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Molecular characterization of *hpuAB*, the haemoglobin–haptoglobin-utilization operon of *Neisseria meningitidis*

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Summary

We previously identified HpuB, an 85 kDa Fe-repressible protein required for utilization of Fe from, and binding to, haemoglobin and the haemoglobin–haptoglobin complex. The gene for *hpuB* was cloned from *Neisseria meningitidis* strain DNM2 and the predicted amino acid sequence indicates that HpuB is an outer membrane receptor belonging to the TonB family of high-affinity transport proteins. A second open reading frame, predicted to encode a 34.8 kDa lipoprotein, was discovered 5′ to *hpuB*, and was designated *hpuA*. HpuA was identified in a total-membrane-protein preparation by construction of a mutant lacking HpuA. Acylation of HpuA was confirmed by [^{3}H]-palmitic acid labelling of meningococci. Consensus promoter sequences were not apparent 5′ to *hpuB*. The *hpuA* insertion mutation exerted a polar effect, abolishing expression of *hpuB*, suggesting that *hpuA* and *hpuB* are co-transcribed. The 3.5 kb polycistronic *hpuAB* mRNA was identified and shown to be transcriptionally repressed by iron. The transcriptional start site was identified 33 nucleotides 5′ to the *hpuA* translational start site, appropriately positioned around consensus promoter and ferric uptake regulator (Fur)-box sequences. The structure of this operon suggests that HpuA–HpuB is a two-component receptor analogous to the bipartite transferrin receptor TbpB–TbpA.

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Introduction

Neisseria meningitidis, the primary cause of bacterial meningitis in the United States (Loughlin *et al*., 1995), can advance from a benign colonization of the nasopharynx to a fulminant bacteremia resulting in death in as little as 8 h (Volk *et al*., 1986). To cause this disease, the meningococcus must overcome the nutritional immunity that results from iron (Fe) limitation in the host. To prevent Fe toxicity, and control the insolubility of the Fe^{3+} ion, the mammalian host sequesters Fe using Fe-binding compounds such as transferrin (TF), lactoferrin (LF) and haem (Hm) (Finkelstein *et al*., 1983; Weinberg, 1984). As a consequence, the concentration of free Fe in mucosal secretions and tissue fluids is maintained below that required to support microbial growth (Bullen *et al*., 1978). Acquisition of Fe from host Fe-binding compounds therefore becomes a crucial determinant of pathogenesis (Finkelstein *et al*., 1983; Weinberg, 1978; 1984). Meningococci possess several distinct Fe-acquisition systems that may be important at different stages of disease. During colonization of the nasopharyngeal mucosal epithelium, LF is thought to be the primary Fe source (Weinberg, 1978). Once meningococci invade the bloodstream, TF initially is the major Fe source available to support growth (Otto *et al*., 1992; Weinberg, 1978). In the latter stages of septicaemic disease, some studies have suggested that Fe–TF may be limiting as a result of the hypoferremic response (Holbein, 1980; 1981; Holbein *et al*., 1979). To obtain Fe and sustain a prolonged septicaemia, meningococci may use haemoglobin (Hb) released as a result of disseminated intravascular coagulation (DIC) (Wyngaarden and Smith, 1985). Haptoglobin (Hp), a plasma glycoprotein, rapidly binds to this released Hb forming a complex (Hb–Hp) that prevents many microorganisms, such as *Escherichia coli*, from using Hb as an Fe source. (Eaton *et al*., 1982; Hershko, 1975; Kinco *et al*., 1980; Muller-Eberhard *et al*., 1968). However, meningococci are capable of using Hb–Hp complexes as the sole source of essential Fe (Dyer *et al*., 1987; Lewis and Dyer, 1995).

N. meningitidis and the related pathogen *Neisseria gonorrhoeae* have responded to host Fe limitation by evolving several highly specialized outer membrane receptors for acquiring Fe from host Fe-binding compounds. Specifically, outer membrane receptors necessary for utilization

of Fe from TF, LF, Hb and Hb–Hp have been described. (Irwin *et al*., 1993; Lewis and Dyer, 1995; Pettersson *et al*., 1994; Quinn *et al*., 1994; Schryvers and Morris, 1988a,b; Stojiljkovic *et al*., 1995). The outer membrane receptors for TF (Tbp1) and LF (LbpA) share a high degree of similarity to each other and to other outer membrane receptors belonging to the TonB family of high-affinity transporters (Cornelissen *et al*., 1992; Legrain *et al*., 1993; Pettersson *et al*., 1993). The TF receptor has been suggested to be unusual among the TonB-dependent receptors, as this receptor may be a bipartite structure composed of the TonB-dependent Tbp1 and a lipoprotein designated Tbp2 (Anderson *et al*., 1994; Cornelissen *et al*., 1992; Cornelissen and Sparling, 1994; Irwin *et al*., 1993; Legrain *et al*., 1993). Stojiljkovic *et al*. (1995) have identified a meningococcal Hb receptor, HmbR, and demonstrated that this protein, which is analogous to Tbp1, is dependent on TonB for high-affinity Fe transport. HmbR is apparently a single-component TonB-dependent receptor capable of removing Hm from Hb and transporting the intact Hm moiety into the periplasm (Stojiljkovic *et al*., 1995).

We previously described Hpu, which is an 85 kDa Fe-repressible protein (FeRP) of *N. meningitidis* strain DNM2 involved in utilization of Fe from Hb and Hb–Hp (Lewis and Dyer, 1995). Our data strongly suggested that Hpu was the outer membrane receptor for Hb and Hb–Hp. A mutant lacking Hpu was deficient in acquisition of Fe from, and surface binding to, Hb and Hb–Hp. In addition, affinity purification demonstrated direct binding of Hpu to Hb–Hp and apo-Hp. In this communication we describe the cloning and sequence analysis of the gene encoding Hpu. We renamed this protein HpuB, because the gene for this protein resides within a two-gene operon, *hpuAB*. Our data suggest that HpuB is also a member of the TonB family of outer membrane receptors. We also identified HpuA, a 35 kDa FeRP encoded 5′ to *hpuB*. The predicted amino acid sequence of HpuA suggests that this protein, which we located in a total-membrane-protein preparation, is a lipoprotein. Labelling experiments using [^{3}H]-palmitic acid demonstrated acylation of HpuA. The *hpuA* and *hpuB* genes are transcribed on a polycistronic 3.5 kb Fe-repressible mRNA; this operon structure is similar to that of the putative *tbp* operon. These data suggest that HpuB is part of a two-component receptor which is similar to that for TF. We suggest that HpuA is the lipoprotein component of this bipartite receptor.

Results

DNA sequence of hpuB

The gene encoding HpuB was cloned from *N. meningitidis* strain DNM2 as described in the *Experimental procedures*, using oligonucleotides derived from the N-terminal amino acid sequence of the protein (Lewis and Dyer, 1995). The *hpuB* open reading frame (ORF) extends for 2430 bp, and is predicted to encode a protein of 810 amino acids (Accession Number U73112). The N-terminal amino acid sequence of HpuB is preceded by a classic signal peptide of 22 amino acids that are predominantly hydrophobic, with one charged residue located at position number six (Fig. 1) (Oliver, 1987). The putative signal peptide ends with the sequence AFA, which conforms to the signal peptidase I processing motif (Oliver, 1987). The mature peptide would thus contain 788 amino acids and have a predicted molecular mass of 88.3 kDa. This molecular mass is in good agreement with the 85 kDa molecular mass of HpuB observed using SDS–PAGE. Upstream of the ATG start codon, we identified a weak potential ribosome-binding site (data not shown), but we were unable to identify any consensus promoter sequences that might direct the transcription of this gene. As described below, this gene appears to be the second gene in a two-component operon. The *hpuB* stop codon is followed by a sequence that is capable of forming a stable hairpin loop and a poly(T) tract characteristic of a rho-independent terminator (Fig. 1; IR2).

HpuB expression is regulated by Fe availability (Lewis and Dyer, 1995). In *E. coli*, Fe-regulated gene expression occurs by transcriptional repression mediated by the Ferric uptake regulator (Fur) protein (Bragg and Neilands, 1987). When the concentration of Fe is sufficient, Fur, along with Fe^{2+} as a co-repressor, binds to a 19 bp consensus sequence upstream of Fe-repressible genes, thus blocking transcription (Bragg and Neilands, 1987). A Fur homologue has been identified in the meningococcus, suggesting that Fe regulation occurs by a similar mechanism (Karkhoff-Schweizer *et al*., 1994; Thomas and Sparling, 1994). However, we did not identify sequences similar to the Fur box, 5′ to *hpuB*.

Similarity of HpuB to TonB-dependent receptors

We previously presented data which strongly suggested that HpuB is an outer membrane receptor for Hb and the Hb–Hp complex (Lewis and Dyer, 1995). A BLASTP search with the predicted amino acid sequence of HpuB revealed a high degree of similarity to outer membrane receptor proteins of the TonB family of high-affinity transporters (Gish and States, 1993). The seven domains described by Cornelissen *et al*. (1992) common to the *E. coli* TonB-dependent outer membrane receptors and the *N. meningitidis* TF receptor, Tbp1, are present in HpuB (Fig. 2). HpuB contains six of the eight amino acids that define the ‘TonB box’ which has been implicated in direct interaction between TonB-dependent receptors and the TonB protein (Fig. 2) (Postle, 1990; Tuckman and Osbourne,

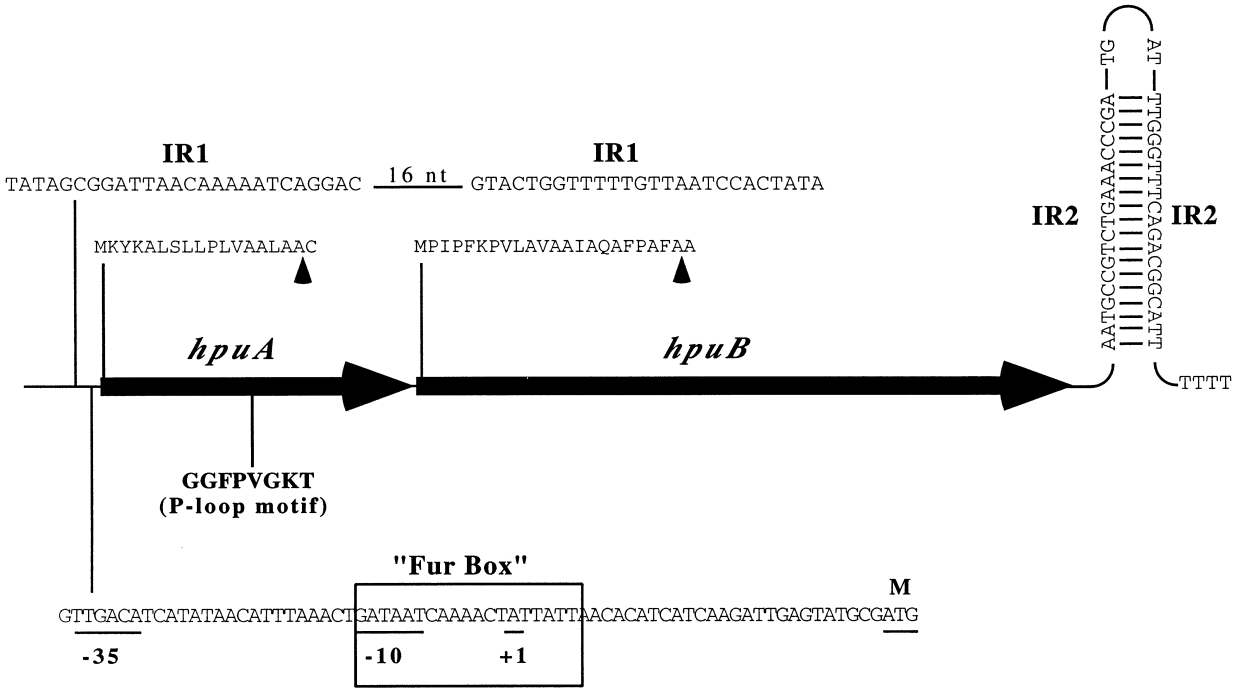


Fig. 1. The nucleotide sequence of *hpuA* and *hpuB* has been deposited in GenBank (Accession Number U73112). A schematic diagram of the *hpuAB* operon is shown. The transcriptional start site (+1), −10 and −35 regions, putative Fur box, inverted repeat regions (IR1 and IR2), putative signal-peptidase-cleavage motifs (the cleavage site is indicated by an arrow), and the P-loop in HpuA are indicated.

1992). These data are consistent with the role of HpuB as an outer membrane receptor.

HpuB is most similar (37% identity and 58% similarity) to the *Haemophilus ducreyi* Hb-binding protein, HgbA, identified by Elkins *et al*. (Elkins, 1995; Elkins *et al*., 1995). An HgbA mutant was deficient in binding to Hb and in utilization of Hb as the sole source of Fe. Although, *H. ducreyi* is able to use Hb–Hp as a sole source of Fe, the role of HgbA in acquisition of Fe from Hb–Hp has not been investigated (Lee, 1991). HgbA, like HpuB, shares a high degree of similarity with TonB-dependent transport proteins and contains the seven domains described by Cornelissen *et al*. (1992) (Fig. 2). HpuB shares 32% identity (53% similarity) with the meningococcal LF receptor IroA (Accession Number X69214) (Pettersson *et al*., 1993; 1994). We previously determined that HpuB is not involved in acquisition of Fe from, or binding to, LF (Lewis and Dyer, 1995). Thus, similarity between HpuB and IroA is not the result of similarities in the ligands for each receptor, but may be due to the probable functional dependence of each protein on TonB. The level of similarity of HpuB to HmbR (Accession Number U18558) (28% identity and 52% similarity), which is a meningococcal Hb receptor identified by Stojiljkovic *et al*. (1995), is less than the similarity of HpuB to other functionally distinct TonB-dependent proteins. This suggests that although both proteins are involved in Hb binding and transport, they are discrete proteins. Preliminary polymerase chain reaction (PCR) data (not shown) suggests that meningococcal strain DNM2 harbours an *hmbR* locus in addition to the *hpuB* locus, suggesting that these proteins are not allelic variants at a single locus, but represent the products of different genes.

DNA sequence of hpuA

DNA sequences 5′ to *hpuB* revealed a second ORF that we designated *hpuA*. The *hpuA* ORF extends for 1023 bp, and is predicted to encode a protein of 341 amino acids (Accession Number U73112). The predicted N-terminus of *hpuA* contains a prokaryotic lipoprotein lipid-attachment motif (PROSITE Number 0013.PDoc), suggesting that HpuA is a lipoprotein (Fig. 1). The mature peptide would contain 324 amino acids and have a predicted molecular weight of 34.8 kDa. Consensus −10 and −35 promoter regions were observed directly upstream of the ATG start codon of *hpuA* (Fig. 1). In addition, a Fur box with 79% similarity (15 of 19 nucleotides (nt)) to the consensus Fur box, GATWATGATWATYATTWTC (where W = A/T and Y = C/T), was located within the putative promoter region, suggesting that *hpuA* is transcriptionally regulated by Fe (Fig. 1) (Neilands, 1990; Pressler *et al*., 1988). There were no hairpin-loop structures indicative of a rho-independent terminator located downstream of the *hpuA* stop codon, which is located 30 nt 5′ to the *hpuB* start codon. A 26 bp inverted repeat was located just upstream

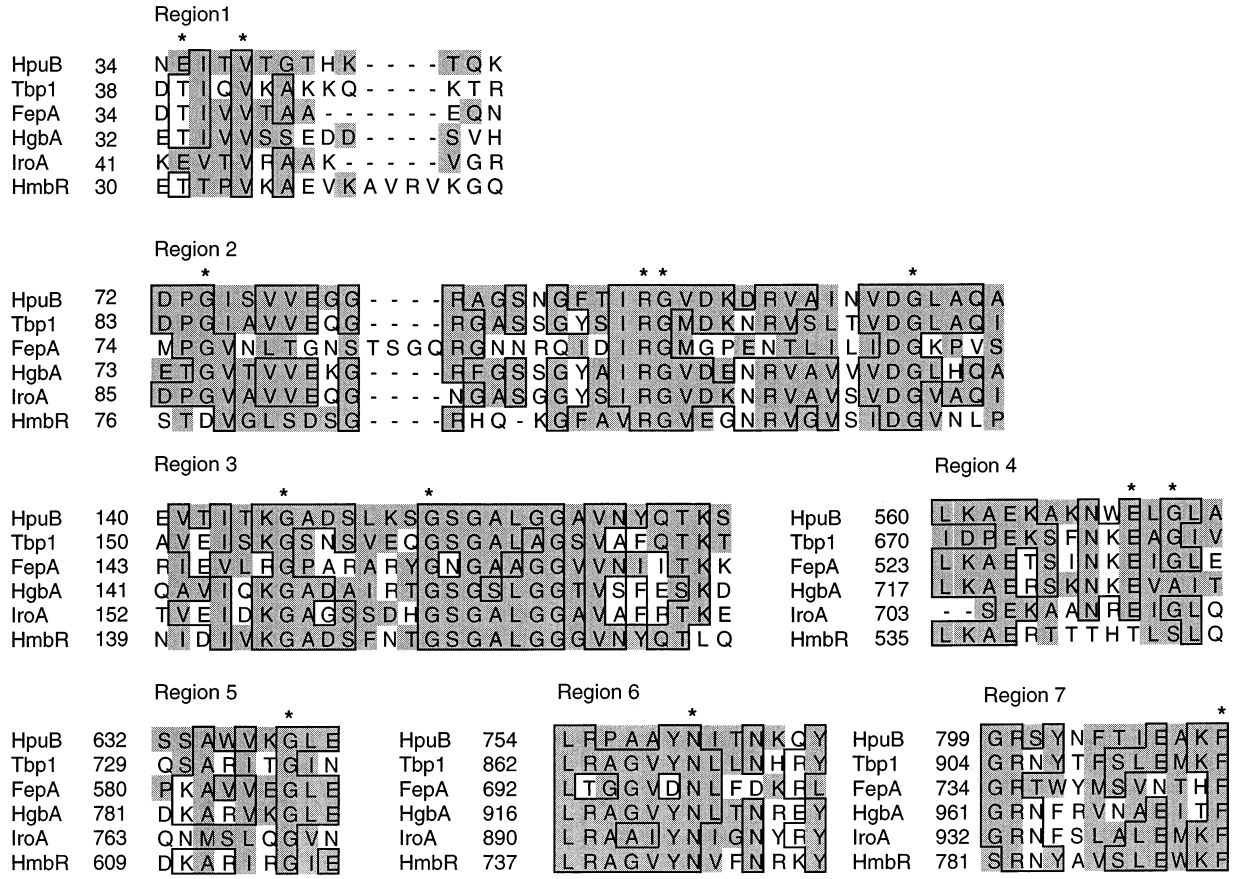


Fig. 2. Peptide alignment of HpuB- and TonB-dependent outer membrane proteins highlighting seven regions of homology described by Cornelissen *et al.* (1992). Tbp1 (*N. gonorrhoeae* TF-binding protein 1 (Cornelissen *et al*., 1992)), FepA (*E. coli* ferric enterobactin receptor (Lundrigan and Kadner, 1986)), IroA (*N. meningitidis* LF receptor (Pettersson *et al*., 1993)) and HmbR (*N. meningitidis* Hb receptor (Stojiljkovic *et al*., 1995)). Boxed regions indicate homologous residues. Asterisks indicate positions of identity described by Cornelissen *et al*. (1992).

of the −35 consensus (Fig. 1; IR1). The function of this inverted repeat is not known, but such structures are commonly involved in gene regulation (Yager and vonHipple, 1987).

Similarity of HpuA to known proteins

A BLASTP search with the predicted amino acid sequence of the HpuA protein revealed no significant similarities to proteins in the non-redundant database. A motif analysis of HpuA suggested that this protein contains a putative phosphate-binding domain or P-loop (Fig. 1) (Fig. 1; PROSITE Number 0017.PDoc). The significance of this is not yet known but several meningococcal Tbp2 lipoproteins also contain this motif: strain M982 (Accession Number Z15130), strain M978 (Accession Number X78941) and strain 6940 (Accession Number X78939).

Identification of HpuA by mutagenesis

We insertionally inactivated the *hpuA* gene with the transposon mTn*3*erm using shuttle mutagenesis as previously described (Lewis and Dyer, 1995; Seifert *et al*., 1986). In brief, mTn*3*erm was randomly inserted into pSM85k, which is pHSS8 containing the 850 bp pTA1 insert. A clone in which the transposon was located in the 3′ end of *hpuA* was identified by restriction enzyme digestion and DNA sequencing. This plasmid, pSM85kG was then used to transform *N. meningitidis* DNM2 to erythromycin resistance. One transformant, DNM2G2, was characterized by Southern hybridization, which indicated that a single cross-over event occurred such that the plasmid and transposon were incorporated into the chromosome at the *hpuA* gene locus (data not shown).

SDS–PAGE analysis of total membrane proteins prepared from DNM2G2 grown in the presence and absence of Fe allowed us to identify the HpuA protein. When membrane proteins were stained with either silver or Coomassie brilliant blue, DNM2G2 appeared to lack a 35 kDa FeRP that was present in both DNM2 and the HpuB mutant, DNM2E4 (Fig. 3). The size of this protein is consistent with the 34.8 kDa molecular mass predicted for the

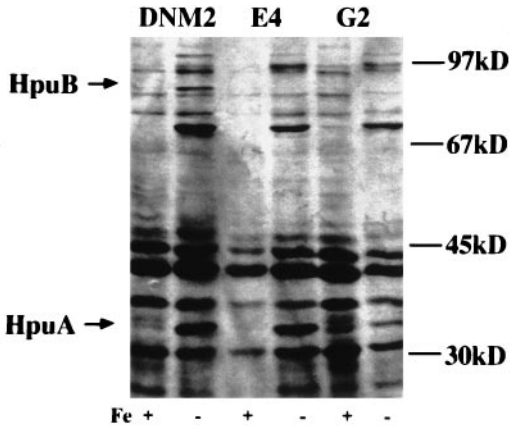


Fig. 3. Comparison of total-membrane-protein profiles obtained from strains DNM2, DNM2E4 (E4) and DNM2G2 (G2). Total membrane proteins isolated from iron-replete (+) or iron-starved (−) DNM2 (lanes 1 and 2, respectively), DNM2E4 (lanes 3 and 4, respectively) and DNM2G2 (lanes 5 and 6, respectively) were separated on a 7.5% SDS–PAGE gel and stained with silver and Coomassie brilliant blue. Note the absence of the 35 kDa FeRP, designated HpuA, in lane 6. Also notice the lack of HpuB in strain DNM2G2 (lane 6).

mature HpuA peptide. The gel shown in Fig. 3 is a representative gel chosen from multiple gels analysed. Note that there is a constitutive protein that migrates very closely with HpuA visible in all lanes of the SDS–PAGE.

SDS–PAGE (Fig. 3) and Western blot analysis (data not shown) confirmed that DNM2G2 also lacked HpuB. This would be expected if *hpuA* and *hpuB* are co-transcribed, as an insertion mutation in *hpuA* would be expected to be polar, and abolish expression of *hpuB*.

[^{3}H]-palmitic acid labelling of HpuA

As noted above, the predicted N-terminal amino acid sequence of HpuA contains a lipoprotein lipid-attachment motif. Lipoproteins frequently migrate aberrantly in SDS–PAGE. Interestingly, the predicted and observed molecular weights of HpuA are remarkably close. To determine if HpuA was a lipoprotein, we metabolically labelled meningococci with [^{3}H]-palmitic acid. SDS–PAGE and fluorographic analysis of labelled meningococci gown in the presence (Fe+) and absence (Fe−) of Fe demonstrates that HpuA is in fact a lipoprotein (Fig. 4). ^{3}H-labelled HpuA is readily apparent in meningococci grown in the absence of Fe (Fig. 4, lane 4) but is not apparent in cells grown in the presence of Fe (Fig. 4, lane 3). ^{3}H-labelled HpuA was not detected in an HpuA mutant (data not shown).

Analysis of the hpuAB *transcript*

The arrangement of the *hpuA* and *hpuB* ORF's suggested that these genes are transcribed as a polycistronic message, beginning at the putative promoter 5′ to *hpuA*. RNA, prepared from Fe-starved and Fe-replete meningococci, was separated on formaldehyde gels and hybridized with *hpuA*- or *hpuB*-specific probes (Fig. 5) to examine the transcription of each of these genes. When an *hpuB*-specific probe (*hpuB* probe or 3′-*hpuB* probe) was used, two distinct mRNAs of 2.5 kb and 3.5 kb were observed in RNA prepared from Fe-starved meningococci (Fig. 6). An *hpuA* probe also hybridized to the 2.5 kb and 3.5 kb mRNAs (data not shown). These messages were not detected, with any probe, in RNA prepared from Fe-sufficient meningococci, indicating that neither *hpuA* nor *hpuB* is transcribed in the presence of Fe (Fig. 6; data not shown). A negative control (*E. coli* rRNA) also did not hybridize with these probes (Fig. 6; data not shown). Methylene blue staining of the RNA after hybridization demonstrated that both Fe+ and Fe− RNA was intact and present in equal concentrations on the membrane (data not shown). We assessed the integrity of the RNA by probing with the gonococcal phosphoglucomutase gene; mRNA of the anticipated size was detected in both Fe− and Fe+ RNA (data not shown).

Reverse transcription-PCR (RT-PCR) was used to confirm that the *hpuA* and *hpuB* ORFs are transcribed as a single message. Primer P4.84 (Table 1), which hybridizes to *hpuB* mRNA, was annealed to RNA prepared from Fe-starved meningococci, and reverse transcriptase was used to generate a complementary DNA (cDNA). This cDNA was used as the template for a PCR reaction with primers chosen such that the 5′ primer (P24.85, Table 1) would anneal within *hpuA*, and the 3′ primer (P26.85, Table 1) would anneal within *hpuB*. Thus, if the *hpuB* message was monocistronic, the cDNA would not contain *hpuA* sequences and the PCR reaction would not amplify a product. However, if the message was polycistronic, then the cDNA would contain *hpuA* sequences and a

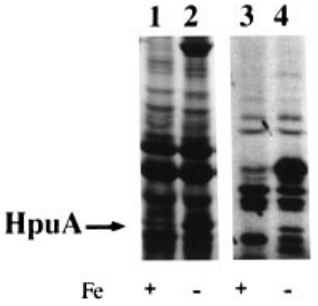


Fig. 4. [^{3}H]-palmitic acid labelling of HpuA. Total membrane proteins and ^{3}H-labelled extracts prepared from iron-replete (+) or iron-starved (−) DNM2 were separated on a 10% SDS–PAGE. Coomassie-brilliant-blue-stained membrane proteins are shown in lanes 1 and 2, and the fluorograph of ^{3}H-labelled extracts is shown in lanes 3 and 4. The position of HpuA is indicated.

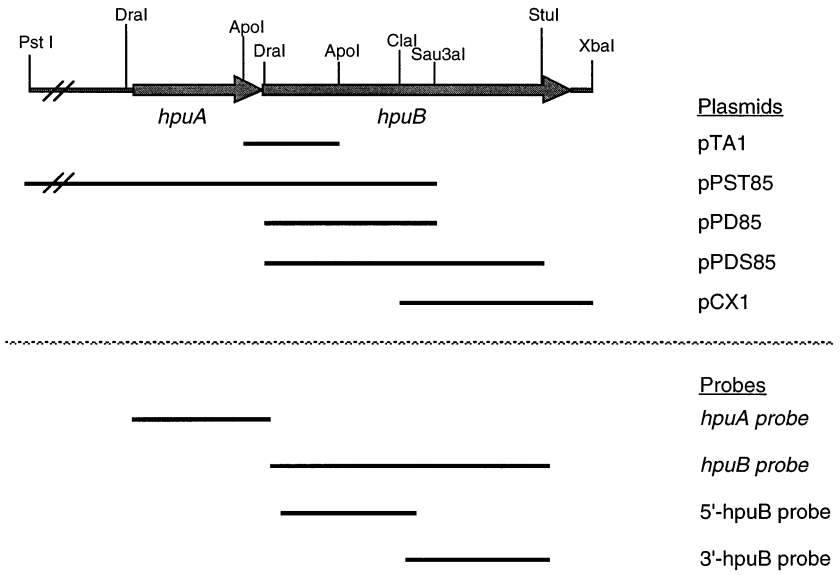


Fig. 5. A schematic diagram of the *hpuAB* operon showing restriction sites relevant to the generation of the plasmid constructs and probes described in the text (see the *Experimental procedures*).

656 bp PCR product would be amplified. Using this assay, we amplified a product of the correct size and confirmed by Southern hybridization, using the *hpuA* probe (Fig. 5), that this fragment was the *hpuAB* junction (data not shown). Control primers, which were chosen to amplify an internal fragment of *hpuB* and were contained on the cDNA in both cases, amplified a product of the correct size (data not shown). As a negative control, RNA annealed to P4.85 and incubated without reverse transcriptase was used as the template for the PCR reactions described above. Amplification from this template was not detected confirming that amplification did not result from trace amounts of DNA contaminating the RNA preparation (data not shown). In addition, positive and negative control reactions used DNM2 chromosomal DNA or double distilled H_2O (ddH_2O) as the template; these controls confirmed that the RT-PCR results described above were due to the *hpuA* and *hpuB* ORF's contained in a single mRNA.

The 5′ end of the *hpuAB* operon was identified by primer extension using P25.85 (Table 1), which is complementary to nucleotides 22 to 46 of the *hpuA* coding sequence. The cDNA product was electrophoresed on a standard denaturing polyacrylamide sequencing gel along with the products of a sequencing reaction using P25.85 and the cloned *hpuA* in pPST85. A 78 nt cDNA was observed (Fig. 7), placing the transcriptional start at the A residue located 33 nt upstream of *hpuA* (Fig. 1). This position is in good agreement with the locations of the putative Fur box and −10 and −35 consensus promoter regions 5′ to *hpuA* (Fig. 1). The 78 nt product was not detected in control reactions in which RNA was excluded (data not shown).

Is hpuB *transcribed independently?*

The nucleotide sequence data and the RNA analysis demonstrated that *hpuA* and *hpuB* are co-transcribed on a single 3.5 kb mRNA. We are presently uncertain as to the nature of the 2.5 kb mRNA. Although the size of the 2.5 kb mRNA is consistent with the size of an independent monocistronic *hpuB* transcript, our data does not support an independent transcription of *hpuB*. First, this message hybridizes with an *hpuA*-specific probe that does not cross-react with *hpuB* on Southern blot analysis. Second, a transposon insertion in *hpuA* has a polar effect on the expression of *hpuB.* Third, there are no consensus promoter elements located 5′ to *hpuB.* Finally, a primer-extension experiment using primer P26.85 (Table 1), which is

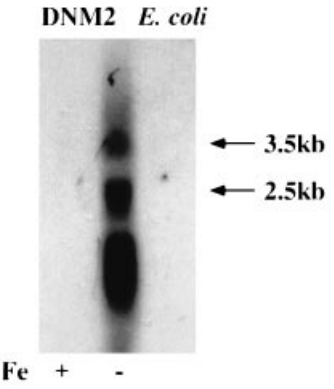
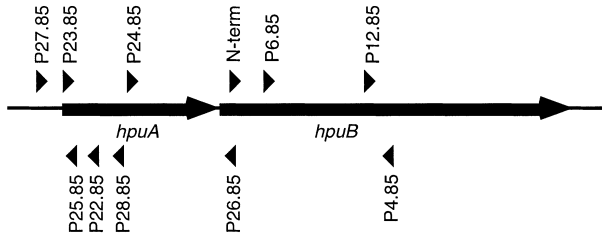


Fig. 6. Northern blot analysis of the *hpuAB* transcript. Total RNA isolated from Fe-replete (+) and Fe-starved (−) DNM2 (lanes 1 and 2, respectively) probed with the 3′-*hpuB* probe. The positions of two Fe-repressed mRNAs are indicated. *E. coli* rRNA (lane 3) was used as a negative control. An identical result was observed when the *hpuA* probe was used (data not shown).

Table 1. Primers used in this work.

Name	Sequence (5'→3')	Gene Location
P4.85	CGGTGTTTTGGCGGATATGC	*hpuB*
P6.85	CTTCAACGCCAACCGCAACA	*hpuB*
P12.85	GAACACTTGGACTTGGGACA	*hpuB*
P22.85	GGTGGGCAGCGGGGTGGC	*hpuA*
P24.85	AAATTCGGGGCGTGGATAG	*hpuA*
P25.85	CAAGGGCAGCGACAAGCGGCAGTAA	*hpuA*
P26.85	GGGAAACGCTTGGGCGATGG	*hpuB*
P27.85	ATCAGGACAAGGCGAGGTAACAAC	*hpuA*
P28.85	ATGGGGTCGGCGTAGGATG	*hpuA*
N-terminal	GCCCAAACCYTNAACGAAATCACCGT	*hpuB*
M13 reverse	TCACACAGGAAACAGCTATGAC	vector



complementary to nucleotides 38 to 58 of the *hpuB* coding sequence, failed to detect an alternative transcriptional start site (data not shown). Further work is necessary to clarify the relationship between these two mRNAs.

Discussion

The genetic organization of the *hpuAB* operon is analogous to the putative *tbpBA* operon (Legrain *et al*., 1993). The *tbpBA* operon is composed of the *tbpB* gene, encoding the outer membrane lipoprotein Tbp2, which is located upstream of the *tbpA* gene, encoding the TonB-dependent outer membrane TF receptor Tbp1 (Cornelissen and Sparling, 1994; Legrain *et al*., 1993). Similarly, the *hpuAB* operon is composed of the *hpuA* gene, encoding the lipoprotein HpuA, which is located upstream of the *hpuB* gene encoding HpuB, the TonB-dependent outer membrane receptor for Hb and Hb–Hp. Northern blot and RT-PCR analysis clearly demonstrated that *hpuA* and *hpuB* are transcribed on a single Fe-repressible polycistronic 3.5 kb mRNA. Primer-extension analysis successfully located the transcriptional start site of the *hpuAB* message 33 nt upstream of the *hpuA* ATG translational start site. This appropriately positions the start of transcription 10 nt downstream from the centre of the putative TATAAT box. It is unlikely that *hpuB* is also transcribed independently from a second promoter. The mTn*3*erm insertion in the 3′ end of *hpuA* exerts a polar effect on *hpuB* expression and we were unable to detect the 5′ end of an independent *hpuB* transcript by primer-extension analysis. Currently, we are uncertain as to the origin of the 2.5 kb mRNA.

Our previous data indicated that HpuB was the outer membrane receptor for Hb and the Hb–Hp complex (Lewis and Dyer, 1995). The data presented in this communication is consistent with that hypothesis. The predicted amino acid sequence of HpuB indicated that this protein is an outer membrane receptor belonging to the TonB family of high-affinity transport proteins. TonB is a 26 kDa protein anchored in the cytoplasmic membrane of possibly all Gram-negative bacteria that transduces energy from the cytoplasmic membrane to the outer membrane, driving high-affinity active transport (Klebba *et al*., 1993; Postle, 1993). TonB spans the periplasm and is believed to directly interact with outer membrane receptors at a region called the TonB box (Bell *et al*., 1990; Heller *et al*., 1988; Postle, 1993; Tuckman and Osbourne, 1992). HpuB contains six of the eight amino acids that define the TonB-box motif. Slight variations in this motif may reflect differences between the *E. coli* and neisserial TonB proteins. Stojiljkovic *et al*. (1995) demonstrated that the HmbR protein of *N. meningitidis* strain 8013 was dependent on TonB in *E. coli*, yet this protein contains only five of the eight amino acids that define the TonB motif. Cornelissen *et al*. (1992) described six additional domains common to the *E. coli* TonB-dependent outer membrane receptors and the *N. meningitidis* TonB-dependent transferrin receptor, Tbp1. These domains are also present in HpuB. The last domain encompasses the C-terminal phenylalanine residue that is found in essentially all outer membrane proteins (Postle, 1990; Struyve *et al*., 1991).

In *E. coli*, TonB-dependent transport systems are composed of a ligand-specific outer membrane receptor protein, a periplasmic binding protein, and a multi-component cytoplasmic permease required for active transport of the ligand across the cytoplasmic membrane (Postle,

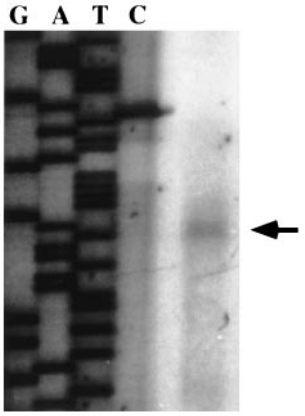


Fig. 7. Primer-extension analysis of total RNA isolated from Fe-starved DNM2 using primer P25.85. The position of the full-length cDNA is indicated by the arrow. Lanes G, A, T and C: the sequencing ladder was obtained using pPST85 (Fig. 5) and primer P25.85.

1993). In the case of the meningococcal *tbpBA* operon, deletion analysis has confirmed that the Tbp2 lipoprotein is required for proper functioning of the TonB-dependent TF receptor (Irwin *et al*., 1993) and it is believed that Tbp1 and Tbp2 act as a complex on the cell surface (Cornelissen *et al*., 1996; Cornelissen and Sparling, 1994; Irwin *et al*., 1993). The requirement of an accessory lipoprotein for TF utilization differentiates this neisserial TonB-dependent transporter from its *E. coli* counterparts.

An important difference between the *E. coli* and neisserial systems is the fate of the ligand that is bound to the receptor. In *E. coli*, the TonB-dependent ferric enterobactin siderophore receptor, FepA, transports the complete ferri–enterobactin complex across the outer membrane, where the complex is bound by the periplasmic binding protein FepB (Klebba *et al*., 1993; Stephens *et al*., 1995). Other TonB-dependent *E. coli* receptors function similarly (Klebba *et al*., 1993). By contrast, the neisserial TonB-dependent TF receptor binds Fe-loaded TF, but only transports Fe to the periplasmic Fe-binding protein, FbpA (Chen *et al*., 1993). Thus, the TF receptor must catalytically remove Fe from TF as well as transport Fe into the periplasm. The accessory lipoprotein Tbp2 may be required for this additional step. By analogy, HpuA may be the Tbp2 homologue in the Hb/Hb–Hp transport system, and may be required for removing Fe or Hm from this ligand for transport. However, we do not yet have direct evidence that HpuA is involved in utilization of Hb or Hb–Hp. We are currently constructing a non-polar *hpuA* deletion mutant to determine the role of this protein in acquisition of Fe from Hb and Hb–Hp.

What might be the function of these accessory lipoproteins? A motif analysis revealed that HpuA and several meningococcal Tbp2 proteins possess a P-loop motif, suggesting that these proteins may bind to ATP or GTP (Saraste *et al*., 1990; Walker *et al*., 1982). This may suggest that ATP hydrolysis at the outer membrane is important for removal of the transported ligand (Fe or Hm) from the bound macromolecular ligand (TF or Hb and Hb–Hp) during transport. We, and others, have recently reported that the DNA 5′ to LbpA (the meningococcal LF receptor) encodes a protein with significant similarity to the Tbp2 lipoprotein (Bonnah *et al*., 1995; 1996; Lewis *et al*., 1996; Pettersson *et al*., 1996). This suggests that the meningococcal LF receptor may also be a bipartite structure that includes an accessory lipoprotein. Thus, the requirement for an accessory lipoprotein may be a common feature of neisserial TonB-dependent receptors, with the exception of HmbR.

Experimental procedures

Bacterial strains and culture conditions

N. meningitidis strains DNM2 (serogroup C, serotype 2a) (Lewis and Dyer, 1995), DNM2E4 (*hpuB*::mTn*3*erm) (Lewis and Dyer, 1995) and DNM2G2 (*hpuA*::mTn*3*erm) were routinely cultured on GC base agar (Difco) at 37°C in a 5.2% CO_2 atmosphere. Strains carrying mini-Tn3erm (mTn*3*erm) were maintained under erythromycin selection (2 μg ml^{-1}). Conditions for Fe-limited growth in Chelex-treated defined medium (CDM) and for examining Fe acquisition were described previously (Lewis and Dyer, 1995). *E. coli* strains were maintained on Luria–Bertani (LB) medium supplemented with antibiotics and Xgal as described (Lewis and Dyer, 1995).

Reagents

All chemicals, unless otherwise indicated, were purchased from Fisher Scientific Co. DNA-modifying enzymes, unless otherwise indicated, were purchased from Promega, New England Biolabs Inc. or Gibco BRL. All radioisotopes were purchased from Dupont, NEN Research Products.

Reagents used for RNA analysis were rendered RNase free by standard procedures. Solutions were treated with 0.10% diethyl pyrocarbonate (DEPC; Sigma) for 18 h at 37°C and autoclaved for 30 min to remove any trace of DEPC. Glassware was baked for a minimum of 18 h at 200°C. Non-disposable plasticware was rinsed thoroughly with 0.1N NaOH, 1 mM EDTA followed with DEPC-treated ddH_2O. RNase-free saturated phenol, pH 4.5, and RNase-free ethidium bromide (10 mg ml^{-1}) were purchased from Amresco.

DNA isolation and manipulation

Chromosomal DNA was prepared from *N. meningitidis* and *E. coli* as described (Lewis and Dyer, 1995). Plasmid DNA was isolated by alkaline lysis (Sambrook *et al*., 1989) or by the use of Qiagen Spin-prep plasmid columns (Qiagen Inc.). Lambda phage DNA was isolated as described by Sambrook *et al*. (1989) or by using a Qiagen lambda column. DNA transformations of *N. meningitidis* and *E. coli* were performed as described previously (Lewis and Dyer, 1995). DNA fragments were purified from agarose gels, where indicated, by the use of Qiaex beads (Qiagen Inc.) according to the manufacturer's instructions.

Primers and probes

Primers used for PCR and DNA sequencing were synthesized by either Biosynthesis Inc. or Integrated DNA Technologies (Table 1). Primers were column purified (desalted) except for those primers used for primer-extension analysis (Biosynthesis, Inc.) which were PAGE purified.

The *hpuA* probe was gel purified as a 1.1 kb *Dra*I fragment from pPST85 (Fig. 5). Three probes specific to *hpuB* were used in separate experiments. The 'full-length' *hpuB* probe (hereafter designated as the *hpuB* probe) was similarly purified from pDS85 (Fig. 5) digested with *Pst*I and *Eco*RI. The 3′-*hpuB* probe was generated by PCR amplification from pDS85 using primer P12.85 and the M13 reverse universal primer (Table 1; Fig. 5). pDS85 was denatured by heating to 94°C for 5 min and PCR was carried out for 30 cycles (1 cycle = denaturation at 94°C for 1 min, annealing at 48°C for 2 min, and primer extension at 72°C for 2 min) using

standard conditions (Boehringer Mannheim Biochemicals). The 1.1 kb product was gel purified using Qiaex beads (Qiagen Inc.). The 5′-*hpuB* probe was generated by PCR amplification of the DNM2 chromosome using primers P4.85 (Table 1) and the N-terminal oligonucleotide (Table 1) (Lewis and Dyer, 1995). PCR was carried out for 30 cycles as described above using a 50°C annealing temperature and Taq DNA polymerase according to the manufacturer's instructions (Perkin–Elmer Cetus). The gonococcal phosphoglucomutase gene was gel purified from *Eco*RI-digested pPGM1.5GK1 (Zhou *et al*., 1994).

Polynucleotide kinase (Promega) was used to label the 5′ end of oligonucleotide probes with [γ^{32}P]-dATP. Double-stranded DNA was labelled using the Random Primer Labelling kit (Bethesda Research Laboratories) according to the manufacturer's instructions, using 25 μCi of [α^{32}P]-dCTP.

Southern transfer and DNA hybridization

Agarose gel electrophoresis, Southern transfer and colony hybridization were performed as previously described (Lewis and Dyer, 1995).

Cloning of the hpuAB *operon*

A 26-mer oligonucleotide probe (Table 1) was designed based on the N-terminal amino acid sequence of HpuB (formerly Hpu) (Lewis and Dyer, 1995). Meningococcal genomic libraries constructed in the lambda cloning vectors λgt11 (Lewis and Dyer, 1995) and λEMBL3 (Quinn *et al*., 1994) were screened using this oligonucleotide as a probe. Hybridizing phage were plaque purified and mapped by restriction-enzyme digestion and Southern hybridization using the N-terminal oligonucleotide as a probe (Lewis and Dyer, 1995). We have previously described pTA1 (Fig. 5), which contains the 850 bp insert from recombinant λgt11 phage λA11-111 (Lewis and Dyer, 1995). Recombinant λEMBL3 phage λ2421, one of several recombinant λEMBL3 phage identified, contained a 13.6 kb insert which included 1.4 kb of the N-terminus of *hpuB*. A 7.5 kb *Pst*I fragment containing all of the cloned *hpuB* and 6.1 kb of upstream DNA was cloned from phage λ2421 into the *Pst*I site of pGEM-3Zf+, creating pPST85 (Fig. 5). A *Dra*I site located 14 nt 5′ to the putative ATG start codon of *hpuB* was used to separate *hpuB* from the upstream DNA by means of subcloning. The 1.4 kb *Dra*I to *Bam*HI fragment containing the 5′ half of *hpuB* was subcloned from pPST85 into *Hinc*II / *Bam*HI-digested pGEM-3Zf+, creating pPD85 (Fig. 5). Sequence analysis revealed a 17 bp cloning artefact at the 3′ end of pPST85 extending from the *Sau*3AI site to the *Pst*I site. This region, which resulted from the ligation of two non-contiguous *Sau*3AI fragments during construction of the λEMBL3 phage library, was not present in the overlapping *hpuB* clones described below and is not depicted in Fig. 5.

The 3′ end of *hpuB* was cloned from the meningococcal chromosome on two overlapping DNA fragments identified by Southern hybridization. A 2.2 kb *Dra*I–*Stu*I fragment of *hpuB* was identified by hybridization to the 5′-*hpuB* probe and cloned into *Hinc*II-digested pGEM-3Zf+ to form pDS85 (Fig. 5). Similarly, a 1.5 kb *Cla*I–*Xba*I fragment was identified by hybridization to the 3′-*hpuB* probe (Fig. 5) and cloned into pGEM-7Zf+ (digested with *Cla*I and *Xba*I), creating pCX1.

DNA sequencing

DNA was sequenced by the dideoxy chain-termination method of Sanger (Sanger *et al*., 1977) using the Sequenase kit version 2.0 (United States Biochemical) according to the manufacturer's instructions. Some regions were sequenced on an Applied Biosystems (ABI) 373A-01 automated DNA sequencer (Perkin–Elmer) as described by Chissoe *et al*. (1991). Mn^{2+} and single-strand binding protein (SSB; United States Biochemical) were used in Sequenase reactions, as directed by the manufacturer, to eliminate compressions. Prior to denaturation and electrophoresis, SSB was digested with proteinase K (1 μg) for 20 min at 60°C. Electrophoresis on 6–8% denaturing polyacrylamide gels was performed as described (Sambrook *et al*., 1989).

Preliminary sequence data suggested that the putative *hpuA* gene in pPST85 appeared to contain a frameshift in the coding sequence, caused by the deletion of a G residue from the poly(G) region located between nucleotides 58 and 67 of the coding DNA. To resolve this frameshift mutation, a 466 bp fragment encompassing this poly(G) region was amplified from the meningococcal chromosome by 30 cycles of PCR (see above; annealing was at 55°C for 2 min) using primers P27.85 and P28.85 (Table 1). The DNA sequence of each strand of the Qiaex-purified PCR product was directly determined using the *fmol* Sequencing Kit as directed by the manufacturer (Promega). End-labelled ([γ^{32}P]-dATP) primers P22.85 and P27.85 were used in sequencing reactions according to the manufacturer's instructions (Table 1). PCR was carried out for 30 cycles (1 cycle = denaturation at 95°C for 30 s, and annealing and extension at 70°C for 30 s) and reactions were analysed on a standard 7% polyacrylamide sequencing gel. Ten G residues were identified in the poly(G) tract, bringing the 5′ and 3′ ends of *hpuA* into the correct reading frame. This indicated that the *hpuA* ORF extends for 1023 bp and encodes a putative protein of 341 amino acids.

DNA sequences were analysed with the GCG package (Devereux *et al*., 1984), and the BLASTP algorithm was used to search non-redundant protein databases (Gish and States, 1993). The DNA sequence of *hpuA* and *hpuB* has been deposited in GenBank under the accession number U73112.

RNA preparation

RNA was isolated from meningococci grown to mid-log phase in CDM (Fe−) or CDM supplemented with 100 μM $Fe(NO_3)_3$ (Fe+) using the RNAgents Total RNA Isolation System from Promega, with the modifications for isolation of bacterial RNA according to the manufacturer's instructions (*Promega Notes* PN023). Cells from 50 ml of culture were suspended in 1 ml of 5 mg ml^{-1} lysozyme and incubated at 4°C for 20 min prior to the addition of the denaturing solution. The RNA yield was determined spectrophotometrically at 260 nm, and this method routinely yielded 2.5 mg of RNA per 50 ml culture. RNA was then treated with RQ1 RNase-free DNase (Promega) (1 units per 30 μg RNA) for 15 min at 37°C in 20 mM HEPES pH 7.5, 10 mM NaCl, 6 mM $MgCl_2$

and 10 mM $CaCl_2$. DNase was removed by phenol extraction as described by Sambrook *et al*. (1989) using acid phenol, pH 4.5.

Northern blotting

RNA (30 μg) was electrophoresed at 40 V on 1% formaldehyde gels in 1× MOPS running buffer as described by Sambrook *et al*. (1989). Gels were then incubated, with gentle agitation, in 0.5 N NaOH for 20 min, and rinsed with three changes of DEPC-treated ddH_2O for 15 min each. Finally, gels were equilibrated in DEPC-treated 20× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate) for 30 min. RNA was transferred to nitrocellulose (Micron Separations, Inc.) in DEPC-treated 20× SSC overnight by capillary action as described by Sambrook *et al*. (1989) and fixed to the nitrocellulose filters by baking at 80°C for 1.5 h prior to hybridization. Hybridization probes were labelled with [α^{32}P]-dCTP as described above. Hybridization was performed exactly as described for Southern blot hybridization except that the concentration of Denhardt's reagent (1% BSA, 1% Ficoll and 1% polyvinyl- pyrolidine) was increased to 2× in the prehybridization and hybridization solutions (Lewis and Dyer, 1995). After hybridization, RNA was visualized on the membrane by staining with methylene blue (Sambrook *et al*., 1989). All reagents used for Northern blots, except Denhardt's solution, were rendered RNase free by either treating with DEPC or baking at 200°C.

RT-PCR

RT-PCR was performed essentially as described (Beverly, 1992). In brief, 5 μg of RNA prepared from Fe-starved meningococci was treated with an additional 16.5 U of RQ1 RNase-free DNase in the presence of 98 U of RNasin and 1 mM dithiothretol (DTT) as described above. Following acid-phenol extraction, RNA was precipitated with sodium acetate, suspended in 20.5 μl DEPC ddH_2O and annealed to 500 ng of primer P4.85 (100 ng μl^{-1}) by heating to 65°C for 15 min, followed by rapid chilling on ice. cDNA was synthesized at 42°C using Superscript II reverse transcriptase (Gibco BRL) in a 50 μl reaction containing 1000 U of enzyme, first-strand buffer (Gibco BRL), 10 mM of each dNTP (dATP, dCTP, dGTP and dTTP), 1 mM DTT and 10 U of RNasin. Reverse transcriptase was omitted from negative-control reactions. After 1 h the reaction was diluted 10-fold with DEPC-treated ddH_2O and stored at −20°C. cDNA or the negative-control reaction (5 μl) was used as a template in a 30 cycle PCR reaction (see above) with a 60°C annealing temperature using either primers P24.84 and P26.85 or P6.85 and P4.85 (Table 1). In addition, reactions containing DNM2 chromosomal DNA (positive control) or ddH_2O (negative control) as the template were prepared as described above.

Primer extension

The MMLV-RT H^- Primer Extension System (Promega) was used to map the transcriptional start site of *hpuAB*. PAGE-purified primer P25.85 (Table 1) or P26.85 (Table 1) was end-labelled with [γ^{32}P]-dATP as described and 6 ng of labelled primer was annealed to 10 μg of total RNA prepared from Fe-starved meningococci. Annealing was carried out at 65°C for 30 min, and then RNA and primer were slowly cooled (at room temperature in 100 ml of 65°C water) to 35°C. When the temperature of the annealing reaction reached 50°C, 10 U of RNasin was added. At 35°C, MMLV-RT was added and the reaction was incubated at 42°C for 30 min as described. Following termination, cDNA products were separated on an 8% denaturing polyacrylamide sequencing gel next to the products from a sequencing reaction using either primer P25.85 or P26.85 and pPST85 (Fig. 5). Gels were dried and exposed to KODAK X-OMAT film overnight at room temperature. Control reactions were prepared exactly as described above except that DEPC-treated ddH_2O was added in place of 10 μg of RNA.

Shuttle mutagenesis

Shuttle mutagenesis to insertionally inactivate *hpuA* was performed as described previously (Lewis and Dyer, 1995).

Membrane-protein preparation, SDS–PAGE analysis and Western blot analysis

Membrane-protein preparation, SDS–PAGE analysis and Western blot analysis were performed as previously described (Lewis and Dyer, 1995).

[^{3}H]-palmitic acid labelling of N. meningitidis

Meningococci from cultures grown for 15 h on CDM (Fe−) or CDM supplemented with 100 μM $Fe(NO_3)_3$ (Fe+) agar plates were used to inoculate CDM broth cultures (Fe− and Fe+, respectively). Cultures were incubated at 37°C with shaking. When the cells entered log phase, [9,10-^{3}H]-palmitic acid (Dupont, NEN Research Products) was added to a final concentration of 50 μCi ml^{-1}, and growth was continued until cells reached late log phase (Theisen *et al*., 1992). Meningococci were then harvested by centrifugation at 67 000 × *g* for 5 min, and stored at −80°C. The cell pellets were thawed and suspended in 0.05 vol. of sterile 10 mM HEPES, pH 7.4. Approximately 0.2 ml of the original culture was analysed on SDS–PAGE (10%, 12.5% and 15%) as previously described (Lewis and Dyer, 1995). Following SDS–PAGE, gels were fixed, stained with Coomassie brilliant blue (Sasse and Gallagher, 1987), and prepared for fluorography using EN^{3}HANCE (Dupont, NEN Research Products) according to the manufacturer's instructions. Gels were exposed to Kodak X-OMAT film at −80°C for 3 or 4 d prior to developing. To aid in localization of the HpuA protein, total-membrane proteins prepared from *N. meningitidis* strains DNM2 and DNM2G2 were electrophoresed on the same gel as ^{3}H-labelled cells and stained with Coomassie brilliant blue.

In a separate experiment, ^{3}H-labelled cells were precipitated with trichloroacetic acid (TCA) and washed with methanol to remove loosely attached lipids, according to the protocol of Theisen *et al*. (1992). The dried pellets were suspended in 10 mM HEPES and analysed by SDS–PAGE and fluorography as described above. The lipoprotein profile observed was indistinguishable from that obtained when the TCA

precipitation and methanol wash was omitted. The fluorographs shown (Fig. 4) were prepared using the first protocol described above.

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