Microsatellite instability regulates transcription factor binding and gene expression

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Microsatellites are tandemly repeated simple sequence DNA motifs widely prevalent in eukaryotic and prokaryotic genomes. In pathogenic bacteria, instability of these hypermutable loci through slipped-strand mispairing mediates the high-frequency reversible switching of phenotype expression, i.e., phase variation. Phase-variable expression of NadA, an outer membrane protein and adhesin of the pathogen *Neisseria meningitidis*, is mediated by changes in the number of TAAA repeats located upstream of the core promoter of *nadA*. Here we report that loss or gain of TAAA repeats affects the binding of the transcriptional regulatory protein IHF to the *nadA* promoter. Thus, phase-variable transcription of *nadA* potentially incorporates interplay between stochastic (mutational) and prescriptive (classical) mechanisms of gene regulation.

phase variation

Neisseria meningitidis is a Gram-negative encapsulated bacterium predominantly found in the human nasopharynx, where it can be a constituent of the normal microbial flora. However, some strains are invasive and penetrate first the blood and then the meninges to cause septicemia and meningitis. *N. meningitidis* has evolved several environmentally induced regulatory systems to control the expression of the virulence factors that are differentially expressed during the establishment of the infection (1–3). Phase variation, which results in high-frequency switching of phenotype expression, relies on environment-independent stochastic changes occurring within repeated simple sequence DNA motifs located in coding or promoter regions of numerous genes potentially involved in pathogenicity (4). Addition or subtraction of repeated units creates frameshifts and premature stop codons (5) or alters the strength of the promoter (6–8).

The *nadA* gene, which encodes an outer membrane protein and adhesin (9), was recently shown to be present in 50% of the disease-associated *N. meningitidis* strains and in 100% of the strains belonging to hypervirulent lineages ET-5, ET-37, and cluster A4, but in only 16% of the strains isolated from healthy people (10). NadA is currently under investigation as a vaccine candidate (11). The phase-variable expression of the *nadA* gene was recently shown to be regulated at the transcriptional level through a variation of the number of reiterated TAAA motifs present in the repeat tract located upstream of the core promoter of the gene (12).

Here we investigate the mechanism of phase variation of NadA, and in particular, the role of the TAAA microsatellite in transcriptional regulation. We show that both the TAAA repeat tract and the sequence located upstream of the microsatellite are involved in varying transcriptional activity and that the *nadA* promoter region also possesses multiple integration host factor (IHF)- and ferric uptake regulatory protein (Fur)-binding sites. Further, changes in the number of TAAA repeats, a requisite for phase variation, alter the binding of IHF to the *nadA* promoter. Thus transcription of *nadA* involves an interplay between mutation (slippage) and classical accessory transcriptional factors.

Methods

***N. meningitidis* Strains.** Strain 64/96 presents $(TAAA)_4$, strain BZ83 presents $(TAAA)_5$, strain CU385 presents $(TAAA)_6$, strain ISS759 presents $(TAAA)_8$, strains MC58, SB25, F6124, and 90/18311 present $(TAAA)_9$, strain 92001 presents $(TAAA)_{10}$, and strain BZ169 presents $(TAAA)_{12}$ in the repeat tract associated with the *nadA* gene. The strains were described in refs. 9 and 13.

DNA Constructs and Mutant Strains. Plasmid pΔ221-108 contains a 930-bp DNA fragment containing the *NMB1993* gene of *N. meningitidis* strain MC58 amplified by PCR with primers 1993-*Sac*I and 1993-*Afl*II (Table 1), and then a 1.3-kb DNA fragment containing the *nadA* gene of strain MC58, its core promoter and the TAAA repeat tract amplified by PCR with primers 1994-*Afl*II.1 and 1994-*Sma*I. Then the 1.25-kb kanamycin-resistance cassette is found, excised from the pUC4K vector (Pharmacia) by digestion with *Bam*HI, followed by a 475-bp DNA fragment containing the *NMB1995* gene of strain MC58 amplified by PCR with primers 1995-*Bam*HI and 1995 (Table 1). Plasmid pΔ221-50 contains the 930-bp DNA fragment containing the *NMB1993* gene, a 1.25-kb DNA fragment containing the *nadA* gene and its core promoter amplified by PCR with primers 1994-*Afl*II.2 and 1994-*Sma*I, and the 1.25-kb kanamycin-resistance cassette followed by the 475-bp DNA fragment containing the *NMB1995* gene. Plasmid pΔ984-108 contains a 750-bp DNA fragment containing the *NMB1992* gene amplified by PCR with primers 1992-*Sac*I and 1992-*Afl*II, the 1.3-kb DNA fragment containing the *nadA* gene, its core promoter and the repeat tract amplified, and the 1.25-kb kanamycin-resistance cassette followed by the 475-bp DNA fragment containing the *NMB1995* gene. Plasmid pΔ984-50 contains the 750-bp DNA fragment containing the *NMB1992* gene, a 1.25-kb DNA fragment containing the *nadA* gene and its core promoter, and the 1.25-kb kanamycin-resistance cassette followed by the 475-bp DNA fragment containing the *NMB1995* gene. Plasmid pΔ86-50 contains a 1,070-bp DNA fragment containing the *NMB1993* gene and the intergenic region located between the *NMB1993* and the *nadA* genes up to the repeat tract, amplified by PCR with primers 1993-*Sac*I and nadAUpRep-*Afl*II, and then the 1.25-kb DNA fragment containing the *nadA* gene and its core promoter and the 1.25-kb kanamycin-resistance cassette, followed by the 475-bp DNA fragment containing the *NMB1995* gene. The plasmids were linearized with *Xmn*I and transformed into strain MC58. The deletions were confirmed by PCR amplification and sequencing using primers 1993-*Sac*I and nadAR for mutants Δ221-108, Δ221-50, Δ984-108, and Δ984-50, and by using primers nadAR and nadAF for mutant Δ86-50. In all of the mutants the deleted DNA sequence was replaced by an *Afl*II restriction site.

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Abbreviations: IHF, integration host factor; Fur, ferric uptake regulatory protein.

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Table 1. Primers used in this study

Primer	Sequence (5′ to 3′)
1993-*Sac*I	GAGCTCTCGGACAACTCTGGCAGGC
1993-*Afl*II	CTTAAGCGGATTTAGATATGGTGG
1994-*Afl*II.1	CTTAAGCGAACTACCTAACTGCAAGAAT
1994-*Sma*I	CCCGGGGCTGCGGTTTCTTTATCG
1995-*Bam*HI	GGATCCTTTATTTTCTATCCGGCCG
1995	TCATTATACGCGCAACGGGG
1994-*Afl*II.2	CTTAAGTTGCGACAATGTATTGT
1992-*Sac*I	GAGCTCCCGTCTGAACGGCCGCC
1992-*Afl*II	CTTAAGGGCGGATGCTTGTCGGGC
nadAUpRep-*Afl*II	CTTAAGATTCTTGCAGTTAGGTAGTTC
nadAR	TCGACGTCCTCGATTACGAAGGC
nadAF	TGGCTGTGGTCAGTACTTTGGATGG
Gel-Shift1	ATCCGACAAAAAGGCCG
Gel-Shift4	GCTGATGTCAATTCTGCCGG
Gel-Shift2	TACATTGTCGCAATTTATTT
Gel-Shift5	CCGGCAGAATTGACATCAGC
Gel-Shift3	GTAAGTATTTTATCCCCACC

Colony Immunoblotting, RT-PCR, and Quantification of the Level of Transcription. Colony immunoblotting and RT-PCR were performed as described (12). The statistical method used for the quantification of the level of *nadA* transcription was also reported (12). In brief, the program IMAGEQUANT (Amersham Biosciences) was used to calculate the amount of *nadA* cDNA normalized with the amount the housekeeping gene *gdh* cDNA. Pairwise comparisons of the data sets from each variant or strain were performed by using GRAPHPAD software (GraphPad, San Diego). With $P < 0.05$ as a cut-off, three distinct classes emerged.

EMSAs. PCR products were generated by using genomic DNA from *N. meningitidis* strain MC58 as a template to yield the −226 to −138 probe by using primers Gel-Shift1 and Gel-Shift4, the −159 to −35 probe by using primers Gel-Shift2 and Gel-Shift5, and −108 to +30 probe by using primers 1994-*Afl*II.1 and Gel-Shift3 (Table 1). PCR products were gel purified by using QIAEXII gel extraction kit (Qiagen). The double-stranded probes were labeled with [γ-^{32}P]ATP by using polynucleotide kinase (Roche). A 16-μl binding reaction mixture containing purified gonococcal IHF or meningococcal Fur, 3 μg of poly[d(I-C)], 5 μg of BSA, 3.2 μl of 5× binding buffer [100 mM Tris·HCl, pH 7.5/200 mM KCl/25 mM $MgCl_2$/0.625 mM $MnCl_2$/50% (vol/vol) glycerol/10 mM DTT], and the labeled probe was incubated at room temperature for 30 min, loaded onto a nondenaturing 5% polyacrylamide gel, and electrophoresed in 1× Bis-Tris/Borate, pH 7.5, at 100 V. Gels were dried and exposed to radiographic film.

Results

Three Distinct Levels of *nadA* Transcription Exhibit Periodicity Related to the Number of Repeated TAAA Motifs. To elucidate the role of the microsatellite located upstream of the *nadA* core promoter in the transcriptional regulation of the gene, transcription of *nadA* was investigated by RT-PCR in variants of *N. meningitidis* strain MC58 and in strains of *N. meningitidis* displaying different numbers of reiterated motifs in the TAAA repeat tract. Three statistically distinct levels of *nadA* transcription, related to the number of reiterated TAAA motifs, were detected: high, low, and intermediate (see ref. 12 and Fig. 1 *A* and *B*). Both in variants of *N. meningitidis* strain MC58 and in strains of *N. meningitidis*, $(TAAA)_8$ and $(TAAA)_{10}$ were associated with a high level of *nadA* transcription, and $(TAAA)_9$ and $(TAAA)_{12}$ were associated with a low level of transcription (Fig. 1 *A* and *B*). Taken together (Fig. 1*C*), these findings indicated a periodicity (low–high–intermediate–low–high) of the level of expression of the *nadA* gene that was dependent on the number of TAAA repeats. We hypothesized that this variable pattern of transcription might reflect altered binding of one or more transcriptional regulatory factors located within or upstream of the TAAA microsatellite or in the core promoter.

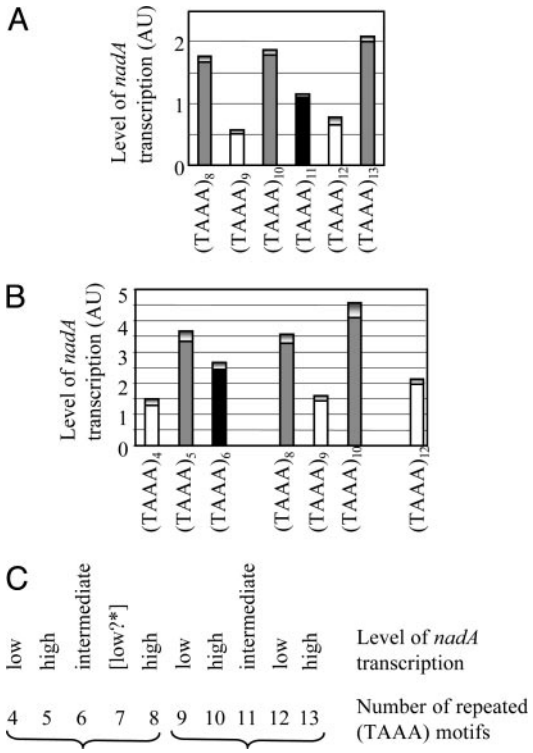

Fig. 1. Level of transcription of the *nadA* gene related to the number of repeated TAAA motifs. (*A* and *B*) Transcription in variants of *N. meningitidis* strain MC58 (*A*) and in strains of *N. meningitidis* (*B*). The bars represent the means and SEs of at least four independent measurements by RT-PCR, performed and analyzed as described (12). AU, arbitrary units. The program IMAGEQUANT was used to calculate the amount of *nadA* cDNA normalized with the amount the housekeeping gene *gdh* cDNA. Pairwise comparisons of the data sets from each variant or strain were performed by using GRAPHPAD software. With $P < 0.05$ as a cut-off, three distinct classes emerged. The gray bars represent high, the white bars low, and the black bars intermediate levels of *nadA* transcription. (*C*) Recapitulation of the level of *nadA* transcription related to the number of repeated TAAA motifs. *, No variant or strain with seven repeats was available for analysis. The repeating pattern of transcription is indicated.

IHF and Fur Bind to the *nadA*-Promoter Region. We showed that the DNA sequence of the *nadA* promoter contained several putative IHF- and Fur-binding sites (Fig. 2). IHF is a histone-like protein involved in the condensation of the bacterial chromosomal DNA that has been shown to act as a transcriptional regulator (14, 15). Fur has been shown to mediate transcriptional regulation of numerous bacterial virulence factors in response to iron (16–18).

We tested whether purified neisserial IHF or Fur proteins bound to their respective putative binding sites in the *nadA*-promoter region (Fig. 3). EMSAs showed that migration of the −159 to −35 DNA fragment (Fig. 3*A*) was retarded when incubated with saturating amounts of gonococcal IHF or meningococcal Fur proteins (Fig. 3*B1*). In support of the functional relevance of the IHF and Fur transcriptional factors and the putative binding sites (Fig. 2), multiple shifted bands were detected when the −159 to −35 DNA fragment was incubated with subsaturating amounts of each of the proteins (Fig. 3*B2*).

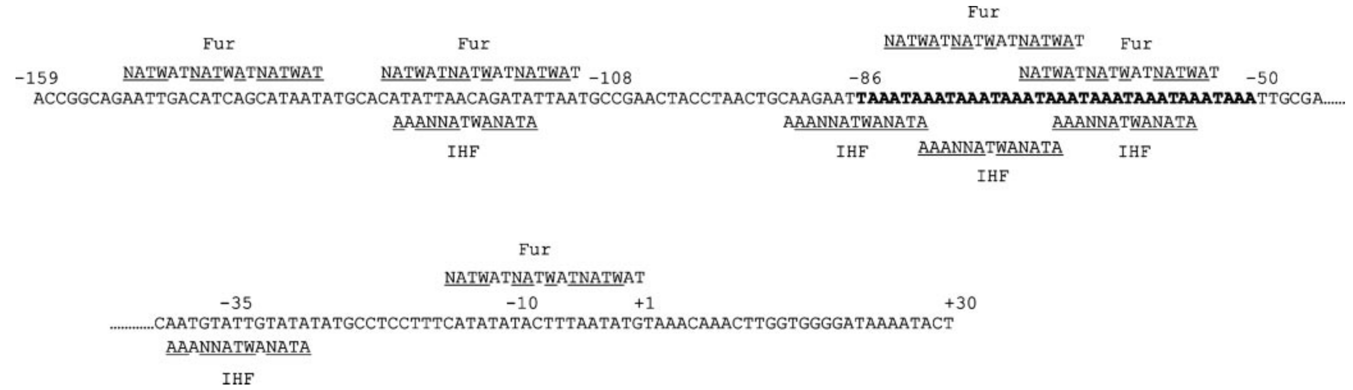

Fig. 2. Identification of putative binding sites for Fur and IHF proteins in the DNA sequence located from nucleotide −159 to nucleotide +30, with respect to the *nadA* transcription initiation site. The repeat tract is indicated in bold. *Neisseria* Fur (17) and IHF (28) core binding site consensus sequences are indicated above and below the DNA sequence, respectively. The matching bases are underlined. N, A, T, C, or G; W, A, or T.

Binding of both IHF and Fur to the −159 to −35 DNA fragment was specific because no shift occurred with the −226 to −138 DNA fragment that lacks putative IHF- and Fur-binding sites (Fig. 3*B6*).

Transcriptional Activity of *nadA* Depends on Both the Repeat Tract and the Region Located Upstream of the Microsatellite. To investigate further the role of IHF and Fur in the transcriptional control of *nadA*, we constructed a series of mutants harboring deletions in the sequence upstream of the *nadA* reading frame that contained the different IHF- and Fur-binding sites (Fig. 4). We showed that the amount of *nadA* transcript synthesized in mutants Δ221–108 and Δ984–108 (Fig. 4 *B* and *C*), in which sequence upstream of the microsatellite is deleted, was increased from low to intermediate compared with that of the intact promoter (Fig. 4*A*). Mutant Δ86–50, in which the repeat tract was deleted, also showed an intermediate level of expression of *nadA* (Fig. 4*D*). Mutants Δ221–50 and Δ984–50, in which the microsatellite and the region upstream of the repeats were deleted, also exhibited an intermediate level of *nadA* transcription (Fig. 4 *E* and *F*). Thus, both the TAAA repeat tract and the region upstream of the repeats are implicated in the negative regulation of *nadA* transcription.

The Phase-Variable Expression of the *nadA* Gene Depends Only on the Microsatellite. We investigated whether phase variation of NadA could still occur in the absence of the upstream region and/or the TAAA repeats. We studied whether the level of transcription could be altered in the mutants showing different types of deletion and all expressing the *nadA* gene at an intermediate level. Mutants lacking the repeat tract, i.e., mutants Δ86–50 (Fig. 4*D*), Δ221–50 (Fig. 4*E*), and Δ984–50 (Fig. 4*F*) constitutively expressed the *nadA* gene at an intermediate level. Deletion of the TAAA tract abrogated phase variation (data not shown). In contrast, phase variation, a switch from $(TAAA)_9$ to $(TAAA)_8$, was observed in mutants Δ221–108 (Fig. 4*B*) and Δ984–108 (Fig. 4*C*) and was associated with an

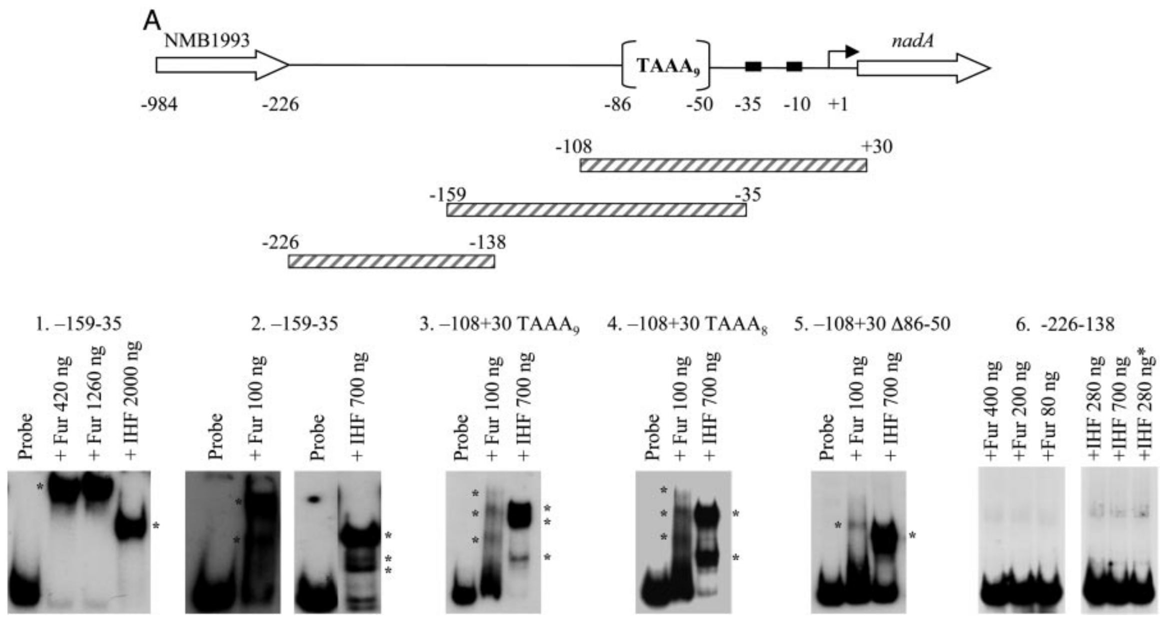

Fig. 3. EMSA of the *nadA* promoter. (*A*) Schematic representation of the DNA region flanking the repeat tract. The transcription initiation nucleotide is indicated by an arrow and labeled +1. The −10 and −35 elements are indicated with black boxes. The coordinates of the repeat tract and of the upstream gene are indicated. The DNA fragments used as probes are indicated as hatched horizontal bars. (*B*) EMSA analysis of the promoter-proximal regions of the *nadA* gene. The probes and the amount of proteins used are specified. *, Retarded bands in analyses 1–5 and an excess of unlabeled probe was added to the reaction in analysis 6.

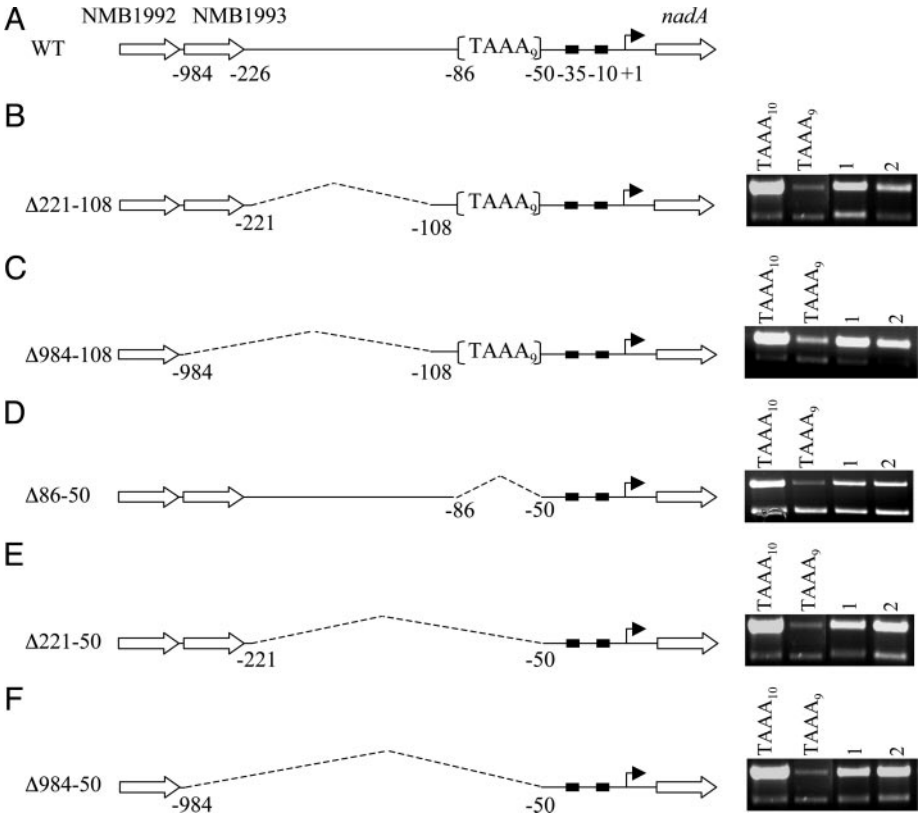

Fig. 4. Deletions of the DNA sequences located upstream from the *nadA* core promoter and level of *nadA* transcription associated with the deletions. (*A*) WT: schematic representation of the DNA region flanking the repeat tract. The symbols used are the same as in Fig. 3. (*B–F*) Deletions are indicated by the dashed lines. The mutant names are indicated to the left. The level of *nadA* transcription was measured by RT-PCR in two independent experiments and is shown to the right. The lanes $TAAA_{10}$ and $TAAA_9$ show the pattern of expression observed when the intact *nadA* gene is expressed at a high and a low level, respectively. Lanes 1 and 2 show the patterns of expression obtained for two independent mutants having the same deletion. The upper band shows the level of *nadA* transcription, and the lower band the level of housekeeping *gdh* gene transcription.

increase in *nadA* transcription from intermediate to high (Fig. 5). The altered phenotype was confirmed by using colony immunoblots (data not shown). Deletion of the upstream region with an intact TAAA tract was therefore permissive for phase variation.

The Instability of the Microsatellite Regulates IHF Binding to the *nadA* Promoter. To investigate the role of the instability of the microsatellite in the binding of IHF or Fur to the *nadA* promoter, EMSAs were performed by using three PCR-amplified DNAs:

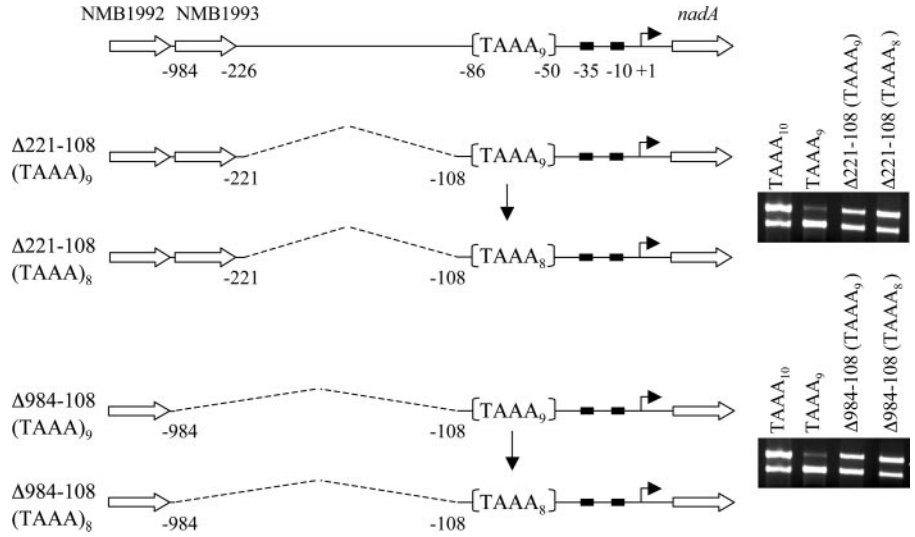

Fig. 5. Level of *nadA* transcription in mutants Δ221–108 and Δ984–108. The deletions are represented as in Fig. 4. The vertical arrow indicates that the deleted $(TAAA)_8$ variants were obtained after passage of the deleted $(TAAA)_9$ mutants.

fragment −108 to +30 containing $(TAAA)_9$, fragment −108 to +30 containing $(TAAA)_8$, and fragment −108 to +30 in which the repeat tract (−86–50) had been deleted. Incubation of the Fur protein with the DNA fragments −108 to +30 $TAAA_9$ and −108 to +30 $TAAA_8$ resulted in similar band shifts (Fig. 3 *B3* and *B4*). In contrast, IHF bound to three sites when there were nine copies of (TAAA) and only two when there were eight copies of (TAAA) (Fig. 3 *B3* and *B4*). When the −108 to +30 Δ86–50 DNA fragment was used, only one band was retarded upon incubation with IHF and Fur (Fig. 3*B5*). Taken together these data showed that changes in the number of copies of TAAA alter the binding of IHF to the *nadA* promoter, either directly through an alteration in the binding of IHF to the repeat tract itself or indirectly as a result of a change in the number of copies of TAAA on neighboring sequence.

Discussion

Under iron-limitation condition, which is a feature of the human host, many pathogens induce the expression of virulence factors that are transcriptionally regulated by iron through the Fur protein. A microarray analysis recently indicated that in *N. meningitis*, several virulence-associated genes were over-expressed under iron-depleted conditions (15). The histone-like protein IHF was shown to be involved in the regulation of the expression of the type IV pilus that is necessary for the establishment of the colonization of the mucosal surfaces by the meningococcus (18, 19). Although it was shown in *Escherichia coli* that Fur and the regulatory factors H-NS and IHF cooperate to regulate the expression of the gene encoding the iron superoxide dismutase (20), in *N. meningitidis* the *nadA* gene is the first indication of a gene that is apparently regulated by both IHF and Fur. The involvement of these transcription factors in the regulation of the *nadA*-promoter activity was inferred from the analysis of mutants deleted for the multiple IHF- and Fur-binding sites and from the specific binding of both proteins to the *nadA* promoter in EMSAs. A similar approach has recently been used to demonstrate the involvement of IHF in the regulation of the *virB* operon in *Brucella abortus* (21).

N. meningitidis has evolved a complex multioperator regulatory system, resulting in the fine tuning of the *nadA* promoter. The transcription of the *nadA* gene was shown to exhibit a periodicity related to the number of TAAA motifs present in the repeat tract. A repeating pattern of transcription was previously described for the *araBAD* operon in *E. coli* (22). Insertion of integral DNA helical turns between an operator and the promoter cyclically hindered repression (22). We show that the instability of the TAAA microsatellite plays a central role in the regulation of the *nadA* transcription. We propose that this occurs through modulation of the binding of the transcription factor IHF. However, our findings are open to other interpretations, e.g., it is possible that the binding of RNA polymerase could be affected by changes in the number of TAAA repeats located immediately upstream of the core promoter. A microsatellite was previously shown to be part of the binding site for a transcriptional regulator of the promoter for cholera toxin in *Vibrio cholerae* (23). Instability of a trinucleotide repeat tract located upstream of a *Mycoplasma gallisepticum* core promoter was reported to alter the spacing between the flanking sequences and to have consequences on the transcription of the gene (24). Several lines of evidence indicate that microsatellites localized in eukaryotic-promoter regions are involved in transcriptional regulation, notably through their binding to nuclear proteins (25, 26). Besides, the DNA phasing generated by TA dinucleotide polymorphisms between two possible control regions in the promoter was recently hypothesized to influence the level of transcription of the gene encoding a subunit of the human NADPH oxidase (27).

The system of regulation described by us, i.e., the interplay between mutational and classical mechanisms of gene regulation, is a striking example of a bacterial contingency locus (4), potentially facilitating fitness of *N. meningitidis* through variable expression of bacterial adherence factor so as to modulate carriage within and transmission between humans. It seems likely that this transcriptional mechanism will not be confined to bacterial pathogens but may be widespread in prokaryotes and eukaryotes. Such a transcriptional mechanism has not previously been described in eukaryotes, but it could be of great relevance to phenotypic diversity (quantitative traits) of cell populations, e.g., during embryogenesis or in the pathobiology of cancers. Further, the function of microsatellites in eukaryotes has proved elusive despite their relative abundance.

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