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Promoter Mapping and Transcriptional Regulation of the Iron-Regulated *Neisseria gonorrhoeae fbpA* Gene

REN-YO FORNG,[1] CHIOMA R. EKECHUKWU,[1] SHAMBAVI SUBBARAO,[2] STEPHEN A. MORSE,[2] AND CAROLINE A. GENCO[1]*

Department of Microbiology and Immunology, Morehouse School of Medicine, Atlanta, Georgia 30310-1495,[1] and Division of AIDS, Sexually Transmitted Diseases, and Tuberculosis Laboratory Research, Centers for Disease Control and Prevention, Atlanta, Georgia 30333[2]

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In this study, we have mapped the promoter region of the *Neisseria gonorrhoeae* ferric iron binding protein-encoding gene *fbpA*, determined the start point of transcription, and examined the accumulation of *fbpA* mRNA. Primer extension analysis of the *fbpA* promoter region indicated a single transcriptional start site located 51 bp upstream of the ATG translational start site. Northern blot analysis with a 200-bp *fbpA* structural gene probe detected one transcript of 1.0 kb in RNAs extracted from gonococcal cultures grown under iron-restricted conditions; the 1.0-kb transcript was observed to accumulate at a steady rate throughout the growth cycle. In comparison, in cultures grown under iron-sufficient conditions, the intensity of the 1.0-kb transcript was reduced considerably. Isolation of total RNA from rifampin-treated cells indicated that the half-life of the 1.0-kb *fbpA* transcript in cells grown under iron-restricted conditions was 1.2 ± 0.2 min, while that of the 1.0-kb *fbpA* transcript obtained from cultures grown under iron-sufficient conditions was 0.5 ± 0.1 min. Taken together, our results indicate that the *fbpA* promoter is regulated by iron and that transcription and translation of FbpA are closely linked.

In *Escherichia coli* and *Salmonella typhimurium* many genes involved in iron acquisition and virulence are transcriptionally regulated by the availability of iron through the ferric iron uptake regulator protein, Fur (16, 19). Regulation of iron-regulated genes by Fur homologs is widespread, and homologs of *fur* have been shown to exist in genetically diverse microorganisms, including *Neisseria gonorrhoeae* and *Neisseria meningitidis* (5, 17, 27). *N. gonorrhoeae* expresses a number of iron-regulated proteins when grown in vitro under iron-limited conditions (15). Some, like the periplasmic binding protein FbpA, which is synthesized by all strains of *N. gonorrhoeae*, are thought to play an essential role in iron acquisition and utilization (21). Previous investigations have established that FbpA shuttles iron from transferrin and ferric citrate through the periplasm (10, 21, 22); recent studies suggest that FbpA may also be involved in the acquisition of iron from heme (14). The gene for the gonococcal and meningococcal FbpA has been cloned and sequenced (6, 7), and the promoter region has been shown to contain two regions that exhibit homology with the *E. coli* Fur binding sequence (15, 16). The *fbpA* promoter region contains a 19-bp sequence with 80% similarity to the primary Fur consensus binding sequence, designated IB1, and a 15-bp sequence with 87% similarity to the secondary Fur consensus binding sequence, designated IB2 (see Fig. 2B). DNase I footprinting studies have recently confirmed binding of *E. coli* Fur to a 42-bp sequence within the *fbpA* promoter sequence encompassing these two regions (13). Studies employing *fbpA-cat* fusions have confirmed that transcriptional regulation of *fbpA* in response to iron occurs via the negative regulator Fur (13, 26). To gain a more complete understanding of the role of iron in expression of *fbpA*, we have examined the accumulation and stability of the *fbpA* mRNA and the regulation of FbpA transcription by iron. We have also mapped the promoter region of *fbpA* and determined the start point of transcription.

Bacterial strains and growth conditions. *N. gonorrhoeae* F62 (obtained from R. P. Williams, Baylor College of Medicine, Houston, Tex.) was maintained on complex medium (gonococcal base) (Difco Laboratories, Detroit, Mich.) agar containing 1% IsoVitaleX (BBL, Baltimore, Md.) and grown aerobically with 5% CO_2 at 37°C. Broth cultures were grown in a shaking water bath in a liquid medium similar in composition to GC agar base but lacking agar and supplemented with 4.2% $NaHCO_3$ and 1% IsoVitaleX. Iron restriction was achieved by the addition of 25 to 50 μM Desferal (Ciba Geigy, Summit, N.J.). All cultures were monitored spectrophotometrically at 660 nm, and samples were removed at 1-h intervals for RNA or protein analysis (Fig. 1).

Promoter mapping of *fbpA*. Total cellular RNA was isolated from early-log-phase gonococcal cells grown at 35°C in gonococcal broth (GCB) or chemically defined medium (CDM) (23) under either iron-restricted (25 μM Desferal) or -sufficient (50 μM ferric nitrate) conditions. Annealing of an 18-bp oligonucleotide complementary to the 5′ end of the noncoding strand of *fbpA* (SS1, 5′GGCGGTCAGGGCTGCGGC3′) and primer extension reactions with reverse transcriptase (20) were carried out with 45 μg of RNA, and the samples were analyzed on a 6% sequencing gel alongside the M13mp18 sequence, which was generated with a standard M13 primer. As shown in Fig. 2A, a single major band was observed under both growth conditions; this band represents a transcriptional start site located 51 bp upstream of the ATG translational start site. Although this major band was also detected with cellular RNA from iron-sufficient cells, a more intense band was observed with RNA from cells grown under iron-restricted conditions.

The *fbpA* promoter contains a typical ribosome binding site as well as degenerate −10 and −35 regions (Fig. 2B). Three nucleotides which exhibit identity to the *E. coli* consensus sequence can be found within both the −35 and −10 regions. Using a multiple-sequence-alignment program, we examined

* Corresponding author. Mailing address: Department of Microbiology and Immunology, Morehouse School of Medicine, 720 Westview Dr., SW, Atlanta, GA 30310-1495. Phone: (404) 752-1874. Fax: (404) 752-1179. E-mail: genco@link.msm.edu.

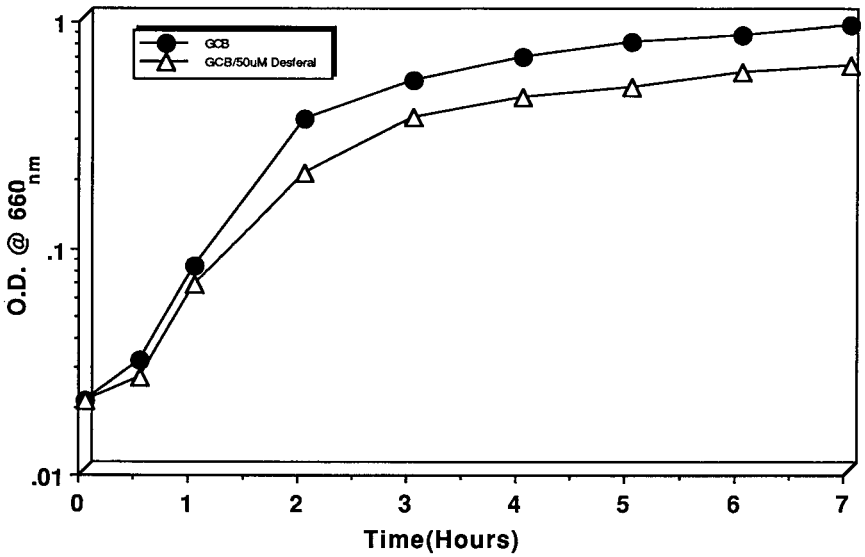


FIG. 1. Growth of *N. gonorrhoeae* under iron-sufficient and iron-restricted conditions. *N. gonorrhoeae* F62 was inoculated from GCB plates into GCB liquid medium, and iron restriction was achieved by the addition of 50 μM Desferal. Samples were removed at 1-h intervals, and growth was monitored spectrophotometrically at 660 nm. Iron-sufficient cultures consisted of GCB; iron-restricted cultures consisted of GCB plus Desferal. O.D., optical density.

the −10 and −35 regions from the gonococcal and meningococcal iron-regulated genes *frpA*, *fbpB*, *fbpC*, *fur*, and *tbpB* (5–9, 11, 17, 24, 25, 28, 29) and determined that the *Neisseria* −10 and −35 consensus sequences have very strong homology with the *E. coli* sigma 70 −10 consensus sequence (Fig. 2C and D), indicating that transcription of these *Neisseria* iron-regulated genes may occur via RNA polymerase containing a sigma 70 subunit.

Analysis of *fbpA* mRNA under iron-sufficient and -restricted conditions. In a preliminary study (21), Northern blot analysis indicated that *fbpA* mRNA was detected at low levels in RNA extracted from gonococci grown under iron-sufficient conditions. To examine the kinetics of *fbpA* mRNA synthesis, we compared the accumulations of *fbpA* mRNA during growth under iron-sufficient and -restricted conditions. Growth of *N. gonorrhoeae* F62 under iron-sufficient conditions (GCB) resulted in a typical growth curve with a final cell density of 10^8 CFU (Fig. 1). Iron restriction was achieved by the addition of 50 μM Desferal to GCB; this was found to effectively limit the growth of *N. gonorrhoeae* as measured by optical density readings and viable counts (Fig. 1 and data not shown). Therefore, for all studies described below, *N. gonorrhoeae* was grown in GCB plus 50 μM Desferal for iron-restricted growth. Samples of cultures grown under iron-sufficient and -restricted conditions were removed at 1-h intervals, total RNAs were extracted, and 50 μg of RNA was examined by Northern blot analysis with a 200-bp *fbpA* structural gene probe. The *fbpA* probe was prepared by PCR amplification of total gonococcal genomic DNA with primers FS1 (5′GACATTACCGTGTAC AACGGC3′) and FS2 (5′CGGAAAGGGTGGCGAGTGCC G3′). The protocol for the rapid isolation of total cellular RNA from gram-negative bacteria and Northern blot analysis were performed as described by Ausubel et al. (4).

In cultures grown under iron-restricted and -sufficient conditions, we typically observed a 1.0-kb *fbpA* transcript (corresponding to the expected size of a transcript proceeding from the *fbpA* promoter to the putative transcription termination signal) (Fig. 3). In cultures grown under iron-sufficient conditions, the 1.0-kb *fbpA* transcript was initially detected at 0.5 h and was found to increase in quantity slowly (Fig. 3B). However, the 1.0-kb *fbpA* transcript was barely detectable in samples obtained at 4 h and was not detected in samples obtained at 6 h (Fig. 3B). Northern blot analysis of mRNA isolated from iron-restricted cultures also detected a transcript of approximately 1.0 kb in size in RNA extracted from gonococci grown under iron restriction (Fig. 3A); however, this transcript accumulated at a steady rate throughout the entire growth cycle, with an increase in the accumulation during exponential growth phase relative to that present during early logarithmic growth phase. Densitometer scans of autoradiographs of the 1.0-kb *fbpA* mRNA obtained from cells after 6 h of growth indicated that the 1.0-kb *fbpA* transcript continued to accumulate during growth under iron-restricted conditions (Fig. 3C).

To examine the production of the *fbpA* transcript in cultures which were iron restricted to a greater extent, we performed an experiment in which we added an additional 50 μM Desferal to cultures of *N. gonorrhoeae* which had been growing for 3 h under iron restriction. For this experiment, *N. gonorrhoeae* was cultured in GCB plus 50 μM Desferal for 3 h, after which an additional 50 μM Desferal was added to the culture and growth continued for an additional hour. Samples were removed at 2, 3 (time of addition of the Desferal boost), 3.5, and 4 h. RNA was extracted, and equivalent amounts of total RNA (50 μg) were loaded on an agarose gel and examined by Northern blot analysis as described above. Under these conditions we found an increase in the accumulation of the 1.0-kb *fbpA* transcript in cultures sampled 30 min after the addition of Desferal compared to amounts of the transcript in cultures sampled prior to the addition of Desferal (2- and 3-h samples) (Fig. 3E and F). These results indicate that the increased accumulation of the 1.0-kb *fbpA* transcript was produced in response to the increase in iron restriction of these cultures.

Analysis of FbpA production. Previous studies have established that less than 20% of FbpA is turned over in a 30-min period during growth under iron-restricted conditions (10). In order to correlate transcription of *fbpA* with the expression of FbpA, samples were removed at 1-h intervals from gonococcal cultures grown under iron-sufficient and -restricted conditions and fractionated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, and the presence of FbpA was de-

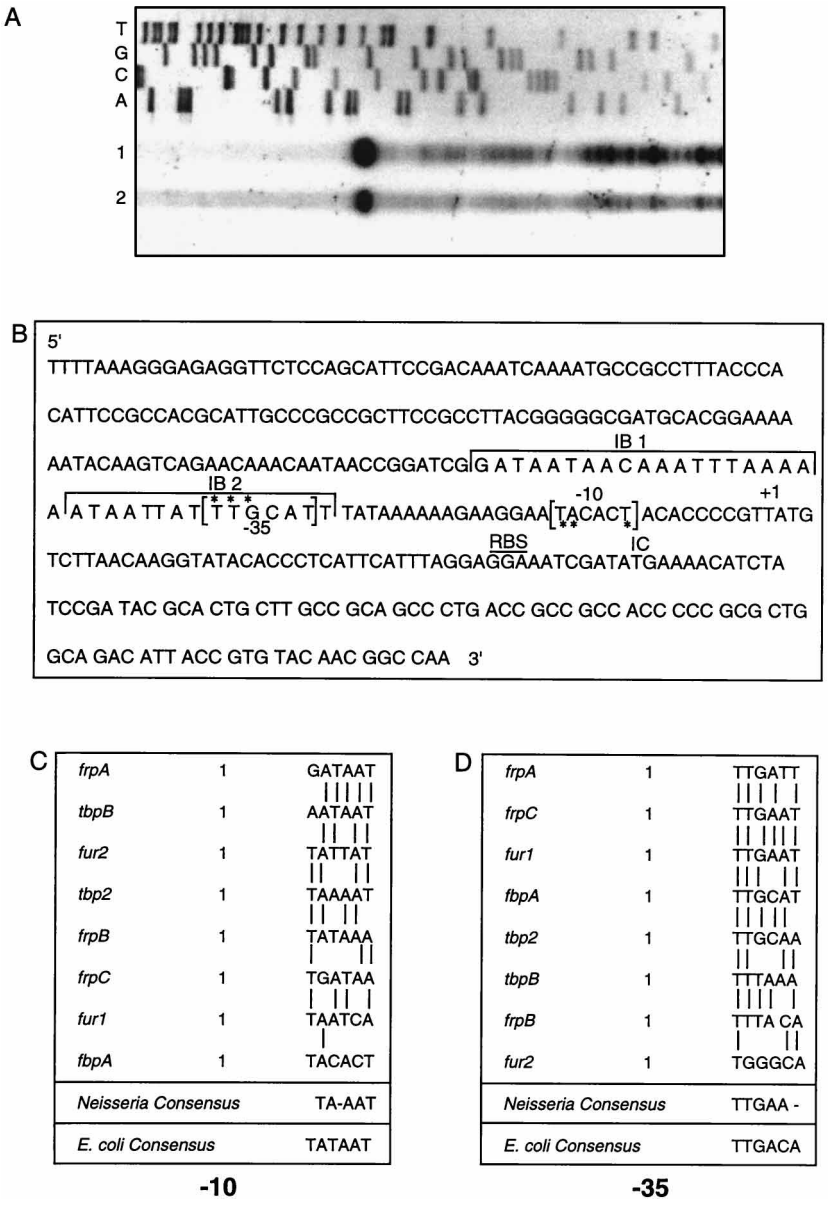
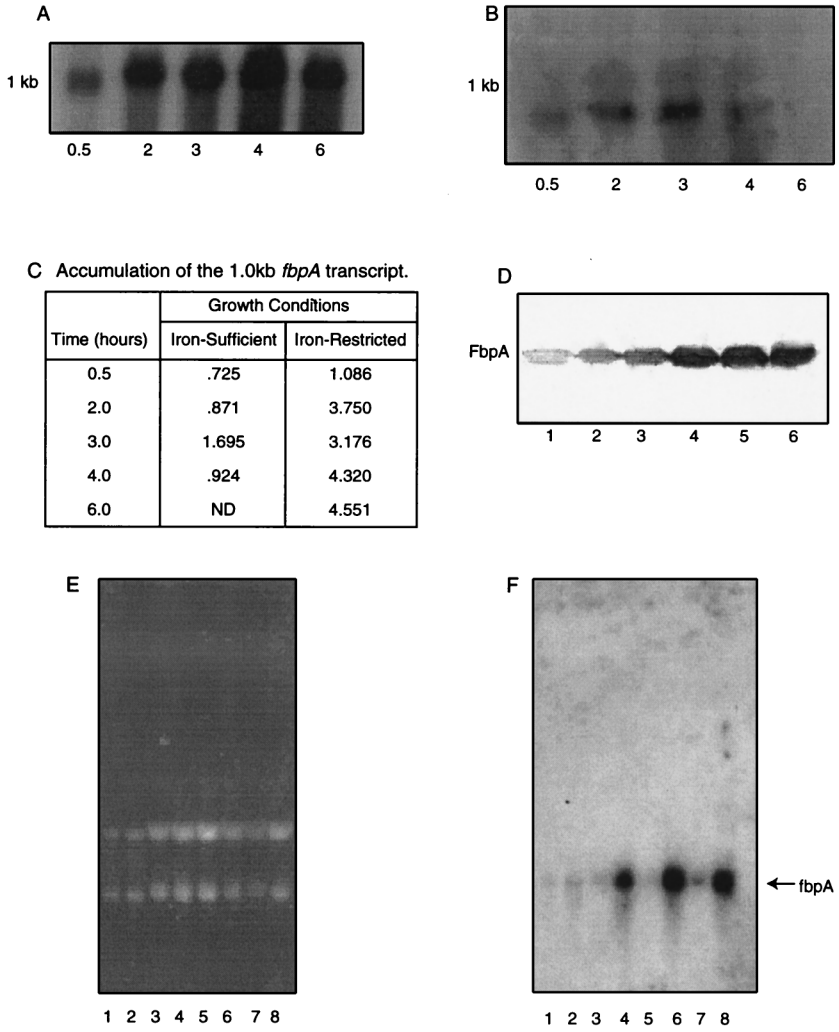


FIG. 2. Primer extension analysis and nucleotide sequence of the promoter region of *fbpA*. (A) Total cellular RNAs were extracted from *N. gonorrhoeae* F62 grown in CDM under iron-sufficient (lane 2) or iron-deficient conditions (CDM plus 25 μM Desferal) (lane 1). Total RNAs were hybridized with a synthetic oligonucleotide (SS1) which is complementary to the sequence within the *fbpA* structural gene. The extension products were analyzed alongside the M13 sequence, which was generated with a standard M13 primer. (B) The nucleotide sequence of the 5′ sequence of the *fbpA* was determined as previously described (6). IB1, iron box 1 region showing homology to the primary Fur consensus binding sequence (16); IB2, iron box 2 showing homology to the secondary Fur consensus binding sequence; *, nucleotides identical to the consensus −10 and −35 sequences; +1, transcriptional start site; IC, initiation codon; RBS, ribosome binding site. (C and D) Sequence alignment of the −10 (C) and −35 (D) regions of gonococcal and meningococcal iron-regulated genes. Vertical lines indicate identity.

termined by Western blot analysis (22). Our results indicate that cultures grown under iron-restricted conditions produced FbpA throughout the entire growth cycle (Fig. 3F). FbpA synthesis increased over time and closely reflected both the increase in optical density over time as well as the increase in the amount of *fbpA* mRNA (Fig. 4A). With the same concentration of total protein, FbpA was barely detectable by Western blot analysis in cell lysates from cultures grown under iron-sufficient conditions (data not shown); however, a slight increase in FbpA was observed at later stages of growth (4 to 6 h). The expression of FbpA in stationary phase may be due to the depletion of iron from the growth media at later time points. Alternatively, glucose or O_2 may be limiting at this stage of growth; this glucose or O_2 limitation may result in decreased iron uptake into the cell and may potentially influence the expression of FbpA. These results confirmed that the expression of FbpA was also regulated by iron.

Stability of *fbpA* mRNA. To examine the stability of the 1.0-kb *fbpA* transcript, rifampin (200 μg/ml) was added to gonococcal cultures (grown under iron-replete or -depleted conditions) which had reached mid-exponential phase of growth (approximately 3 h) prior to RNA isolation. Aliquots (1.0 ml) were removed at 1-min intervals and immediately placed on ice. Total RNA was isolated, and equivalent quan-



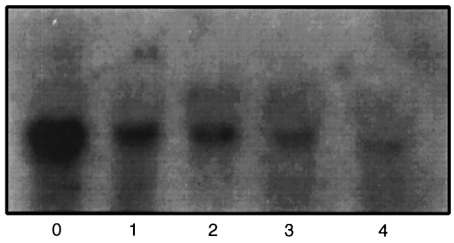
Time (hours)	Growth Conditions	
	Iron-Sufficient	Iron-Restricted
0.5	.725	1.086
2.0	.871	3.750
3.0	1.695	3.176
4.0	.924	4.320
6.0	ND	4.551

FIG. 3. Analysis of *fbpA* mRNA and FbpA accumulation. Total RNAs were isolated over the entire growth curve of cells grown under iron-restricted (GCB plus 50 μM Desferal) (A) or iron-sufficient (GCB) (B) conditions. RNAs were fractionated, blotted, and hybridized to a probe corresponding to 200 bp of the *fbpA* structural gene. Total RNAs were transferred overnight to a Hybond N^+ nylon membrane (Amersham, Arlington Heights, Ill.) in 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), and membranes were prehybridized overnight at 65°C in prehybridization solution (2), followed by hybridization with a ^{32}P-labeled *fbpA* probe overnight at 65°C in the prehybridization solution (containing 1% dextran sulfate). Following hybridization, membranes were washed four times at 65°C in 2.5× SSC–1% SDS for 15 min followed by one wash at 65°C in 0.25× SSC–0.1% SDS. The sizes of the *fbpA* transcripts are indicated to the left. The times (in hours) at which samples were removed from growing gonococcal cultures are indicated below the lanes. (C) Densitometer scans of signals on the autoradiograms that had been exposed for identical times are expressed as band intensities. ND, not detectable. (D) Cellular protein from iron-restricted gonococcal cultures was isolated at the same time as total RNA, samples were separated by SDS–12% polyacrylamide gel electrophoresis and transferred to nitrocellulose, and rabbit anti-FbpA serum was added. The membrane was washed, goat anti-rabbit serum conjugated to alkaline phosphatase was added, and the membrane was incubated at room temperature for 30 min. Numbers under the lanes indicate time points (in hours) during growth at which samples were obtained. Results are from one experiment and are representative of three separate experiments. (E and F) Total RNAs were isolated at 2, 3, 3.5, and 4 h postinoculation from *N. gonorrhoeae* cultures grown under iron-restricted (GCB plus 50 μM Desferal; lanes 2, 4, 6, and 8) and iron-sufficient (GCB; lanes 1, 3, 5, and 7) conditions. An additional 50 μM Desferal was added to iron-restricted cultures after the 3-h sample was removed. Total RNA (50 μg) was extracted and separated by agarose gel electrophoresis; 23S and 16S rRNAs were used as internal controls for even loading of all lanes. Agarose gels (E) were blotted onto nitrocellulose membranes and hybridized with an *fbpA*-specific probe (F).

tities were analyzed by Northern blot analysis as described above. Northern blots of RNAs extracted from iron-sufficient and -restricted cultures were exposed to X-ray film for identical times prior to quantification of signals. Following quantification of the selected signals on the autoradiogram by densitometry, the half-life of the transcript was determined as the time point at which 50% of the signal had decayed by plotting densitometry readings versus time of isolation on a semilogarithmic plot.

Northern blot analysis with an *fbpA*-specific probe indicated that the majority of the 1.0-kb *fbpA* transcript was degraded within 1 min, with little detectable mRNA present 4 min after the addition of rifampin under iron-restricted conditions (Fig. 4). At 1 min, following the addition of rifampin, only 7% of the 1.0-kb *fbpA* transcript was detected in cultures grown under iron-sufficient conditions; in contrast, 44% of the *fbpA* transcript was detected in samples obtained from cultures grown under iron-restricted conditions (Fig. 4B). In addition, there was approximately 12% of the 1.0-kb *fbpA* transcript in RNA extracted from cells grown under iron-sufficient conditions compared to the amount in RNA extracted from cells grown under iron-restricted conditions. The half-life of the 1.0-kb *fbpA* transcript under iron-restricted conditions was calculated as 1.2 ± 0.2 min, whereas the half-life of the 1.0-kb transcript

A



B Stability of the 1.0kb *fbpA* transcript.

Time (Minutes)	Growth Conditions	
	Iron-Sufficient	Iron-Restricted
0	.469	3.362
1	.035	1.491
2	.043	.954
3	.040	.527
4	.034	.405

FIG. 4. Decay of *fbpA* mRNA. (A) Total cellular RNAs were isolated at various time intervals after rifampin treatment of *N. gonorrhoeae* F62 grown under iron-restricted conditions, fractionated, blotted, and hybridized to a probe corresponding to 200 bp of the *fbpA* structural gene. The size of the *fbpA* transcript is indicated to the left. The times (in minutes) at which samples of cells were removed from cultures incubated with rifampin are indicated below the lanes. (B) Densitometer scans of signals on the autoradiograms that had been exposed for identical times are expressed as band intensities. Results are from one experiment and are representative of three separate experiments.

under iron-sufficient conditions was calculated as 0.5 ± 0.1 min. These results indicate that the 1.0-kb *fbpA* mRNA was relatively unstable and are consistent with the stability of mRNAs for other bacterial genes that are regulated by environmental conditions (18, 22).

Concluding remarks. Although a number of iron-regulated genes have been identified in both *N. gonorrhoeae* and *N. meningitidis*, detailed studies of the transcriptional regulation of these genes have not been reported. Recently, two additional open reading frames downstream of the previously characterized gonococcal *fbp* gene locus designated *fbpB* and *fbpC* have been identified (2). The *fbpABC* locus has been proposed to encode an *N. gonorrhoeae* periplasmic-protein-dependent transport system analogous to the *Serratia marcescens sfuABC* and *Haemophilus influenzae hitABC* systems (1–3). The results presented here indicate that *fbpA* is transcribed as a monocistronic message independently of *fbpB* and *fbpC*. Although it was proposed that the *fbpABC* gene cluster may form an operon regulated by iron (2), we have failed to detect any *fbpB*- or *fbpC*-specific polycistronic or monocistronic message by using different hybridization stringencies in Northern analysis (data not shown). Sequencing data has confirmed the presence of a strong stem-loop structure which precedes the *fbpA* stop codon (2). Since stem-loop structures can function in message stability (30), the presence of this structure may function to regulate the level of *fbpA* expression; however, the specific role of this stem-loop structure remains to be determined.

In conclusion, we have demonstrated that the *fbpA* promoter is regulated by iron and that transcription and translation of the gonococcal FbpA are closely linked. We have also confirmed that *fbpA* is transcribed as a monocistronic message. The results presented here indicate that at least one mechanism for the regulation of the expression of *fbpA* is at the level of mRNA stability. Interestingly, the stability of the *fbpA* transcript was higher in cultures grown under iron-restricted conditions, suggesting that during times of iron excess when FbpA is not needed, the *fbpA* transcript is rapidly degraded. It is of interest that to date, research indicates that most of the gonococcal genes that are functionally related are distributed throughout the genome (12). These functionally related gonococcal genes include the majority of iron-regulated genes that have been described to date. Our results lend further support to the lack of an operon type of control in the gonococcus. The elucidation of the specific mechanisms of control of the iron-regulated *fbp* locus and the role of the gonococcal Fur in the regulation of this process should provide valuable information on the expression of FbpA in vivo.

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