

# MOLECULAR CLONING AND CHARACTERIZATION OF THE STRUCTURAL GENE FOR THE MAJOR IRON-REGULATED PROTEIN EXPRESSED BY *NEISSERIA GONORRHOEAE*

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Pathogenic microorganisms synthesize a unique class of proteins when confronted with an iron-restricted environment such as the human host. These proteins, termed iron-regulated proteins, function either in the acquisition of growth-essential iron (1) or as mediators of microbial virulence such as toxins (2). Similarly, *Neisseria gonorrhoeae* responds to an iron-limited environment by expressing a repertoire of iron-regulated proteins (3–6). One of these is synthesized in greater amounts than the other iron-regulated proteins and has been called the major iron-regulated protein (MIRP).<sup>1</sup> The designation of this protein has recently been changed to Fbp (for iron-binding protein), according to the nomenclatural recommendations arising at the Sixth International Pathogenic *Neisseria* Conference (7). The Fbp has an apparent molecular weight of ~37,000, as determined by SDS-PAGE (5), and is antigenically conserved among the pathogenic species of the genus *Neisseria* (8). It has been purified to homogeneity (9), and many of the biochemical properties associated with the purified protein have been defined. Most notable is the observation that iron co-purifies with Fbp and is bound by the protein as a ferric ion at an approximate molar ratio of 1:1 (9–11). These observations have led to the hypothesis that the Fbp functions in a central iron-binding capacity essential for the assimilation of iron by *N. gonorrhoeae* (2, 3, 6). This study reports the cloning and sequencing of the structural Fbp gene from *N. gonorrhoeae* strain F62 and demonstrates characteristics of this protein consistent with a role in iron acquisition.

## Materials and Methods

*Bacterial Strains, Plasmids, and Media.* *N. gonorrhoeae* strain F62 was kindly provided by R. P. Williams (Baylor College of Medicine, Houston, TX). pUC13 and pUC19 were obtained from Bethesda Research Laboratories, Gaithersburg, MD. *Escherichia coli* TB1 and 71.18 were obtained from L. Mayer (Centers for Disease Control, Atlanta, GA). The expression vector

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$\lambda$ gt11 and *E. coli* Y1090 were purchased from Promega Biotec (Madison, WI). *N. gonorrhoeae* was routinely grown as described previously (9). Cultures were incubated at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. *E. coli* was propagated and maintained as described elsewhere (12).

**Generation of Amino Acid Sequence from an Arginine-specific Cleavage.** Fbp, 135 nmol (5 mg in 5 ml) purified as described elsewhere (9), was denatured by dialysis overnight in 0.1 M phosphate buffer, pH 7.4, containing 6 M guanidine-HCl. Lysine residues were modified by the addition of a 50-fold molar excess of maleic anhydride (Sigma Chemical Co., St. Louis, MO) for 4 h at room temperature. The modified protein was then dialyzed against two changes of 0.05 M phosphate buffer, pH 7.2, containing 1 mM CaCl<sub>2</sub>. Modified Fbp was digested by the addition of 4 U of agarose-bound trypsin (Pierce Chemical Co., Rockville, IL), followed by incubation for 4 h at 37°C. Agarose-bound trypsin was removed from the mixture by centrifugation and the digest was lyophilized. Lysine residues were deprotected by rehydrating the lyophilized material in 1 ml of 5% (vol/vol) acetic acid, pH 3.5, followed by incubating the mixture at 37°C for 48 h. After removal of the blocking groups, the suspension was lyophilized and suspended in 1 ml of distilled water. Chromatographic separation of the cleavage products was accomplished by reversed-phase HPLC, using an Altex RP-18 column (Beckman Instruments, Palo Alto, CA). Fragments were eluted with a gradient between distilled water containing 0.1% (wt/vol) trifluoroacetic acid and acetonitrile containing 0.08% trifluoroacetic acid. Peaks were collected manually, dried by lyophilization, and sequenced by Edman degradation.

**Synthetic Oligonucleotides.** Synthetic primers and probes (summarized in Table I) were synthesized by standard phosphoramidate chemistry (14, 15). Crude oligonucleotides were purified by reverse-phase chromatography as described elsewhere (16).

**Southern Blotting and DNA Hybridization.** Restriction endonuclease-digested genomic DNA was transferred to nitrocellulose, according to the method of Southern (17). Bacterial colonies were transferred to nitrocellulose by the method of Grunstein and Hogness (18). The Southern blots and colony blots were hybridized for 16 h in 6× SSC and 5× Denhardt's solution (19) with 0.5 × 10<sup>6</sup> cpm of <sup>32</sup>P-labeled oligonucleotide per milliliter at room temperature (20). The filters were washed at room temperature with 6× SSC containing 0.1% SDS, dried and exposed to XAR 5 x-ray film (Eastman Kodak, Rochester, NY).

**DNA Sequencing.** The hybrid gonococcal DNA inserts in pUC13 and pUC19 were excised by digestion with Eco RI and Hind III restriction endonucleases. The liberated fragments were purified by agarose gel electrophoresis, ethanol-precipitated, and ligated into the M13 phage vectors mp8 and mp9 (21). The DNA sequence was determined by the dideoxy method of Sanger et al. (22). Single-stranded DNA was prepared, and the nucleotide sequence was established using Sequenase (United States Biochemicals, Cleveland, OH).

**Enzyme Amplification of Genomic DNA Using the Inverse Polymerase Chain Reaction (IPCR).** The sequence of the 5' flanking sequence was obtained by a modification of the method described by Triglia et al. (23) and Ochman et al. (24) and is summarized in Fig. 4. For circularization, 0.1 μg of Sau 3A-digested genomic DNA was diluted to a final concentration of 1 μg/ml in ligation buffer (50 mM Tris HCl, pH 7.4; 10 mM MgCl<sub>2</sub>; 20 mM dithiothreitol; 1 mM ATP, and 5 μg/ml BSA). T<sub>4</sub> DNA ligase (0.2 U/μl) was added and the mixture was incubated at 14°C for 16 h. After ligation, Sma I (Bethesda Research Laboratories) was added to linearize the DNA. The 5' flanking sequence was then flanked by two areas of known sequence that could be primed for amplification by PCR.

The PCR amplification was carried out in a Perkin-Elmer Cetus Thermal Cycler (Emoryville, CA). The reaction mixture consisted of 0.1 μg DNA obtained as described above in the presence of 60 pmol of oligonucleotides BP5 and BP7 and 200 μM dNTPs. The procedure consisted of 25 cycles of denaturation at 95°C for 1.5 min, primer annealing for 2 min at 60°C, and elongation by Taq polymerase (Perkin-Elmer Cetus) at 70°C for 3 min. The amplification reaction mixture (100 μl) was mixed with 1 ml H<sub>2</sub>O in a Centricon-30 (Amicon Corp., Danvers, MA) and centrifuged at 5,500 rpm to desalt and remove excess dNTPs.

**Cloning of the Amplified IPCR Fragment and DNA Sequencing.** The IPCR-generated DNA was directly ligated into the Sma I site of M13 phage vector mpl1 (21), transformed into *E. coli* host 71.18 using the method of Hanahan (25), and plated on B agar plates. The resulting

plaques were screened with  $^{32}\text{P}$ -end-labeled oligonucleotide BP6. Single-stranded DNA was prepared from a clone hybridizing to this probe.

## Results

*Identification of a Fragment Encoding the Gonococcal Fbp Sequence from Genomic Digests.* Previous studies have suggested that a 1,020-bp coding sequence would be required to encode the mature Fbp. Initial attempts to clone a fragment of this size in  $\lambda\text{GT11}$ , based upon reactivity with affinity-purified polyclonal Fbp-specific antibodies, were unsuccessful. As an alternative, oligonucleotide probes corresponding to known amino acid sequences were used. The  $\text{NH}_2$ -terminal 48 residues of the gonococcal Fbp have been reported previously (6, 9), and accounted for  $\sim 15\%$  of the entire protein. To obtain sequence information outside this region, peptides isolated from an arginine-specific cleavage were subjected to  $\text{NH}_2$ -terminal sequence analysis (Fig. 1). Addi-

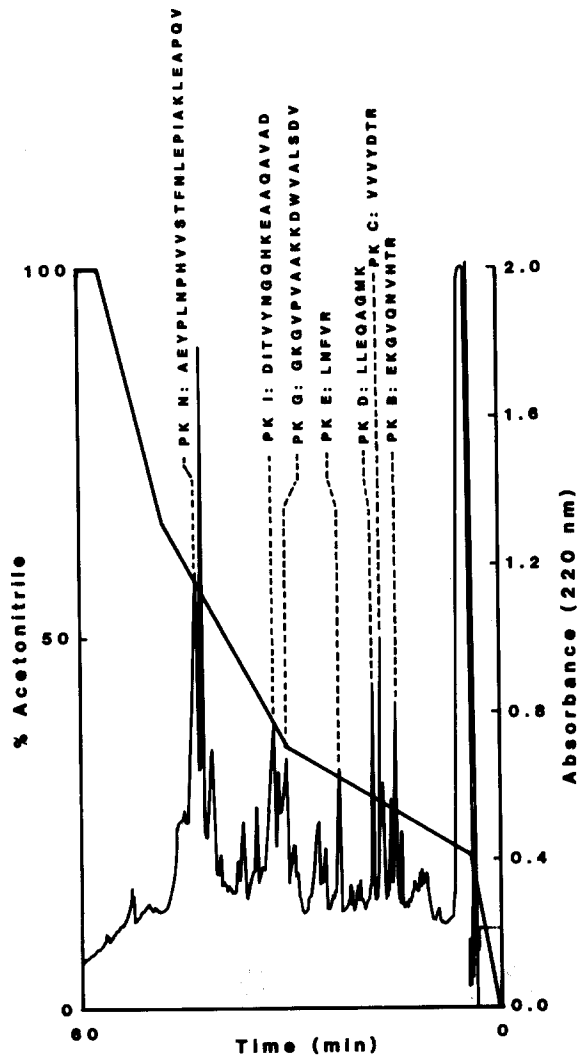


FIGURE 1. Chromatographic profile of an arginine-specific tryptic digestion of the purified Fbp of *N. gonorrhoeae* strain F62 as described in the text. Deprotected peptides were separated by HPLC, using a C18 column and a gradient of acetonitrile (demonstrated by the solid line corresponding to the scale on the left). The chromatographic profile was monitored at 220 nm and is shown relative to the right-hand scale. Major peaks, noted above the chromatograph as PK B, C, D, E, G, I, and N, were manually collected, repurified using a similar acetonitrile gradient, and subjected to  $\text{NH}_2$ -terminal sequence analysis. The sequence of each peak fraction is given above.

tional sequences corresponding to ~20% of the molecule were generated by this technique. From these data, two synthetic oligonucleotides were derived and synthesized as described in Table I. Oligonucleotide BP1 was based on the NH<sub>2</sub>-terminal amino acids 6-11; oligonucleotide BP2 was derived from a portion of the amino acid sequence of the peptide in peak G (Fig. 1).

Southern blot analysis of *N. gonorrhoeae* F62 DNA and pUC13 showed that both BP1 and BP2 hybridized strongly to the DNA from strain F62, but not to pUC13 (data not shown). Furthermore, both synthetic oligonucleotides recognized a 900-bp Sau 3A genomic fragment. Using BP1 or BP2 as a screen, no recombinants could be identified when these 900-bp fragments were cloned into the Bam HI site of pUC13. In addition, positive clones were not obtained when a λGT11 library was screened with end-labeled BP1 or BP2 (data not shown). Thus, it is possible that a large fragment of DNA containing the entire gene or a substantial portion thereof was lethal to *E. coli*.

Southern blot analysis of genomic DNA digested with Rsa I revealed that the oligonucleotide probes BP1 and BP2 hybridized to a 600-bp fragment (data not shown). Genomic Rsa I fragments ranging in size from 400 to 800 bp were ligated into the Sma I site of pUC13 and transformed into *E. coli* TB1. 1 colony out of 1,500 hybridized with the end-labeled oligonucleotide, BP1. Restriction analysis of the plasmid isolated from this clone, pSB3, revealed a 600-bp insert that hybridized strongly to

TABLE I  
Oligonucleotides Used in This Study

Designation	Sequence	Derived from
BP1*	5' TCTTTITGTTGICCIIT 3'	NH <sub>2</sub> -terminal sequence corresponding to positions 6-11 of the mature protein (9)
BP2†	5' TTTTTTCTAACCCA 3'	Amino acid sequence "KKDWV" contained within the sequence of the peptide in peak G (Fig. 1)
BP3	5' AACAACTACTACTGGCAGCT 3'	DNA sequence of a 21-bp segment within 56-bp sequence defined by the Taq I and Rsa I sites of pSB3 (Fig. 2)
BP5	5' TTGTACACCGGTAATGTCTGCC 3'	Complement of the DNA sequence shown in Fig. 3, spanning the 5' Rsa I site of pSB1 (Fig. 2)
BP6	5' TGGCGGCGGTCAGGGCTGCGGC 3'	Complement of the DNA sequence shown in Fig. 3, corresponding to a sequence within the leader peptide region on pSB1
BP7	5' TGTCCACACCGCCTGAATTTTCGT 3'	DNA sequence corresponding to a 24-bp sequence lying within a 42-bp fragment defined by the Rsa I and Sau 3A sites on pSB7 (Fig. 2)

\* Oligonucleotide BP1 was corrected for degeneracy by the replacement of multiple bases with inosine (I), or by inclusion of a single base derived from codon usage data (13), indicated by "T".

† Oligonucleotide BP2 was synthesized as a mixture of the eight possible combinations that could encode this amino acid sequence.

both BP1 and BP2 (Fig. 2). This fragment was cloned into M13 mp8 and mp9, and both strands consisting of 601 bases were sequenced. An open reading frame predicting the known NH<sub>2</sub>-terminal amino acid sequence was identified, starting at residue 5 and extending to residue 206.

*Identification of Overlapping Fragments Containing the Entire Fbp Gene Sequence.* To obtain additional sequence information for the gonococcal *fbp*, an oligonucleotide probe (BP3) was synthesized that corresponded to the 21-bp sequence between the Taq I and Rsa I sites (Fig. 2). When BP3 and BP1 were hybridized to Taq I digested genomic DNA, both oligonucleotides recognized fragments of ~650 bp. A new DNA library was constructed by digesting gonococcal genomic DNA with Taq I and ligating fragments ranging in size from 500 to 750 bp into the Acc I site of pUC13. This ligation mixture was transformed into *E. coli* TB1 and 2,000 transformants were separately screened with oligonucleotide probes BP1 and BP3. Each probe hybridized to a single colony out of 2,000. Restriction analysis of the plasmid hybridizing to BP1 revealed a 630-bp insert (clone pSB1). This insert was isolated and cloned into M13 mp8 and mp9. Sequence analysis demonstrated that the pSB1 insert overlapped with the insert from pSB3 and contained additional 5' sequence that encoded a typical leader peptide region (Fig. 2). The clone hybridizing with BP3 contained a plasmid with a 556-bp insert (clone pSB7). Sequence analysis of this insert was obtained upon cloning into M13 mp8 and mp9. The data revealed that it also overlapped with the insert in pSB3 but encoded the 3' end of *fbp* and included a potential transcription termination signal (Fig. 2).

The complete sequence of *fbp* is contained on the recombinant plasmids pSB1, pSB3, and pSB7 and is shown in Fig. 3. The 5' end of the gonococcal insert in pSB1 begins 4 bases upstream from the initiation codon. The 3' end of the gonococcal insert in pSB7 is 192 bp downstream from the termination codon. The mature protein consists of 308 amino acids with a signal peptide of 22 residues. The consensus amino acid sequence agreed completely with the NH<sub>2</sub>-terminal amino acid se-

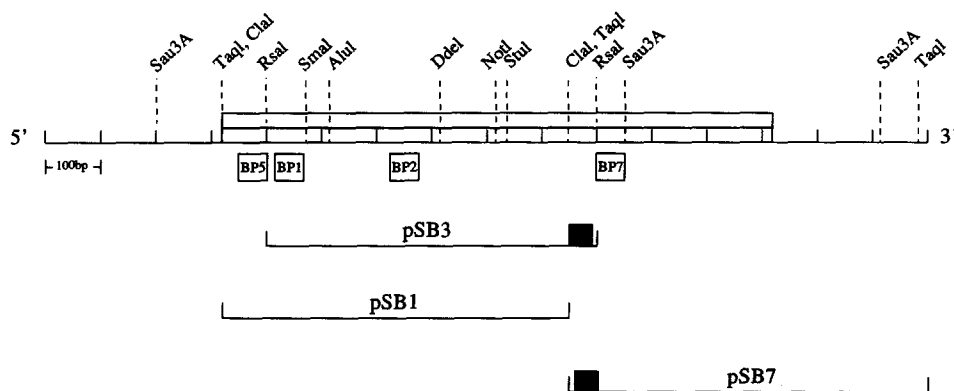


FIGURE 2. Physical map and location of the *fbp* gene sequence derived from inserts of pSB1, pSB3, and pSB7. Clone pSB3 was initially characterized and sequenced as described in text. From this clone, oligonucleotide BP3 was synthesized to correspond to 21 bases of DNA located between the Taq I and Rsa I sites. Genomic Taq I fragments ligated into pUC13 were screened with BP1 and BP3 to obtain clone pSB1 and pSB7. Also shown are the oligonucleotides BP5 and BP7, which were used in the IPCR reaction to obtain the sequence of the promoter region.

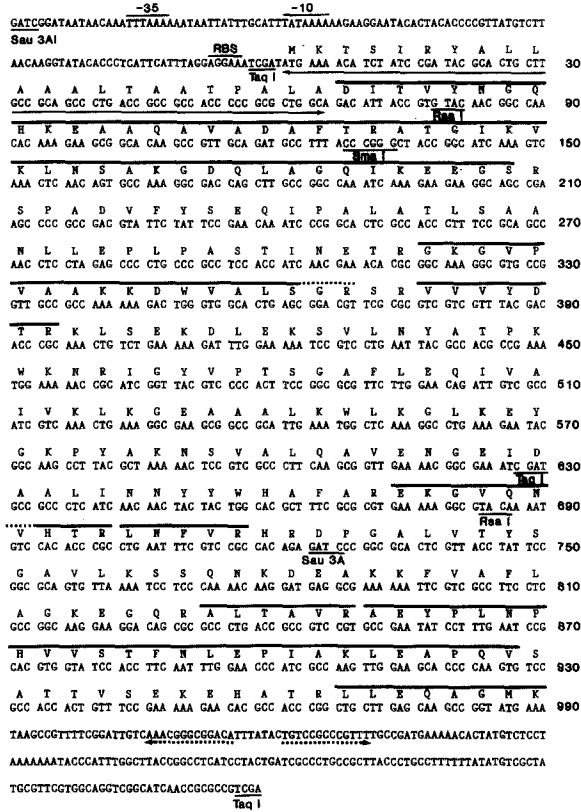


FIGURE 3. Nucleotide sequence and predicted amino acid sequence of the Fbp of *N. gonorrhoeae* strain F62. The proposed promoter sequences (-35 and -10) and ribosome binding site (RBS) are shown. The solid arrow designates the signal peptide; the dotted arrow indicates the 13 bp loop that may serve as the transcription termination signal. The solid lines above the amino acid symbols correspond to the identical sequences of peptides determined from the arginine-specific cleavage of purified Fbp (Fig. 1). The dotted lines above 366-369 bp and 691 bp indicate discrepancies in the peptide sequence.

quencing data (9). The locations of the peptide sequences generated from the arginine-specific cleavage were found within the consensus amino acid sequence (Fig. 3). 17 bp downstream from the stop codon, a 13-bp inverted repeat was located (Fig. 3). It is possible that this sequence corresponded to a transcription termination or attenuation signal.

*Amplification and Sequencing of the 5' Flanking Sequence of the Gonococcal Fbp Gene.* DNA fragments containing the 5' flanking sequence could not be identified by the previous technique. However, based on the occurrence of a Sau 3A site within the Fbp gene sequence and the observation that both BP1 and BP2 hybridized to a 900-bp Sau 3A fragment (Fig. 2), we considered it likely that this fragment contained ~200 bp of the 5' flanking DNA sequence. IPCR is a technique used to amplify fragments containing unknown sequences bounded by only one region of known DNA sequence. This technique was used to amplify and sequence the unknown DNA sequence contained on the 900-bp Sau 3A fragment. The strategy for obtaining and amplifying this fragment is described in Materials and Methods and outlined in Fig. 4. After amplification, agarose gel electrophoresis demonstrated a single 250-bp band of DNA (data not shown). This fragment hybridized strongly to an end-labeled oligonucleotide derived from the leader peptide sequence (BP6) (data not shown). The PCR fragment was ligated into the Sma I site of M13 mp11, and the resulting plaques

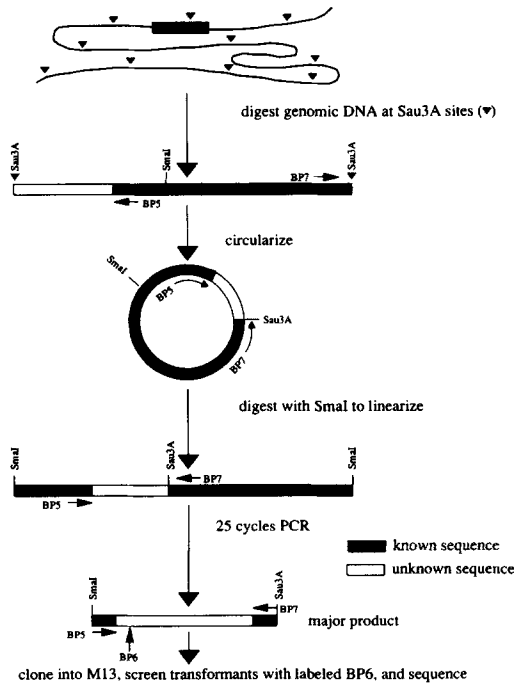


FIGURE 4. Outline of the IPCR procedure used to amplify the Fbp promoter. The genome is cut with restriction endonuclease Sau 3A, circularized, and cleaved with a restriction endonuclease (Sma I) that has a site in between the two oligonucleotides BP5 and BP7. The linear DNA is amplified with 25 cycles of PCR. The 250-bp product was ligated into the Sma I site of M13, and transformants were screened with internal oligonucleotide BP6.

were screened with end-labeled BP6. Only one reactive plaque was observed. The sequence of the insert revealed complete fidelity between known sequences from the 3' end of Fbp, recognized by BP7 (Fig. 4), and the 5' end of Fbp, recognized by BP5 (Fig. 4), with the 5' flanking sequence in between. This 5' sequence is included in Fig. 3 and shows a potential ribosomal binding site located upstream from the initiation codon, as well as potential -10 and -35 consensus sequences.

**Determination of the Number of Fbp Gene Copies per Genome.** The number of copies of the Fbp gene within the genome of *N. gonorrhoeae* strain F62 was examined using nick-translated pSB3 to probe Southern blots of genomic DNA digested with various restriction endonucleases. The results (Fig. 5) show a single hybrid band with Alu I-, Eco RI-, Hind III-, Sau 3A-, Rsa I-, and Taq I-digested DNA. Two hybrid bands were observed with Cla I-digested DNA, reflecting the internal Cla I site in pSB3 (Fig. 2). Thus, it can be concluded that there is only a single copy of the Fbp gene in strain F62.

## Discussion

This article describes the cloning of the gene encoding the gonococcal Fbp that has allowed us to elucidate its structure at the level of primary sequence. The cloning of the complete gonococcal Fbp gene in plasmids and an expression vector has not been possible; most likely the intact gene or a flanking sequence is lethal in the *E. coli* host. Similar observations have been made for the genes encoding the gonococcal porin protein, protein I (26, 27) and the outer membrane protein macromolecular complex (28). To circumvent this phenomenon, oligonucleotides derived from the

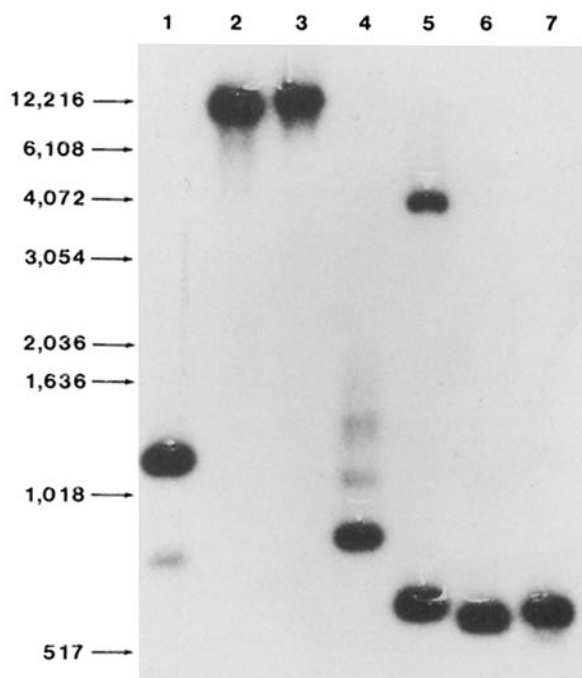


FIGURE 5. Hybridization analysis of the Fbp gene sequence from *N. gonorrhoeae* strain F62. Genomic DNA was digested with Alu I, Eco RI, Hind III, Sau 3A, Cla I, Rsa I, and Taq I, (lanes 1-7, respectively). Clone pSB3 was nick-translated and hybridized to the above genomic digests. Size markers (bp, at left) are the 1-kb ladder.

known NH<sub>2</sub>-terminal amino acid sequence and from a peptide sequence known to lie outside the NH<sub>2</sub>-terminal 48 residues were used to identify DNA fragments encoding portions of the Fbp gene. Sequence analysis of three overlapping clones provided the information needed to define completely the structural gene. The molecular weight of the Fbp, based on the consensus amino acid sequence of the predicted mature protein is 33,571. This value is less than the estimated molecular mass of 36,000 to 37,000 that was obtained by SDS-PAGE (4-6), and more consistent with the estimations of molecular mass obtained by gel filtration chromatography of 26,000 to 32,000 (9). The anomalous behavior of this protein in SDS-PAGE gels may reflect its exceptionally high isoelectric point of >9.35 (9) or features unique to its structure that have yet to be defined.

Potential regulatory sequences juxtaposed 5' to the Fbp structural gene were obtained by IPCR. A poor cloning efficiency of the PCR-amplified fragment was observed and is notable in light of our inability to identify antigenically related Fbp from an expression library. This may in fact reflect the apparent inefficiency with which this sequence can be introduced into *E. coli* or represent the selection of a degenerate sequence generated by a mutagenic event during PCR-amplification (29). These possibilities are presently being explored. For the sequence obtained by the current method, a typical ribosomal binding site as well as degenerate -10 and -35 regions can be proposed (Fig. 3). The degeneracy of the potential -35 region (30) is similar to other promoters that match the -35 consensus sequence poorly, and require an activator that substitutes as part of a polymerase binding site (31).

A precise function for the gonococcal Fbp has yet to be described. To date, specu-



lation has focused on the possibility that this protein may be a central component in the iron-acquisition system (4, 5, 9). Gonococci differ from other pathogenic microorganisms which utilize siderophores for the acquisition of growth-essential iron (32-35). Rather, they appear to directly bind endogenous host iron-containing proteins such as transferrin or lactoferrin as a first step in the assimilation of iron (36). The steps required after this initial binding event have yet to be described, but will certainly involve: (a) the removal of iron from these proteins; (b) the transport of iron across the outer membrane; (c) the transport of iron across the cytoplasmic membrane; and (d) assimilation within the cytoplasm. Several elements of the Fbp, described herein and elsewhere (4, 5, 9), are consistent with its participation in the gonococcal high-affinity iron-acquisition system. For one, the DNA sequence predicts a 22-residue leader peptide based upon the presence of an initiator methionine and 21 intervening amino acids before the known NH<sub>2</sub>-terminal residue of the purified protein (9). The presence of a leader peptide suggests that the mature Fbp functions beyond the cytoplasmic membrane similar to components of other well-defined high affinity nutrient uptake systems (37). Second, elements of high affinity nutrient uptake systems are often regulated by the bioavailability of the target nutrient (38). Therefore, an important indication that the Fbp participates in iron acquisition is its differential expression under iron-replete and iron-limited conditions (5). However, the most compelling evidence that the gonococcal Fbp is involved in iron assimilation is that this protein stoichiometrically binds a single ferric cation (10). The availability of the primary sequence of the gonococcal Fbp allows for a more precise understanding of the biological properties of this protein. Evidence obtained from electron paramagnetic resonance studies indicates that this protein coordinates with iron in a manner similar to the iron-binding proteins transferrin and lactoferrin (Chen, C. Y., and S. A. Morse, unpublished data). These latter proteins are approximately twice as large as the gonococcal Fbp and have the capacity to bind 2 moles of iron per mole of protein. They exist as closely related bilobed structures, each lobe binding a single ferric cation as well as a bicarbonate anion (39). For human lactoferrin, the residues coordinating with iron include the phenolate oxygens of two tyrosines, the imidazole nitrogen of a single histidine, and the carboxylate oxygen of an aspartic acid (40). While no apparent homology exists between lactoferrin and the gonococcal Fbp, it is possible that analogous residues participate in the binding of iron by the Fbp. These studies are in progress.

The precise role of the gonococcal Fbp in iron acquisition awaits a clearer picture of the mechanism by which these organisms obtain iron from their environment. The importance of the Fbp for *N. gonorrhoeae* is underscored by the observation that this protein is found in all pathogenic *Neisseria* spp. studied to date (8). That this protein is synthesized *in vivo* is indicated by the presence of specific antibodies that develop during the course of a natural infection (41). Studies have demonstrated that when iron-starved gonococci are pulsed with <sup>55</sup>Fe-transferrin, followed by a chase with unlabeled transferrin, there is an initial association of the <sup>55</sup>Fe with the Fbp, followed by a decrease over time (Chen, C. Y. and S. A. Morse, unpublished data). These data suggest that Fbp is a transient participant in passing the iron bound to transferrin, a property consistent with a role in iron transport. The data presented here and elsewhere will be used in future studies to uncover the role of the Fbp and its importance to gonococcal pathogenesis.

### Summary

This report describes the cloning and sequencing of the major iron-regulated protein (termed Fbp) of *Neisseria gonorrhoeae* strain F62. Attempts to identify recombinants expressing the Fbp using specific antibody proved unsuccessful. Therefore, an alternative cloning strategy using oligonucleotide probes derived from NH<sub>2</sub>-terminal and tryptic fragments of this protein was used to identify short fragments of the gene. Using this methodology, the gene encoding the precursor of Fbp was cloned on three separate overlapping fragments and sequenced, and the amino acid sequence was deduced. These data were unambiguously confirmed by the known NH<sub>2</sub>-terminal amino acid sequence and were supported by the sequences from tryptic fragments that lie outside of this region. Using oligonucleotide probes, we were unable to obtain clones encoding the potential regulatory region of this protein. Therefore, the technique of inverse polymerase chain reaction was used to amplify a fragment containing an additional 200 bp. This fragment was cloned and sequenced and found to contain a consensus ribosome binding site and potential -10 and -35 sequences. Hybridization analysis of genomic DNA from gonococcal strain F62 indicated that only a single copy of the Fbp gene exists per genome. These results complement the biochemical characterization of the Fbp expressed by gonococci and further suggest that it has a role in iron-acquisition.

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