The necessity to sequester essential biochemical processes poses a challenge for living cells. Physical compartmentalization created by semipermeable membrane partitions achieves this segregation, but at the predictable expense of imposing a restrictive barrier to the cellular ingress and exit of solutes. Prokaryotes and eukaryotes have surmounted this obstacle by evolving transport systems, termed ATP-binding cassette (ABC) transporters, that couple ATP hydrolysis with substrate translocation across biological membranes. These systems exhibit a modular organization comprised of four structural domains that may be expressed as individual polypeptides or may be fused into single multidomain proteins. Two membrane-integral domains span the membrane multiple times and form the passageway through which the solute flux occurs. Two ATP-binding cassettes reside on the cytosolic face of the membrane.

The binding-protein-dependent transporters of gram-negative bacteria represent the best-characterized members of this superfamily. These transporters recruit an auxiliary component, a periplasmic binding protein, that constitutes the major determinant in conferring substrate specificity. Two membrane-integral domains span the membrane multiple times and form the passageway through which the solute flux occurs. Two ATP-binding cassettes reside on the cytosolic face of the membrane.

The neisserial fbpABC locus has been proposed to constitute a single transcriptional unit. To confirm this operonic arrangement, transcription assays using reverse transcriptase PCR amplification were conducted with Neisseria meningitidis. The presence of fbpAB and fbpBC transcripts obtained by priming cDNA synthesis with an fbpC-sequence-specific oligonucleotide indicates that fbpABC is organized as a single expression unit. The ratio of fbpA to fbpABC mRNA was approximately between 10- to 20-fold, as determined by real-time quantitative PCR.

DNA isolation and manipulations. Meningococcal genomic DNA was recovered by standard methods. DNA fragments were purified from agarose gels by using either the GeneClean II Purification Matrix kit (Bio 101, Inc., Vista, Calif.) or by passage through NENSORB 20 (NEN, Boston, Mass.) cartridges. DNA sequencing was performed according to the dideoxynucleotide chain-termination method (22) with an Applied Biosystems) with fluorescence-labelled synthetic oligonucleotide primers based on the known fbpABC sequence (1). All sequence reactions were run and analyzed on an Applied Biosystems 377XL automated DNA sequencer.

The fbpABC locus from N. meningitidis B16B6 chromosomal DNA was PCR amplified with primer set 5' fbpA and 3' fbpCstop with Pfu DNA polymerase (Stratagene, La Jolla, Calif.). The amplification product was ligated into the TA cloning vector pCR2.1 (Invitrogen, San Diego, Calif.), creating pCR2FABC. This construct was used to generate the standard curves in the quantitative PCR assay.
cycles of PCR amplification were performed with primers (Table 1) initiated first-strand cDNA synthesis. Thirty-six manufacturer’s instructions. The indicated gene-specific primer oligonucleotides (N6) as primers, RT buffer (50 mM Tris [pH 8.3], 75 mM KCl, 1.5 mM MgCl2), 10 mM dithiothreitol, and 1 mM (each) deoxynucleoside triphosphates (dNTPs; dATP, dGTP, dCTP, and dTTP), 100 U of Superscript II RT (Gibco BRL), and 17 U of RNase inhibitor (RNAguard; Am- 

<table>
<thead>
<tr>
<th>Strain, plasmid, or primer</th>
<th>Relevant characteristic(s)</th>
<th>Source or reference</th>
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<tr>
<td>Neisseria meningitidis B16B6</td>
<td>Clinical isolate, serogroup B, serotype 2a</td>
<td>C. Frasch</td>
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<td>pCR2FABC</td>
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<td>This study</td>
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<td>5'fbpA</td>
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<td>3'fbpAint</td>
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<td>3'fbpCint</td>
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RNA isolation and RT-PCR. Total cellular RNA was extracted from mid-logarithmic-phase (OD600 of 0.685) meningococcal cultures by using the RNeasy Midi kit (Qiagen, Inc., Clarita, Calif.) according to the manufacturer’s recommendations. To eliminate contaminating genomic DNA, total RNA was subjected to DNase I (amplification grade, Gibco BRL, Life Technologies, Burlington, Canada) treatment as specified by the manufacturer. RNA concentrations were determined by measuring the A260; samples were immediately stored at −70°C. Reverse transcription was performed with the SuperScript II RNase H− RT-PCR kit (Gibco BRL) following the manufacturer’s instructions. The indicated gene-specific primers (Table 1) initiated first-strand cDNA synthesis. Thirty-six cycles of PCR amplification were performed with Taq polymerase (Gibco BRL) on a Perkin-Elmer model 480 DNA thermal cycler with denaturation at 94°C for 30 s, primer annealing at 52°C for 30 s, and extension at 72°C for 3 min. Identical aliquots were processed in parallel without the addition of RT, in order to ensure that residual genomic DNA was not serving as the template in the PCR amplification. PCR amplification products were electrophoresed on 1% agarose gels and stained with ethidium bromide. The identity of all RT-PCR amplification fragments was verified by nucleotide sequencing.

QRT-PCR. For quantitative RT-PCR (QRT-PCR), cDNA synthesis was performed in a 20-μl final volume that included 2 μg of meningococcal total RNA, 100 pmol of random hexamer oligonucleotides (N6) as primers, RT buffer (50 mM Tris [pH 8.3], 75 mM KCl, 1.5 mM MgCl2), 10 mM dithiothreitol, and 1 mM (each) deoxynucleoside triphosphates (dNTPs; dATP, dGTP, dCTP, and dTTP), 100 U of Superscript II RT (Gibco BRL), and 17 U of RNase inhibitor (RNAguard; Am-
ersham Pharmacia Biotech, Inc., Baie d’Urfe, Canada). The RT reaction was performed in an MJ Research minicycler PTC-150 at 22°C for 5 min, followed by incubation at 4°C for 50 min. The samples were heated for 5 min at 95°C to terminate the reaction. Real-time quantitative PCR was performed in 10-μl final volumes in glass capillaries in a LightCycler Instrument (Roche Diagnostics, Laval, Canada) (29). The PCR master mix comprised 1× PCR buffer, 3 mM MgCl₂, 1 mg of bovine serum albumin per ml, 0.2 mM dNTPs, 0.5 mM both forward and reverse primers, a 1:3,000 dilution of SYBR Green I (Molecular Probes), and 0.4 U of Platinum Taq (Gibco BRL). Into each capillary tube, 9 μl of PCR master mix and 1 μl of template target DNA (cDNA or pCR2FABC) were loaded. Sealed capillaries were centrifuged prior to placement into the LightCycler carousel. PCR amplification was performed with an initial denaturation at 95°C for 30 s followed by 45 cycles of denaturation at 95°C for 2 s (ramping at 20°C/s), annealing at 52°C for 5 s (ramping at 20°C/s), and elongation at 72°C for 42 s (ramping at 5°C/s). Amplicon specificity was verified by melting curve analyses with the LightCycler software, version 3.39. The identity of the amplicons was also established by confirmation of the expected molecular weight by agarose gel electrophoresis. Optimal conditions for amplification were determined by preliminary experiments with meningococcal genomic DNA. The quantitative PCR experiment was repeated three times, and each experiment produced similar results.

Transcription assays using RT-PCR. The integrity of the total RNA preparation was assessed by demonstrating the presence of the transcript from the housekeeping gene asd (10) (Fig. 1B, lane h; gene-specific primer 3'9HKasdstop for the RT step, primer pair 5'9HKasdint and 3'9HKasdstop for PCR amplification) and the presence of the iron-regulated transcript tbpA (Fig. 1B, lane d; gene-specific oligonucleotide 48 for the RT step, primers 385 and 48 for the PCR step). The latter result also suggests that the starting RNA preparation is unlikely to be selectively biased against iron-regulated transcripts. The requirement for such a representative mRNA library arises from two considerations. First, the transcription of fbpA is enhanced under iron-limiting conditions (9). Second, given the proposed operonic organization of fbpABC, the expression of the putative polycistronic transcript encompassing this gene cluster would be anticipated to exhibit the same property.

The RT-PCR strategy used in the transcription assays was based on the general premise that upstream gene sequences within a given transcript would be readily detected by PCR amplification if these regions formed a continuous message. First-strand synthesis was initiated with gene-specific primers designed to anneal to intragenic sites within fbpB or fbpC and to the region encompassing the fbpC stop codon. Primer pairs were then selected to bracket the intergenic junctions between fbpAB and fbpBC (Fig. 1A). PCR amplification products generated by these oligonucleotides would therefore be contiguous and would be derived from a polycistronic transcript.
Using a cDNA template reverse transcribed from primer 5′fbpBint engineered for sequences situated within fbpB, an amplicon spanning the fbpAB junction was detected (Fig. 1B, lane I; oligonucleotides 5′fbpAint and 3′fbpBint). This result indicates that fbpA and fbpB are cotranscribed.

Similarly, the presence of the predicted PCR fragments straddling the fbpAB junction (Fig. 1B, lane r; primers 5′fbpAint and 3′fbpBint) and fbpBC (Fig. 1B, lane q; primers 5′fbpBint and 3′fbpCint) intergenic regions, when cDNA generated from the fbpC-specific oligonucleotide 3′fbpCstop was used as template, indicates that fbpA, fbpB, and fbpC are cotranscribed. Thus, the aggregate RT-PCR data illustrate that fbpABC is organized as a single polycistronic transcriptional unit.

The results from a series of control experiments conducted concurrently with each of the four sets of RT-PCR assays confirmed the substrate quality and guaranteed the specificity of each component of the RT-PCRs. First, signals for the expected size for primer pairs 560 and 561 and 5′fbpBint and fbpBC were observed. Second, the absence of a PCR product when genomic DNA acted as the template ensured that residual genomic DNA had not contaminated the starting RNA preparations. Second, the presence of the predicted PCR fragments (Fig. 1B, lanes e, i, n, and t) verified the authenticity of each DNA template concentration, a single PCR product of the expected size for primer pairs 560 and 561 and 5′fbpBint and fbpBC was detected by gel electrophoresis, and amplicon fidelity was confirmed by melting curve analysis (data not shown).

The kinetic PCR assay exhibited a dynamic range of at least 4 orders of magnitude. Each assay was performed in triplicate, and representative data from one such experiment are shown. The figure was imaged with a Hewlett-Packard ScanJet HP, edited by using Adobe Photoshop 3.0, and labelled by using Microsoft PowerPoint 97.

Transcript quantity. Real-time PCR studies were used to compare the relative abundance of fbpA-, fbpAB-, and fbpBC-bearing transcripts.

The test samples were cDNA primed with random hexamer, and 10-fold serial dilutions of pCR2FABC DNA were employed to generate the standard curves. Kinetic curves are shown for four concentrations of DNA (Fig. 2A and 3A). For each DNA template concentration, a single PCR product of the expected size for primer pairs 560 and 561 and 5′fbpBint and fbpBC was detected by gel electrophoresis, and amplicon fidelity was confirmed by melting curve analysis (data not shown). Each kinetic curve was defined by a cycle threshold \( C_t \) which marks the fractional cycle number during the logarithmic phase at which the fluorescence of a given sample becomes significantly different from the baseline signal. The \( C_t \) value also represents the crossover point between the kinetic curve and an arbitrary fluorescence level, which for all of the experiments presented is 1.5. \( C_t \) values are inversely proportional to the log of the initial template concentration and thus are used to calculate transcript copy number. The target message in the unknown sample is quantified by measuring \( C_t \) and by using the calibration curve performed during the same experiment to determine the starting target message quantity. As depicted in the calibration curves for each primer pair (Fig. 2B and 3B), the kinetic PCR assay exhibited a dynamic range of at least 4 orders of magnitude.

These experiments revealed that fbpA mRNA was expressed at a 10- to 20-fold-higher level than the fbpAB transcript (Fig. 2 and 3). Similar ratios were observed when the level of fbpA transcript was compared to that of the fbpBC-expressing transcript (data not shown). These results indicate a preferential accumulation of fbpA transcript relative to full-length fbpABC mRNA.

The evidence provided in this study unequivocally shows that the meningococcal fbpABC locus is transcribed as a single contiguous message, and, therefore, this gene cluster is organized as a polycistronic operon. A prior report employing RT-PCR amplification was unable to detect either fbpC or fbpBC transcripts (25). The reasons for the discordant results are...
unclear, but differences in the primer design and in the RT-PCR amplification protocol represent two potential explanations.

The \( fbpAB \) and \( fbpBC \) transcripts detected in this study are likely translationally active, because transcription and translation are coupled processes in prokaryotes. Implicit in this observation is a functional role for both FbpB and FbpC in neisserial periplasmic iron transport from human transferrin and human lactoferrin. However, the mandatory participation of an unidentified subsidiary ABC transporter involved in neisserial periplasmic iron transport. Such an explanation is unlikley, since in an antecedent study, an \( fbpBC \) mutant is unimpaired in the ability to access iron from human transferrin and human lactoferrin for growth (25).

There are several possible explanations for this observation, but no version supplies an immediately patent answer. First, functional disruption of \( fbpC \) may have unmasked the presence of an unidentified subsidiary ABC transporter involved in neisserial periplasmic iron transport. Such an explanation is unlikely, since in an antecedent study, an \( fbpABC \) mutant, which might also be anticipated to exhibit an iron acquisition phenotype, similar to that of the \( fbpC \) mutant, is incompetent in iron utilization (14). Second, iron transport in the \( fbpC \) mutant may have been restored by the presence of another chromosomal wild-type copy of \( fbpABC \). The absence of other gonococcal gene loci displaying significant sequence homology to \( fbpABC \) in an analysis of the assembled contigs deposited in the ongoing gonococcal and meningococcal genome projects renders this explanation unlikely. Third, iron transport in the \( fbpC \) mutant may have been rescued by complementation with a heterologous ATPase subunit. Such functional exchange has occurred only in the context in which the heterologous complementing ATPase component is significantly overexpressed with respect to its respective cognate integral membrane protein (11, 28). Because this requirement has not been directly satisfied in the defined \( fbpC \) mutant, this explanation also appears unlikely to apply.

The enhanced amount of the \( fbpA \)-bearing transcript compared to the full-length \( fbpABC \) mRNA has several significant implications. First, this result supplies a molecular correlate for the observation that FbpA is synthesized in excess of the periplasmic binding-protein-dependent importers is the preferential production of the periplasmic binding protein constituent (27). This characteristic is functionally relevant because the efficiency of the transport process is critically dependent upon the preservation of such a stoichiometry (16).

Second, this result suggests that segmental differences in transcript stability may account for the differential expression of individual genes in the \( fbpABC \) operon. Such differential rates of transcript decay underlie the preferential accumulation of the periplasmic binding protein MalE in the \( E. coli \) maltose transporter \( malEFGK \) (7). The increased stability of the \( malE \) transcript is a consequence of a stem-loop structure located in the \( malEF \) intergenic region (18, 19). Many stem-loop structures serve as barriers to 3′→5′ exonucleases (13, 19) by impeding the processive action of these enzymes, thereby increasing the chemical longevity of upstream mRNA. The sequence comprising the neisserial \( fbpAB \) intercistronic junction exhibits the potential to adopt a similar conformation (1), raising the intriguing speculation that this secondary structure represents the structural determinant of \( fbpA \) transcript stability.

In summary, we have established that the meningococcal \( fbpABC \) locus exhibits an operonic organization. The genetic (4), structural, and immunological (17) conservation of \( fbpABC \) in the pathogenic \( Neisseria \) spp. suggests that the results from this investigation apply to \( N. gonorrhoeae \).

This work was supported by a grant (MT-15111) from the Medical Research Council of Canada. V.D. is the recipient of a summer studentship from the Alberta Heritage Foundation for Medical Research.
We thank R. Chalus for excellent technical assistance with the use of the LightCycler Instrument.

REFERENCES

Editor: E. I. Tuomanen