JOURNAL OF BACTERIOLOGY, Sept. 1992, p. 5916–5922
0021-9193/92/185916-07$02.00/0

Vol. 174, No. 18

Characterization of the *Campylobacter fetus sapA* Promoter: Evidence that the *sapA* Promoter Is Deleted in Spontaneous Mutant Strains

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Received 6 April 1992/Accepted 6 July 1992

Wild-type *Campylobacter fetus* cells possess S-layer proteins (S^+ phenotype), whereas after laboratory passage, spontaneous stable mutants that do not express these proteins (S^- phenotype) arise. To determine the molecular mechanisms by which *C. fetus* changes to the S^- phenotype, we studied wild-type strain 23D, from which the *sapA* gene encoding the 97-kDa S-layer protein has been cloned, and strain 23B, a spontaneous S^- mutant. We compared these strains with another pair of strains, LP (S^+) and HP (S^-). Southern analysis with the cloned *sapA* gene as a probe indicated that both pairs of strains have multiple *sapA* homologs. Using gene disruption and replacement techniques, we constructed an isogenic strain of 23D that differed only in *sapA* expression (strain 23D:401:1). A 6.0-kb *Hin*dIII fragment from 23D:401:1 containing 3.4 kb of *sapA* upstream region then was cloned into pBluescript to produce pBG101. Nucleotide sequence analysis of *sapA* upstream region revealed a consensus promoter at −121 bp from the translational start site. Primer extension analysis placed a single in vivo transcription initiation site at the −114-bp position of *sapA*. A DNA probe derived from the *sapA* promoter region hybridized to a 5.5-kb *Hin*dIII fragment of chromosomal DNA from strain 23D but not to DNA from strain 23B. Northern RNA blot analysis showed no *sapA* mRNA in strain 23B. These data indicate that the lack of S-layer protein expression in spontaneous mutant strains is caused by the deletion of promoter sequences.

Many microorganisms possess regular surface layers (S-layers) that are noncovalently attached to membrane structures (35). The superficial location of these proteins suggests that these layers are important mediators of interactions with hosts (4, 5, 21, 24). Although the morphological properties of S-layers have been extensively studied for a wide range of microorganisms (22, 34, 35), relatively little is known about the regulatory mechanisms involved in the biosynthesis, transport, and assembly of S-layer proteins.

Campylobacter fetus is an important veterinary pathogen that causes infertility and infectious abortion in sheep and cattle (36) and extraintestinal infections (16) and a thrombotic diathesis (8) in humans. The ability of *C. fetus* to produce these diseases is associated with the presence of surface array proteins (4, 5, 11, 21, 24). The *C. fetus* S-layer is composed of high-molecular-mass protein subunits that migrate at approximately 97, 127, and 149 kDa; usually a subunit of a single size predominates for a given strain (10, 25). *C. fetus* strains may contain either type A or type B lipopolysaccharide, and their S-layer proteins are lipopolysaccharide type specific (38). The type A S-layer proteins are antigenically related and have identical N-terminal amino acid sequences (25) but differ from the type B proteins (10, 38).

S-layer protein expression in *C. fetus* is subject to antigenic variation (10, 25, 30–32). It is possible to isolate *C. fetus* variants that express from zero to up to three different S-layer proteins (10, 25), but the determinants of which protein is predominantly expressed are not known. The various S-layer proteins have different crystalline structures and include different epitopes (15). Wild-type *C. fetus* strains carrying S-layer proteins (S^+ phenotype), but not spontaneous mutants lacking them (S^- phenotype), resist complement-mediated killing by normal or immune serum and resist phagocytosis (4, 5, 21). Strain 23B is an S^- spontaneous mutant of the wild-type S^+ strain 23D (21).

The mechanisms by which pathogens alter their surface antigens are varied but often involve programmed DNA rearrangements (7). As a first step in elucidating the molecular mechanisms involved in the antigenic variation of the *C. fetus* S-layer proteins, we recently cloned and sequenced the strain 23D *sapA* gene, which encodes a polypeptide of 97 kDa (3). The aim of our present studies is to assess the molecular principles of *C. fetus* S-layer protein production. We now report that Southern hybridization with the *sapA* gene as a probe revealed the existence of multiple *sapA* homologs in both the wild-type and mutant strains. We also present an analysis of the promoter region for the *sapA* gene in strain 23D. We provide evidence that the lack of S-layer protein expression in *C. fetus* results from the absence of a *sapA* promoter.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *C. fetus* 84-32 (23D), 84-54 (23B), 82-40LP (LP), and 82-40HP (HP) used in this study were from the culture collection of the Vanderbilt University Campylobacter Laboratory and have been extensively characterized (4, 5, 21). Stock cultures were maintained at −70°C in Brucella broth (BBL Microbiology Systems, Cockeysville, Md.) supplemented with 15% glycerol. *Escherichia coli* DH5α and XL-1 Blue (Stratagene, LaJolla, Calif.) were used for transformation. Plasmid pBluescript SK^- (Stratagene) was used as a cloning vector. Plasmid pBG1 contains the *sapA* gene on a 4.0-kb insert in

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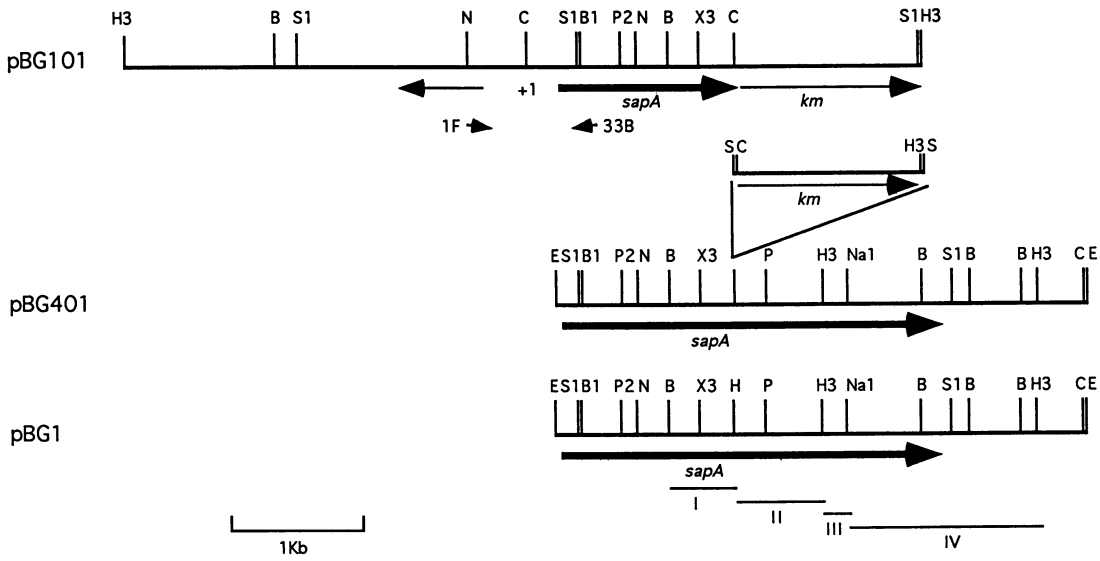

FIG. 1. Physical maps of *sapA*-containing recombinant plasmids. The physical map of pBG1 (3) (4.0-kb *Eco*RI fragment with the *C. fetus* 23D *sapA* gene) is depicted. Plasmid pBG401 represents pBG1 in which a 1.3-kb *C. coli* kanamycin-resistance gene was inserted at the *Hin*cII site. Plasmid pBG101 contains a 6.0-kb *Hin*dIII fragment from *C. fetus* 23D:401:1, which was created by gene replacement with pBG401 as the donor and which expresses the 50-kDa amino-terminal portion of its truncated S-layer protein. Arrows beneath pBG1, pBG401, and pBG101 represent the locations of the genes and directions of transcription. The transcriptional start site (+1) for *sapA* is indicated. The small leftward arrow on the pBG101 map indicates the start of a cryptic ORF. Restriction endonuclease cleavage sites: B, *Bgl*II; BI, *Bst*nI; C, *Cla*I; E, *Eco*RI; H, *Hin*cII; H3, *Hin*dIII; N, *Nde*I; NaI, *Nae*I; P, *Pst*I; P2, *Pvu*II; SI, *Ssp*I; X3, *Xma*III. 1F and 33B represent the primers used for the PCR. Numbers I through IV indicate the regions of *sapA* used as DNA probes.

pUC9 (3). *E. coli* strains were routinely cultured in LB medium (20) with shaking at 37°C, and *C. fetus* strains were cultured in Brucella broth in a microaerobic atmosphere (5% oxygen, 10% carbon dioxide, 85% nitrogen) at 37°C for 48 h. Final concentrations of antibiotics were 100 and 40 μg/ml for ampicillin and kanamycin, respectively.

Chemicals and enzymes. Isopropyl-β-D-thiogalactopyranoside was purchased from Sigma Chemical Co. (St. Louis, Mo.) and used at 57 μg/ml. 5-Bromo-4-chloro-3-indolyl-β-D-galactoside (final concentration, 40 μg/ml) was from Boehringer-Mannheim (Indianapolis, Ind.). Restriction enzymes, T4 DNA ligase, *E. coli* DNA polymerase large (Klenow) fragment, and Sequenase were from Promega and U.S. Biochemicals (Cleveland, Ohio). [α-^{32}P]dATP (650 Ci/mmol) and [γ-^{32}P]ATP (4,500 Ci/mmol) were from ICN Radiochemicals (Irvine, Calif.).

Genetic techniques and DNA sequencing analysis. Chromosomal DNA was prepared as described previously (29). Plasmids were isolated by the procedure of Birnboim and Doly (2), and purification was completed by precipitation in the presence of 800 mM NaCl and 6.5% polyethylene glycol. All other standard molecular genetic techniques were performed as described previously (20). The nucleotide sequence was determined unambiguously on both strands by using double-stranded DNA templates and the dideoxy chain termination procedure as described previously (28). Oligonucleotide primers were synthesized at the Vanderbilt University DNA Core Facility with a Milligen 7500 DNA synthesizer. Nucleotide sequences were compiled and analyzed with the aid of the DNA-Star program (DNA Star, Inc., Madison, Wis.).

Isolation of *sapA* upstream region. The gene disruption and replacement methods were basically those described by Labigne-Roussel et al. (19). Briefly, the *sapA* gene in pBG1 was disrupted by inserting a *Campylobacter coli* kanamycin resistance gene (19) into the *Hin*cII site to create pBG401 (Fig. 1). Similarly, a 0.7-kb *oriT* fragment (18) was cloned into the *Bam*HI site (in polylinker region of the vector) of pBG401, and this construct was moved into *C. fetus* 23D by conjugal transfer under the mating conditions described previously (19). *C. fetus* transconjugants were selected on Mueller-Hinton agar supplemented with trimethoprim (10 μg/ml), polymyxin (2.5 U/ml), vancomycin (5 μg/ml), and kanamycin (50 μg/ml). Construction and characterization of the mutants are the subjects of a separate investigation (6). DNA from wild-type (23D) and mutant (23D:401:1) strains was digested with *Hin*dIII. After separation of the digested DNA on agarose gel, the DNA was transferred to a nylon membrane and hybridized to a *sapA* probe consisting of the 1.65-kb *Pst*I fragment from pBG1 (3). The patterns of hybridization between the wild-type and mutant strains were similar, except that the 5.5-kb band of *sapA* in strain 23D was not present in 23D:401:1 but new bands at 6.0 and 0.8 kb were present, as expected, because of the kanamycin resistance gene insertion. The size of this fragment increased only 0.5 kb as opposed to 1.3 kb (the size of the kanamycin cassette) because of an internal *Hin*dIII site located at the end of the kanamycin cassette (0.8 kb from the downstream *Hin*dIII site). The kanamycin gene probe hybridized only with the 6.0-kb *Hin*dIII fragment in 23D:401:1, which confirms that replacement had occurred in the *sapA* gene only. This was also evident from Western immunoblot analysis, which showed that the mutant strain no longer produces a 97-kDa S-layer protein but instead produces a 50-kDa truncated protein, as expected. This *C. fetus sapA* mutant strain (23D:401:1) was used to clone the *sapA* upstream region. A 6.0-kb *Hin*dIII fragment that hybridized with both kanamycin and *sapA* probes was cloned into pBluescript to create pBG101 (Fig. 1).

RNA isolation and Northern RNA blot analysis. For RNA

isolation, *C. fetus* strains were grown overnight in 80 ml of Brucella broth, cells were harvested, and RNA was recovered by two rounds of hot phenol extraction as described previously (1). The aqueous phase was reextracted once with phenol-chloroform saturated with 0.02 M sodium acetate (pH 5.2) and twice with chloroform, and then the RNA was precipitated in 3 volumes of absolute ethanol at −70°C for 30 min. For Northern hybridization, RNA samples (10 μg) were subjected to electrophoresis on formaldehyde-agarose gels and then transferred to nylon membranes (20). The 4.0-kb *Eco*RI fragment of pBG1 was radiolabeled with [α-^{32}P]dATP as described previously (12) and used to probe Northern blots in the presence of 50% formamide (20). A formamide-formaldehyde-denatured *Hin*dIII digest of phage DNA was used as a molecular weight marker.

Primer extension analysis. For end labeling of oligonucleotides, 12.5 pmol of primer was mixed with 30 pmol of [γ-^{32}P]ATP and 20 U of polynucleotide kinase in 50 mM Tris (pH 8.8)–10 mM $MgCl_2$–5 mM dithiothreitol–0.1 mM spermidine–0.1 mM EDTA for a total volume of 30 μl. After incubation at 37°C for 60 min and EDTA addition, the DNA was precipitated as described previously (14). Primer extensions were done as described by Fouser and Friesen (14). A 2-μl sample of each reaction was subjected to electrophoresis on an 8% polyacrylamide gel simultaneously with a sequence ladder generated with the same primer.

Southern hybridizations with 23D *sapA* regions as probes. *C. fetus* chromosomal DNA was digested with *Ssp*I or *Hin*dIII, and the resulting fragments were electrophoresed on a 0.7% agarose gel in 0.04 M Tris-acetate–2 mM EDTA buffer (pH 8.2). After electrophoresis, the DNA was transferred to a nylon membrane. Hybridizations were carried out at 68°C for 16 h in buffer containing 6× SSC (1× SSC is 0.15 M NaCl–0.015 M sodium citrate), 20 mM sodium phosphate (pH 7.2), 0.05% sodium dodecyl sulfate, 5× Denhardt's solution, (1× Denhardt's solution is 0.02% Ficoll, 0.02% polyvinyl pyrrolidone, 0.02% bovine serum albumin), and 100 μg of salmon sperm DNA per ml. Probes were gel-purified DNA fragments derived from pBG1 (5) or pBG101 and were radiolabeled by primer extension with random hexameric oligonucleotides (12). Filters were washed three times for 15 min each in 1× SSC at 65°C and then in 0.5× SSC for 1 h and exposed to XAR-2 X-ray film.

PCR amplification. Polymerase chain reaction (PCR) amplification of the *sapA* upstream region was performed in 50-μl reaction volumes containing the oligonucleotide primers 1F (5′-TCCGTACAGTTGCACGCTAT-3′) and 33B (5′-TTAATCATAGCACCTATTAA-3′) at 0.5 μM each; dATP, dCTP, dTTP, and dGTP at 200 μM each; 1× reaction buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.3], 1.5 mM $MgCl_2$); 0.01% (wt/vol) gelatin; 1.25 U of *Taq* polymerase (Perkin-Elmer Cetus, Norwalk, Conn.); and 100 ng of genomic DNA. The tubes were overlaid with mineral oil. Thirty cycles of amplification were performed in a DNA thermal cycler (Perkin-Elmer Cetus). Each cycle consisted of a 2.4-min denaturation step at 95°C, a 2.0-min annealing step at 49°C, and a 4-min extension step at 72°C. The PCR-amplified products were analyzed by electrophoresis on 0.8% agarose gels.

RESULTS

Distribution of *sapA* in *C. fetus*. Our initial goal was to determine whether the *sapA* gene from strain 23D (3) is present in its spontaneous mutant strain, 23B, which has no S-layer protein expression. Genomic DNA from strains 23D and 23B was digested with *Ssp*I or *Hin*dIII, transferred to a nylon membrane, and hybridized with the 1.65-kb *Pst*I fragment of pBG1 (which represents the 5′ region of the *sapA* open reading frame [ORF]). The *sapA* probe hybridized to multiple bands present in both the *Hin*dIII and *Ssp*I digestions of chromosomal DNA from each of the strains (Fig. 2). However, the probe hybridized to three bands present in the *Hin*dIII digest from strain 23D that were not present in strain 23B (Fig. 2A). Similarly, with this digestion, there was hybridization to at least two bands in strain LP (S^+) that were not present for HP (S^-), and strain HP contained a new hybridizing band at approximately 4.5 kb. Results of the hybridizations to *Ssp*I-digested chromosomal DNA also showed that the S^- strains lacked hybridizing bands (Fig. 2B). These observations indicate that multiple *sapA* homologs are present in each strain and imply that strains 23B and HP carry unexpressed genes that are homologous to *sapA*.

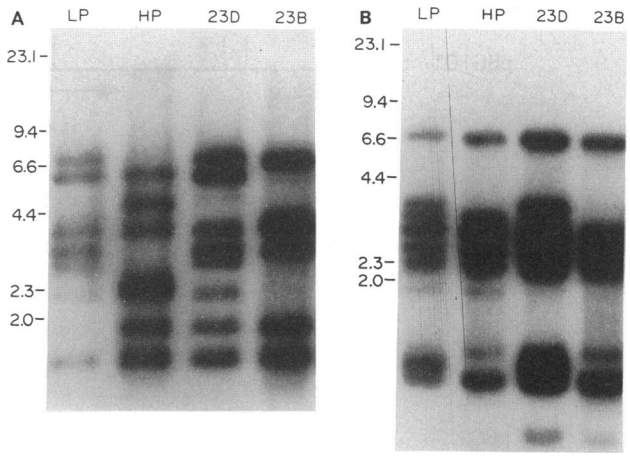

FIG. 2. Southern hybridization of the 1.65-kb *Pst*I *sapA* fragment to genomic DNA from *C. fetus* strains. Chromosomal DNA from S^+ *C. fetus* strains LP and 23D and their spontaneous S^- mutants HP and 23B were digested with *Hin*dIII (A) or *Ssp*I (B), and the restriction fragments were separated on a 0.7% agarose gel. The DNA was then transferred to a nylon membrane and hybridized with the 1.65-kb *Pst*I fragment from pBG1 as previously described (3). The positions of *Hin*dIII-digested λ phage DNA size markers (in kilobases) are indicated to the left of each panel.

To gain further insight into the conservation of the full-length *sapA* gene and to define the regions of divergence among the *sapA* homologs, we used different regions of *sapA* as probes (designated I through IV) and hybridized these to *Hin*dIII-digested *C. fetus* chromosomal DNA. When probes from the middle of the *sapA* ORF were used (Fig. 1, probes I and II), a 5.5-kb major band was hybridized in strain 23D, whereas a 4.0-kb fragment was hybridized in strain 23B, which indicated that *sapA* or adjacent sequences in strain 23B had undergone deletion or rearrangement (Fig. 3). The smaller (~3.2-kb) hybridizing band present in strain 23D with probe II is less intense than that observed with probe I and suggests that the probe II region is less homologous than the probe I region in one of the *sapA* homologs. However, probes derived from the carboxy-terminal region of the *sapA* ORF (Fig. 1, probes III and IV) hybridized to similarly sized fragments (Fig. 3) in both strains 23D and 23B, suggesting that the carboxy terminus and the adjacent downstream sequences were conserved. Taken together, these data suggested that differences in S-layer protein expression by

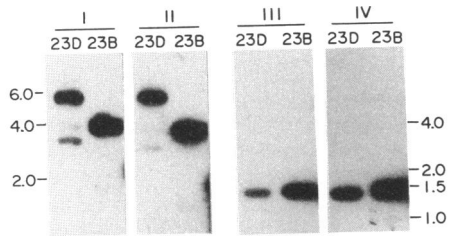

FIG. 3. Southern hybridization of four *sapA* probes to genomic DNA from *C. fetus* 23D and 23B. Chromosomal DNA from these strains was digested with *Hin*dIII; the restriction fragments were separated on a 0.7% agarose gel, transferred to a nylon membrane, and hybridized with the ^{32}P-labeled *sapA* probes (I through IV in Fig. 1).

strains 23D and 23B may be related to the 5′ end of the *sapA* gene or adjacent upstream sequences.

Cloning and sequencing of the *sapA* upstream region. Although the *sapA* structural gene for 23D encoding the full-length protein was cloned in pBG1, the insert begins only 23 bp upstream of the ORF (3). Since from Southern hybridization data we now know that all *C. fetus* strains have multiple *sapA* homologs (Fig. 2), we used a different approach to clone the *sapA* upstream region. First, we identified the *sapA* locus by using gene disruption and replacement techniques (6, 19). After characterizing the *sapA* mutation in *C. fetus* 23D (6), we cloned the mutant *sapA* with an intact upstream region into pBluescript to create pBG101. The cloned gene expressed a truncated SAP (~50 kDa) that was similar in size to the SAP produced by the mutant strain (23D:401:1) and that cross-reacted with the polyclonal antibody directed against the 97-kDa SAP (data not shown). Digestion of pBG101 with several restriction endonucleases indicated that the fragments occupied the positions they would have occupied in pBG1 (Fig. 1). Using oligonucleotide primers and pBG101 as the template DNA, we generated 800 bp of sequence upstream of the *sapA* ORF (Fig. 4).

Sequence analysis. The sequence 121 bp upstream of the *sapA* translational start site exhibits the promoter sequence TATAATT (Fig. 4), which matches the Pribnow consensus sequence TATNATN (17). This putative −10 region is associated with a −35 region, TTGAAT, which shares 4 of 6 bases with the corresponding consensus sequence, TTG ACA (17). The 23D *sapA* upstream region includes an open reading frame beginning 468 bp upstream from the *sapA* translational start site, which runs in the opposite direction to *sapA*; the sequence for the complete ORF has not been determined.

To identify whether the *sapA* promoter region is present on the 23B chromosome, we performed Southern blotting of *Hin*dIII-digested chromosomal DNA with a 0.5-kb *Cla*I-*Nde*I probe (−599 to −94 bases from the *sapA* translational start site). These studies revealed that this promoter region is present on the same restriction fragments (5.5 kb) from both wild-type S$^+$ *C. fetus* strains but that both S$^-$ strains show no hybridization signals (Fig. 5A). To confirm the Southern blot analyses, which indicated that the promoter region was deleted in mutant strains, we performed PCR analysis with primers 1F, located at bases −632 to −613 of the ORF, and 33B, located at bases 365 to 346 (conserved region of *sapA*) of the ORF (Fig. 1). For strain 23D, we generated a fragment of approximately 1 kb, as expected from the choice of primers (Fig. 5B). An identical PCR product was generated with DNA from the other S$^+$ wild-type strain, but no product was observed for the two S$^-$ mutants. These studies indicated that the promoter region of *sapA* in S$^-$ mutants had undergone deletion.

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ATAGTTATTACGCTTTCTTATAAAAGAAGTATTAACTATCTGCATAAATT    50

TAGATAATTTCTCGCATTCTAAAAATATAAAAACTTCGTTATTATTTAGT   100

 1F ——————————►                          NdeI
ATATATCCGTACAGTTGCACGCTATATTTATGTGCTACCATATGCAATAC   150

ATCTTCATATAACGACTTTTCATCAAAATTTTGGAAAAAATCACCTTTTT   200

GTCTCACAGCAACTTTTCCAGAATCTATCAGTCTTAGTTTTCTGCCCAAA   250

ACCCTTACTCCGAGATTAATTTACTTCTTATGTTTAATGATTTTATCATC   300

 BclI          ◄——— O.R.F
TATGATCACTTTTATATAATTTCTATATATCTATATAATTATAAAAAGCA   350

ATCAAAGTTATACTTCGTAAGCAAAAAGTAACAATTTACTAAAATCATAT   400

TACAATTTACCACAAAAAACTATTTTTCTTGCCAAAAAATGCATTTTACT   450

GCATATATACTTAAGTATAACTGAAAAAGTGCCAGACTAAATGCAAAAAT   500

TTAATTTTTTTTCAAAAAATAACATATAGAAATTGATTTTCTAATAATTT   550

                                        -35
TAAAAAGCGAAATTTGCATAATTTAAATGAATTAAATTTGAATTTTATAA   600

          -10       +1
AAAATTATGTTATAATTCGCGCAGTATTTGCAAAAATACTATCGATAGTA   650

AGGTAAGCAATCCGTATATAGATAACTATATATATGGTTGGTTGCTTTTG   700

                          S.D    sapA ——►
CTGGTGATTTTATTTTATTTTATTAAGGAGTCCTTAAATGTTAAACAAAA   750
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FIG. 4. Promoter region of *sapA*. The DNA sequence of the upstream region of *sapA* is shown. Putative promoter elements (−35 and −10 sequences) and the transcription start site (+1) for *sapA* are indicated. A potential ribosome-binding site (Shine-Delgarno sequence) is indicated (S.D). The location of primer 1F utilized for the PCR and the start codon for the cryptic ORF that is encoded by the complementary strand are shown.

Analysis of the promoter region. For further characterization of potential promoters of *sapA* in strain 23D, primer

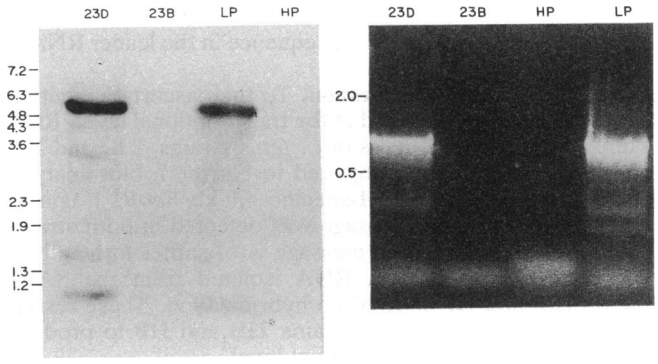

FIG. 5. (A) Southern blot of chromosomal DNA with the 23D *sapA* promoter region as a probe. DNA from *C. fetus* LP, HP, 23D, and 23B was digested with *Hin*dIII, electrophoresed in 0.7% agarose, transferred to a nylon membrane, and hybridized with the promoter region of *sapA* (0.5-kb *Cla*I-*Nde*I fragment). (B) Agarose gel electrophoresis of the amplified PCR products from *C. fetus* strains. Amplification of 23D and LP DNAs with primers 1F and 33B (Fig. 1) resulted in 1-kb product.

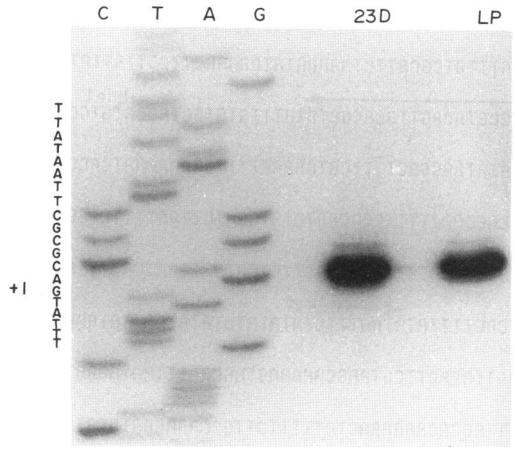

FIG. 6. Primer extension of the *sapA* transcript. A ^{32}P-end-labeled oligonucleotide primer that binds to the coding strand of the *sapA* ORF (beginning 25 bp downstream of the start codon) was annealed to total cellular RNA from S$^+$ wild-type strains 23D and LP and extended with reverse transcriptase and deoxynucleoside triphosphates. The corresponding sequencing ladder is shown, and the nucleotide position of the +1 site (G) is marked.

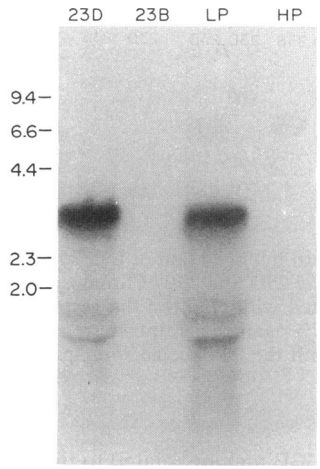

FIG. 7. Northern blot of the *sapA*-specific transcript. RNA (10 μg) from *C. fetus* 23D, 23B, LP, and HP was separated on an agarose-formaldehyde gel, transferred to a nylon membrane, and hybridized to the radiolabeled *sapA*-specific 4.0-kb *Eco*RI fragment of pBG1. The positions of *Hin*dIII-digested λ DNA size markers (in kilobases) are indicated to the left.

extension analysis was used to localize an in vivo transcriptional initiation site for *sapA*. Strain 23D was grown overnight, and total cellular RNA was extracted and then hybridized to a 5′-end-labeled oligonucleotide primer, 2754 (5′-TTGAAACATCTGTTTTGTTTAACA-3′), which binds to the coding strand of the *sapA* gene beginning 25 bp downstream of the ATG codon. For 23D *sapA*, extension from the oligonucleotide primer resulted in a product (Fig. 6) corresponding to an initiation site at the G residue 114 nucleotides upstream from the start of the *sapA* coding region. The *sapA* initiation site is spaced 7 nucleotides downstream from the hexamer TATAAT, which exhibits the most highly conserved nucleotides for *E. coli* −10 promoter elements (17). For the wild-type S$^+$ strain LP, the +1 site was identical to that for 23D (Fig. 6). For S$^-$ strains 23B and HP, no primer extension product was evident (data not shown). The untranslated leader RNA contains 72% AT bases, which is not significantly different from the percentage observed in the *C. fetus* genome (23). Another unusual feature of the leader RNA is a thrice-repeated pentameric unit (ATTTT) (Fig. 4). The significance of this repeat sequence in the leader RNA is not yet known.

Regulation of *sapA* expression. To then ascertain whether *sapA* expression is blocked at the transcriptional level, RNA samples from the two pairs of *C. fetus* strains, 23D and 23B and LP and HP, were subjected to Northern blot analysis and probed with the *sapA*-specific 4.0-kb *Eco*RI fragment from pBG1. A 2.8-kb message was detected in both strains 23D and LP (Fig. 7); this message is of sufficient length to encode the 97-kDa SAP. RNA isolated from the SAP$^-$ strains 23B and HP showed no hybridization. These results indicate that the failure of strains 23B and HP to produce SAP occurs at the transcriptional level.

DISCUSSION

From these studies, we have broadened our understanding of the genetic organization of loci involved in S-layer protein expression in *C. fetus*. First, and most important, from the Southern hybridization studies, it is clear that both wild-type S$^+$ and spontaneous mutant S$^-$ strains contain multiple copies of *sapA* homologs. This observation may help explain why *C. fetus* cells can simultaneously produce more than one S$^+$ phenotype (15) and how the S$^+$ phenotype can shift from one predominant form to another (10, 15, 25). It is notable that two unrelated wild-type S$^+$ strains (23D and LP), isolated from cattle and humans, respectively, decades apart from each other (4), show nearly identical hybridization patterns (Fig. 1 and 5A) and identical primer extension results (Fig. 6), indicating that the genomic location of the *sapA* homologs in *C. fetus* must be well conserved. Differences between the spontaneous S$^-$ mutants (Fig. 1) suggest that more than one genomic localization for the antigenic variability may be operative.

Second, organization of the upstream region of *sapA* was determined and putative promoter sequences resembling the *E. coli* sigma-70 promoter (17) were identified. That these sequences represent the actual promoter utilized is supported by the close approximation of the transcriptional initiation site, determined by primer extension analysis, with the promoter sequence. Identical +1 sites and transcript lengths for wild-type strains 23D and LP indicate that transcriptional mechanisms for *sapA* also may be well conserved.

Third, the S$^-$ phenotype for both spontaneous mutant strains 23B and HP is associated with failure to produce a transcript for *sapA* or a homologous gene. This indicates that *sapA* expression is blocked at the transcriptional level and explains why there has been absolutely no evidence for S-layer protein production in the S$^-$ strains (4, 13, 15, 21). For strains 23B and HP, the absence of transcripts is accompanied by a lack of primer extension products. In addition, our PCR analysis suggests that organization of the *sapA* upstream region in strains 23B and HP is different from that of the wild-type strains. This observation may indicate deletion or rearrangement of the promoter region. The former possibility is suggested by the results of the Southern hybridization with the promoter region as a probe (Fig. 5A). Again, the two wild-type strains show identical patterns, whereas the same hybridization signal is absent in the two

mutants. That hybridizations with probes I and II but not those with probes III and IV show differences between 23D and 23B chromosomal DNA (Fig. 3) is also consistent with the hypothesis that the promoter region is centrally involved in the variation between the two strains.

Antigenic variation is found in many bacteria and is a mechanism for creation of a heterogeneous population that can survive environmental fluctuations. Regulation of antigenic variation may involve gross DNA rearrangements (translocation or inversion) (7, 26) or small mutations in reiterated sequences (9, 33). Both types of regulation have been observed in the expression of surface structures in *Neisseria gonorrhoeae* (37), and the latter type has been observed for *Yersinia pseudotuberculosis* (27). However, we do not yet understand whether rearrangement occurs in vivo or whether the S^- phenotype is advantageous for *C. fetus* in vitro. Efforts are currently under way in this laboratory to clone and sequence the *sapA* homolog from strain 23B. Application of detailed molecular knowledge of S-layer protein structure and variability will help us to understand the role of S-layers in *C. fetus* pathogenesis.

ACKNOWLEDGMENTS

We thank Charlene Oakley for preparation of the manuscript and Agnes Labigne for review of this work.

This work was supported in part by Public Health Service grant R01 AI24145 from the National Institutes of Health.

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