZ* reporter located in *synA* in SA1 (diamonds) or SA2 (squares) or in *ctrA* in CA2 (triangles) during growth. Each time point shows the average value of three experimental measurements, and data are representative of three independent assays. (B) Expression of *synA*::*lacZ* reporters during mid-log growth phase ($OD_{550} \approx 0.5$) in different meningococcal backgrounds: 1, SA2, wild type; 2, SA2 in a *ctrA*::Ω background; 3, SA295, *synA*::*lacZ* with −95 to +339 promoter fragment of *synA* fused with *lacZ-ermC* and integrated into a noncoding chromosomal locus and retaining an intact capsule A and C region; 4, SA295 in a *synA*::Tn*916* background; 5, *lacZ-ermC* cassette without promoter integrated into the same locus as SA295.

addition, the production and expression of capsule did not influence *syn* operon expression, since the distant-site SA295 *syn* construct had similar activity in encapsulated and unencapsulated (*synA*::Tn*916*) backgrounds (Fig. 4B). Finally, the inactivation of polysialyltransferase (*synD*::Tn*916*) did not affect the transcription of the *syn* (SA2) reporter construct (data not shown). Overall, these results indicated that, at least under in vitro growth conditions, the *ctr* and *syn* operons did not exhibit evidence of product or feedback regulation.

5′ UTR of *synA* inhibits both *syn* and *ctr* operon transcription. A transcription-regulatory role of the 5′ UTR sequence of *synA* was suggested because of the difference in transcription between the SA1 and SA2 *syn* constructs (Fig. 4A). Additional mutations were generated in this region to further characterize important elements. A 16-bp direct repeat (+78 to +93 relative to the *synA* transcriptional start site) and a 20-bp inverted repeat (+108 to +127) were identified within the sequence between the *Sna*BI and the *Eco*RI sites (Fig. 1B). In addition, several putative integration host factor (IHF) binding sites were identified in the intergenic region using the *E. coli* IHF consensus sequence 5′-YAANNNNTTGATW-3′ (where Y is C/T, W is A/T, and N is G/A/T/C), three of these overlapped either the direct or inverted repeat. Specific deletions that removed either the direct (mutants SA293 and CA293) or inverted (mutants SA233 and CA233) repeat or both repeats (mutants SA286 and CA286) were made in both *syn* and *ctr* reporter strains (Fig. 1B and 5). In addition, a 5-base substitution disrupting the symmetry of the direct repeat (mutants SA247 and CA247) was also generated in the reporter strains.

Figure 5 shows the activities of these mutants relative to those of the wild-type parent strain. Deletion of the 20-bp inverted repeat (mutants SA233 and CA233) modestly decreased *syn* transcription but had no effect on *ctr* expression. In contrast, deletion of the direct repeat, which precedes the ribosome-binding site, increased *ctr* (mutants CA293 and CA247) and, to a lesser degree, *syn* expression (mutants SA293 and SA247). Removing both repeats (mutant CA286) enhanced *ctr* expression ~3-fold, while *syn* expression increased 50% (mutant SA286). Furthermore, the increase in transcription seen with deletions of both repeats (mutants SA286 and CA286) was more significant than with the individual deletion of the repeats, implying a synergistic effect between these structural elements.

No inhibition of *synA* and *ctrA* transcription by the 5′ UTR of *synA* in an *E. coli* K1 background. The identical capsule [(α2→8)-linked polysialic acid] expressed by serogroup B meningococci is expressed in *E. coli* K1 strains. The *E. coli* K1 strain is the etiologic agent of human neonatal meningitis, and the K1 capsule locus has been extensively investigated (38). Meningococcal capsular biosynthesis and transport gene homologues have been identified in the K1 *E. coli* strain. In order to examine if the meningococcal regulation machinery was present in this genetic background, the intergenic region was fused with a promoterless *lacZ* gene (pBlue-Topo), subcloned into a pBR322-derived plasmid (pHP45), and transformed into a clinical isolate of the K1 strain, CAB1. The 5′ end of the promoters contained the sequence downstream of the *Ssp*I site in *ctrA*, defined above as being of sufficient promoter length for controlling *syn* expression. The 3′ end of the promoters corresponded to either the SA1 or SA2 meningococcal construct, respectively. The SA1 construct should have higher activity than the SA2 construct if analogous regulation is present in the K1 strain. However, SA1 and SA2 produced similar activity in the *E. coli* K1 background (Fig. 6), indicating that the regulatory mechanisms that yield higher activity in the SA1 construct were absent in the *E. coli* K1 strain.

DISCUSSION

Recent studies have genetically and biochemically characterized the capsule transport *ctrABCD* and biosynthesis *syn ABCD(E, F)* operons of the *cps* gene complex in serogroups B, C, Y, and W-135 *N. meningitidis* (5, 9, 30–33). Insertional mutagenesis of genes required for capsule biosynthesis and capsule transport produces unencapsulated phenotypes, confirming the necessity of these genes (30). A number of studies have indicated that the expression of meningococcal capsules is closely associated with virulence (16–18) but that capsule may not be constitutively expressed on the meningococcal surface (27, 35–37). These data indicate that expression of capsule is subject to regulation.

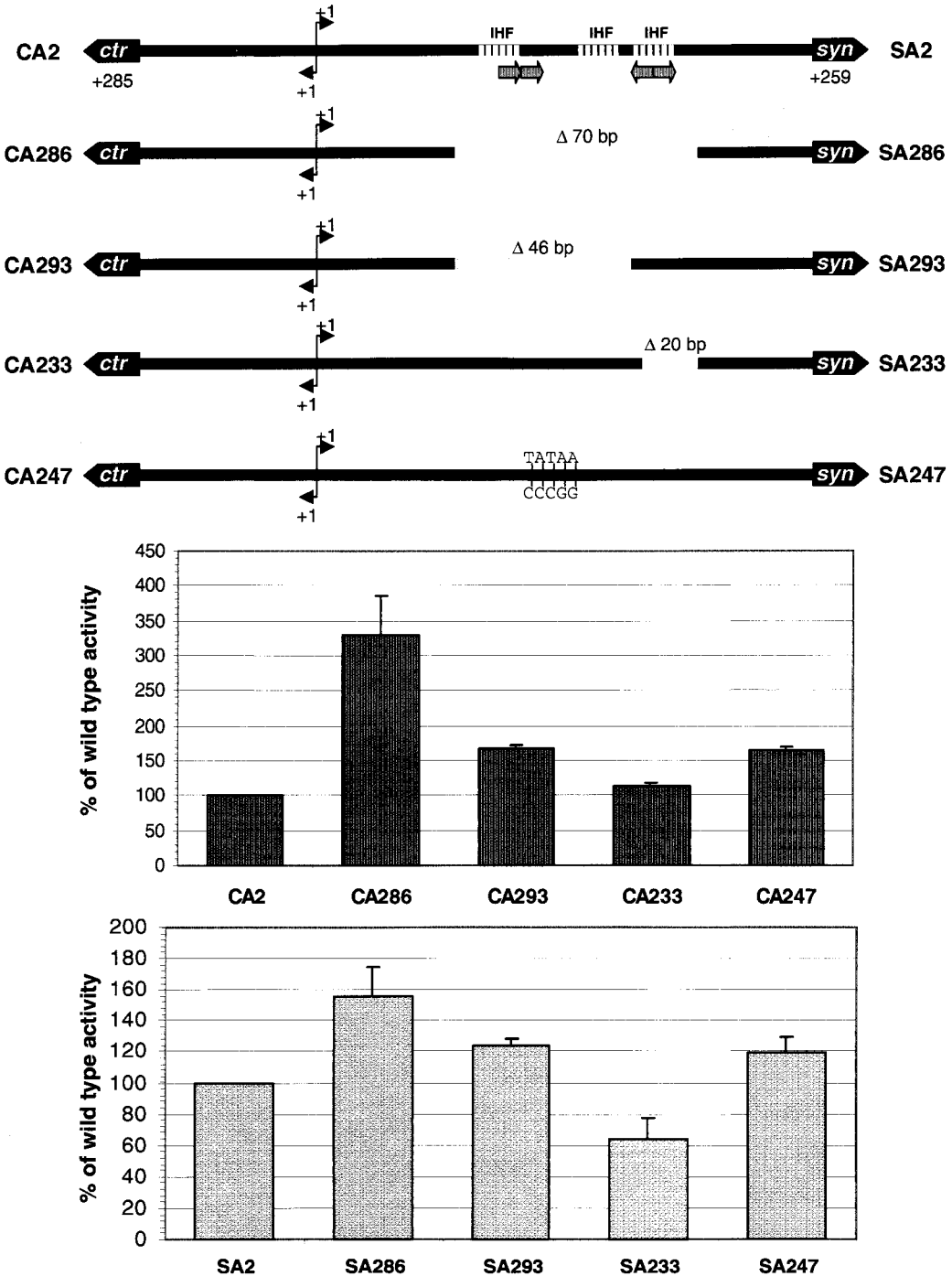

FIG. 5. Transcriptional activities of *synA*::*lacZ* (SA2) and *ctrA*::*lacZ* (CA2) reporters in *N. meningitidis* containing mutations within 5′ UTR of *synA*. The mutations are shown in the diagram at the top and also noted in Fig. 1B. Each strain was grown in GC medium to mid-exponential phase, and β-galactosidase activities were assayed by the method of Miller (21). The values are presented as the percentage of the wild-type activity (SA2, 929.2 ± 158.0 U; CA2, 227.0 ± 29.8 U). Data shown are the averages of at least three independent experiments. Two-tailed unpaired Student's *t* test indicated that values for all mutants except SA293 and CA233 are statistically significantly different from that of the wild-type parent strain ($P < 0.05$).

Capsule expression in *N. meningitidis* is known to be controlled through genetic on-off switch mechanisms and possibly through posttranscriptional modification. Two kinds of on-off switch mechanisms of meningococcal capsule expression have been defined. Reversible insertion and the excision of a naturally occurring insertion element, IS*1301*, into *synA* have been shown in a serogroup B strain, B1940 (11). Other IS*1301* insertions in the capsule biosynthesis operon eliminate capsule expression (32; J. Dolan-Livengood and D. S. Stephens, unpublished data). In addition, a poly(C) track within *synD*, the

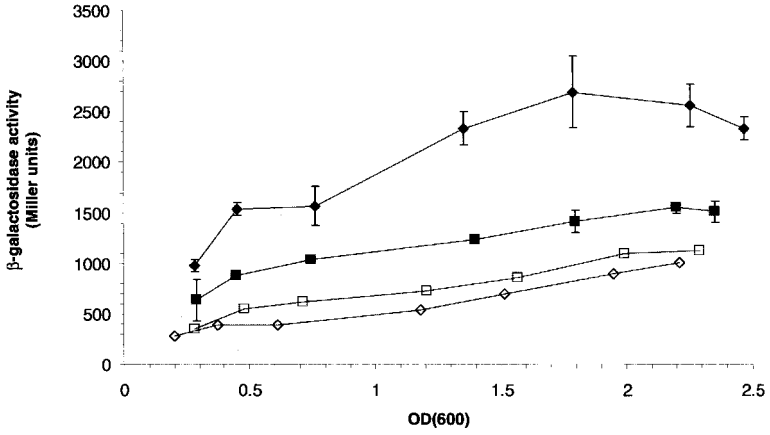

FIG. 6. Expression of *synA*::*lacZ* transcriptional fusions in genetic backgrounds of meningococcal strain NMB (solid symbols) and *E. coli* K1 strain CAB1 (open symbols). Meningococcal reporter strains SA2 (solid squares) and SA1 (solid diamonds) have a *lacZ-ermC* cassette recombined in the *synA* locus on the chromosome, while *E. coli* strains contain a low-copy-number plasmid with the promoter fusion of *lacZ* corresponding to that of SA2 (open squares) and SA1 (open diamonds). Each time point was measured in triplicate, and the results of one representative experiment are presented.

polysialyltransferase, of serogroup B strains alters capsule expression at a frequency of 10^{-3} through a slipped-strand mispairing mechanism (12). Finally, enzymatic activity of a CMP-sialic acid hydrolase has been detected in meningococcal cell extracts and is proposed to be a posttranscriptional regulatory mechanism for meningococcal capsular polysaccharide (20).

We found that transcriptional regulation is also a pathway for control of sialic acid capsule expression in *N. meningitidis*. We have previously shown that the first genes of the biosynthesis and transport operons, *synA* and *ctrA*, respectively, are transcribed divergently from abutting start sites located in a 134-bp intergenic region. Furthermore, the sequence of this intergenic region is identical among all serogroups synthesizing capsules containing sialic acid (30). The data presented in this report confirm that the 134-bp intergenic region contains the promoters of these operons and identifies other transcriptional regulatory elements of serogroup B meningococcal capsule expression. Coordinate transcriptional regulation of divergent promoters in bacterial pathogens is well described. In *Vibrio cholerae*, for example, two virulence genes, *acfA* and *acfD*, are transcribed in opposite directions from a 173-bp intergenic region (8). Expression of these genes is under the coordinate control of ToxR, which activates the promoter of *acfD* and represses *acfA* transcription.

The *syn* and *ctr* promoters present in the 134-bp intergenic region were both capable of actively transcribing *lacZ* in the meningococcus. The *ctr* promoter produced lower levels of *lacZ* transcription than the *syn* promoter in a meningococcal background, confirming previous work with these promoters in *E. coli* (30). The *syn* promoter is a near consensus σ^{70}-type promoter, while the *ctr* promoter is similar to the −10 extended promoter class, originally identified in work with lambdoid phages in *E. coli*, and lacks a consensus −35 recognition sequence (19). Under most conditions, RNA polymerase would be predicted to bind tightly to the consensus σ^{70} *syn* promoter, instead of the −10 extended *ctr* promoter, and thus transcribe the biosynthesis operon more efficiently. Sialic acid, which is synthesized by SynA/B/C proteins of the biosynthesis operon, is used for lipooligosaccharide (LOS) sialylation as well as capsule polymerization. This might explain why the *syn* promoter is constitutively expressed at a high level. Indeed, a mutation that inactivates the serogroup B meningococcal polysialyltransferase shifts the partially sialylated LOS of the wild-type parent strain to a fully sialylated LOS (18) and indicates shunting of sialic acid into the LOS pathway when capsule expression is blocked. Since the only known function of the proteins encoded by the capsule transport operon appears to be transport of the assembled capsule polymers, these proteins may not need to be expressed in high quantity.

A relatively small (<200 bp) upstream region controls the *syn* operon. This mechanism is not uncommon. The production of an exopolysaccharide by the phytopathogen *Pseudomonas solanacearum* is accomplished by an 18-kb gene cluster that is controlled by a 140-bp region upstream of the transcription start site and a multicomponent regulatory network (14). The regulation of bundle-forming pili in enteropathogenic *E. coli* is controlled by the sequence within −95 bp upstream of the transcriptional start point (24).

In addition to the promoters, we identified within the 134-bp region a 70-bp sequence of the 5′ UTR of *synA* that influences both *ctr* and *syn* promoter activity. A 20-bp inverted repeat of this region, which includes the *synA* start codon, was suggested as a possible regulatory element for transcription of *syn* and *ctr* operons (6). In *Neisseria gonorrhoeae* and *N. meningitidis*, a 16-bp inverted repeat encompassing the putative ribosome-binding site of the *rfaC* gene, encoding the LOS α1,5-heptosyltransferase I, is involved in the transcriptional regulation of this gene (40). Deletion of the 20-bp inverted repeat sequence moderately decreased *syn* transcription but had no effect on *ctr* expression. In contrast, deletion of a 16-bp direct repeat which precedes the ribosome-binding site enhanced both *ctr* and *syn* expression. Interestingly, our laboratory had previously shown that clinical meningococcal isolates occur with deletions in the direct repeat. For example, a serogroup W-135 strain was found to have an 8-bp deletion and 2-nucleotide substitution in the direct repeat (30). The elimination of the direct repeat element in vivo would be predicted to upregulate *syn* and *ctr* promoter activities and capsule expression in meningococci.

A 70-bp deletion that removed both the inverted and direct repeats produced the most significant increase in transcription, suggesting that the combination of the direct and inverted repeats facilitated the greatest repression of transcription of the *syn* and *ctr* operons. An arrangement similar to this direct-inverted repeat motif is found downstream of the *vrg* promoters in *Bordetella pertussis* and is thought to be the target for a transcriptional repressor that binds to this region and prevents transcription (2). This direct-inverted repeat sequence may also assume a topology that interferes with RNA polymerase binding, and the removal of this region enhances the efficiency of the transcription complexes. Alternatively, the sequence within the 5′ UTR of *synA* may influence the stability of mRNA and therefore modulate the transcriptional level of *syn* genes. Interestingly, three putative IHF binding motifs were also identified within the 70-bp region. IHF is known to bend DNA within the promoter region and modulate transcription (10). The involvement of IHF in the regulation of K5 capsule gene clusters in pathogenic *E. coli* has recently been reported (26).

Two-component regulatory systems have been identified that control capsular polysaccharide production and expression in bacterial pathogens. For example, the colanic acid capsule of *E. coli* is controlled by the RcsB-RcsC two-component system (29), while the AlgQ-AlgR system controls the alginate capsule in *Pseudomonas aeruginosa* (7) and the CsrR-CsrS system is involved in hyaluronic acid capsule production in *Streptococcus pyogenes* (13). In preliminary studies, we have not found that transcription of the sialic acid-containing meningococcal capsules is influenced by environmental conditions that act as triggers for two-component regulatory systems. Changes in temperature, pH, osmolarity, iron, carbon source, and serum exposure have not affected *syn* or *ctr* transcription. In support of these observations, only four putative response regulators in the serogroup A and serogroup B meningococcal genomes have been noted. In contrast, *E. coli* and *Bacillus subtilis* have 34 and 35 response regulator proteins, respectively. Mutations in three of the putative meningococcal two-component regulatory systems showed no effect in *syn* or *ctr* transcription (Y.-L. Tzeng and D. S. Stephens, unpublished data).

We did find that transcription of the *syn* operon occurs at the highest levels during the stationary phase of the meningococcal growth curve. The increase in synthesis of sialic acid for incorporation into the LOS or the polysialic acid capsule pathway appears greatest during the later stages of the bacterial growth curve. In the plant pathogen *Erwinia stewartii*, a homoserine lactone autoinducer is required for the induction of capsule biosynthesis (3). The possibility of a quorum-sensing mechanism regulating capsule expression in *N. meningitidis* will require further study.

In summary, expression of the sialic acid capsule of *N. meningitidis* is initiated by divergent promoters located within a 134-bp intergenic region separating the biosynthesis and transport operons. Transcription of these operons is repressed by a 70-bp 5′ UTR of *synA*, is increased during stationary growth, and shows species-specific regulation. The 134-bp *synABCD-ctrABCD* intergenic region is an important control point for the transcriptional regulation of sialic acid capsule expression.

ACKNOWLEDGMENTS

We thank Lane Pucko for administrative assistance.

This work was supported by Public Health Service grant AI/40247 (to D.S.S.) from the National Institute of Allergy and Infectious Diseases.

REFERENCES

1. **Ali, S. A., and A. Steinkasserer.** 1995. PCR-ligation-PCR mutagenesis: a protocol for creating gene fusions and mutations. Biotechniques **18:**746–750.
2. **Beattie, D. T., S. Knapp, and J. J. Mekalanos.** 1990. Evidence that modulation requires sequences downstream of the promoters of two vir-repressed genes of *Bordetella pertussis*. J. Bacteriol. **172:**6997–7004.
3. **Beck von Bodman, S., and S. K. Farrand.** 1995. Capsular polysaccharide biosynthesis and pathogenicity in *Erwinia stewartii* require induction by an *N*-acylhomoserine lactone autoinducer. J. Bacteriol. **177:**5000–5008.
4. **Chung, C. T., and R. H. Miller.** 1988. A rapid and convenient method for the preparation and storage of competent bacterial cells. Nucleic Acids Res. **16:**3580.
5. **Claus, H., U. Vogel, M. Muhlenhoff, R. Gerardy-Schahn, and M. Frosch.** 1997. Molecular divergence of the *sia* locus in different serogroups of *Neisseria meningitidis* expressing polysialic acid capsules. Mol. Gen. Genet. **257:** 28–34.
6. **Collado-Vides, J., B. Magasanik, and J. D. Gralla.** 1991. Control site location and transcriptional regulation in *Escherichia coli*. Microbiol. Rev. **55:** 371–394.
7. **Deretic, V., and W. M. Konyecsni.** 1989. Control of mucoidy in *Pseudomonas aeruginosa*: transcriptional regulation of *algR* and identification of the second regulatory gene, *algQ*. J. Bacteriol. **171:**3680–3688.
8. **DiRita, V. J., C. Parsot, G. Jander, and J. J. Mekalanos.** 1991. Regulatory cascade controls virulence in *Vibrio cholerae*. Proc. Natl. Acad. Sci. USA **88:**5403–5407.
9. **Frosch, M., C. Weisgerber, and T. F. Meyer.** 1989. Molecular characterization and expression in *Escherichia coli* of the gene complex encoding the polysaccharide capsule of *Neisseria meningitidis* group B. Proc Natl. Acad. Sci. USA **86:**1669–1673.
10. **Goosen, N., and P. van de Putte.** 1995. The regulation of transcription initiation by integration host factor. Mol. Microbiol. **16:**1–7.
11. **Hammerschmidt, S., R. Hilse, J. P. van Putten, R. Gerardy-Schahn, A. Unkmeir, and M. Frosch.** 1996. Modulation of cell surface sialic acid expression in *Neisseria meningitidis* via a transposable genetic element. EMBO J. **15:**192–198.
12. **Hammerschmidt, S., A. Muller, H. Sillmann, M. Muhlenhoff, R. Borrow, A. Fox, J. van Putten, W. D. Zollinger, R. Gerardy-Schahn, and M. Frosch.** 1996. Capsule phase variation in *Neisseria meningitidis* serogroup B by slipped-strand mispairing in the polysialyltransferase gene (*siaD*): correlation with bacterial invasion and the outbreak of meningococcal disease. Mol. Microbiol. **20:**1211–1220.
13. **Heath, A., V. J. DiRita, N. L. Barg, and N. C. Engleberg.** 1999. A two-component regulatory system, CsrR-CsrS, represses expression of three *Streptococcus pyogenes* virulence factors, hyaluronic acid capsule, streptolysin S, and pyrogenic exotoxin B. Infect. Immun. **67:**5298–5305.
14. **Huang, J., and M. Schell.** 1995. Molecular characterization of the *eps* gene cluster of *Pseudomonas solanacearum* and its transcriptional regulation at a single promoter. Mol. Microbiol. **16:**977–989.
15. **Janik, A., E. Juni, and G. A. Heym.** 1976. Genetic transformation as a tool for detection of *Neisseria gonorrhoeae*. J. Clin. Microbiol. **4:**71–81.
16. **Jarvis, G. A.** 1995. Recognition and control of neisserial infection by antibody and complement. Trends Microbiol. **3:**198–201.
17. **Jarvis, G. A., and N. A. Vedros.** 1987. Sialic acid of group B *Neisseria meningitidis* regulates alternative complement pathway activation. Infect. Immun. **55:**174–180.
18. **Kahler, C. M., L. E. Martin, G. C. Shih, M. M. Rahman, R. W. Carlson, and D. S. Stephens.** 1998. The (α2→8)-linked polysialic acid capsule and lipo-oligosaccharide structure both contribute to the ability of serogroup B *Neisseria meningitidis* to resist the bactericidal activity of normal human serum. Infect. Immun. **66:**5939–5947.
19. **Kumar, A., R. A. Malloch, N. Fujita, D. A. Smillie, A. Ishihama, and R. S. Hayward.** 1993. The minus 35-recognition region of *Escherichia coli* sigma 70 is inessential for initiation of transcription at an "extended minus 10" promoter. J. Mol. Biol. **232:**406–418.
20. **Masson, L., and B. E. Holbein.** 1983. Physiology of sialic acid capsular polysaccharide synthesis in serogroup B *Neisseria meningitidis*. J. Bacteriol. **154:**728–736.
21. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
22. **Monod, M., C. Denoya, and D. Dubnau.** 1986. Sequence and properties of pIM13, a macrolide-lincosamide-streptogramin B resistance plasmid from *Bacillus subtilis*. J. Bacteriol. **167:**138–147.
23. **Prentki, P., and H. M. Krisch.** 1984. In vitro insertional mutagenesis with a selectable DNA fragment. Gene **29:**303–313.
24. **Puente, J. L., D. Bieber, S. W. Ramer, W. Murray, and G. K. Schoolnik.**

1996. The bundle-forming pili of enteropathogenic *Escherichia coli*: transcriptional regulation by environmental signals. Mol. Microbiol. **20:**87–100.
25. **Raleigh, E. A., K. Lech, and R. Brent.** 1995. Selected topics from classical bacterial genetics, p. 1.4. *In* F. M. Ausubel et al. (ed.), Current protocols in molecular biology. John Wiley & Sons, New York, N.Y.
26. **Rowe, S., N. Hodson, G. Griffiths, and I. S. Roberts.** 2000. Regulation of the *Escherichia coli* K5 capsule gene clusters: evidence for the roles of H-NS, BipA, and integration host factor in regulation of group 2 capsule gene clusters in pathogenic *E. coli*. J. Bacteriol. **182:**2741–2745.
27. **Stephens, D. S., P. A. Spellman, and J. S. Swartley.** 1993. Effect of the (α 2→8)-linked polysialic acid capsule on adherence of *Neisseria meningitidis* to human mucosal cells. J. Infect. Dis. **167:**475–479.
28. **Stephens, D. S., J. S. Swartley, S. Kathariou, and S. A. Morse.** 1991. Insertion of Tn*916* in *Neisseria meningitidis* resulting in loss of group B capsular polysaccharide. Infect. Immun. **59:**4097–4102.
29. **Stout, V., and S. Gottesman.** 1990. RcsB and RcsC: a two-component regulator of capsule synthesis in *Escherichia coli*. J. Bacteriol. **172:**659–669.
30. **Swartley, J. S., J. H. Ahn, L. J. Liu, C. M. Kahler, and D. S. Stephens.** 1996. Expression of sialic acid and polysialic acid in serogroup B *Neisseria meningitidis*: divergent transcription of biosynthesis and transport operons through a common promoter region. J. Bacteriol. **178:**4052–4059.
31. **Swartley, J. S., L. J. Liu, Y. K. Miller, L. E. Martin, S. Edupuganti, and D. S. Stephens.** 1998. Characterization of the gene cassette required for biosynthesis of the (α1→6)-linked *N*-acetyl-D-mannosamine-1-phosphate capsule of serogroup A *Neisseria meningitidis*. J. Bacteriol. **180:**1533–1539.
32. **Swartley, J. S., A. A. Marfin, S. Edupuganti, L. J. Liu, P. Cieslak, B. Perkins, J. D. Wenger, and D. S. Stephens.** 1997. Capsule switching of *Neisseria meningitidis*. Proc. Natl. Acad. Sci. USA **94:**271–276.
33. **Swartley, J. S., and D. S. Stephens.** 1994. Identification of a genetic locus involved in the biosynthesis of *N*-acetyl-D-mannosamine, a precursor of the (α2→8)-linked polysialic acid capsule of serogroup B *Neisseria meningitidis*. J. Bacteriol. **176:**1530–1534.
34. **Troy, F. A.** 1992. Polysialylation: from bacteria to brains. Glycobiology **2:**5–23.
35. **Virji, M., K. Makepeace, D. J. Ferguson, M. Achtman, and E. R. Moxon.** 1993. Meningococcal Opa and Opc proteins: their role in colonization and invasion of human epithelial and endothelial cells. Mol. Microbiol. **10:**499–510.
36. **Virji, M., K. Makepeace, D. J. Ferguson, M. Achtman, J. Sarkari, and E. R. Moxon.** 1992. Expression of the Opc protein correlates with invasion of epithelial and endothelial cells by *Neisseria meningitidis*. Mol. Microbiol. **6:**2785–2795.
37. **Virji, M., K. Makepeace, and E. R. Moxon.** 1994. Distinct mechanisms of interactions of Opc-expressing meningococci at apical and basolateral surfaces of human endothelial cells: the role of integrins in apical interactions. Mol. Microbiol. **14:**173–184.
38. **Whitfield, C., and I. S. Roberts.** 1999. Structure, assembly and regulation of expression of capsules in *Escherichia coli*. Mol. Microbiol. **31:**1307–1319.
39. **Zhou, D., and M. A. Apicella.** 1996. Plasmids with erythromycin resistance and catechol 2,3-dioxygenase- or beta-galactosidase-encoding gene cassettes for use in *Neisseria* spp. Gene **171:**133–134.
40. **Zhou, D., A. Zaleski, B. Buscher, A. Preston, and M. A. Apicella.** 1996. A sixteen bp palindrome sequence encompassing the putative ribosomal binding site is conserved in pathogenic *Neisseria rfaC* genes and is involved in the regulation of expression of meningococcal rfaC, p. 356–357. *In* W. D. Zollinger, C. E. Frasch, and C. D. Deal (ed.), Tenth International Pathogenic Neisseria Conference, 8 to 13 September 1996, Baltimore, Md.

Editor: E. I. Tuomanen