Regulation of *Streptococcus gordonii* sspB by the sspA Gene Product

AZZA EL-SABAENY,1,2 DONALD R. DEMUTH,3 AND RICHARD J. LAMONT1*

Department of Oral Biology, University of Washington, Seattle, Washington 98195; Department of Biochemistry, University of Pennsylvania, Philadelphia, Pennsylvania 19104; and Department of Botany and Microbiology, University of Alexandria, Alexandria, Egypt

Received 12 February 2001/Returned for modification 17 April 2001/Accepted 10 July 2001

*Streptococcus gordonii* expresses two related adhesins, SspA and SspB, the genes for which are adjacent on the chromosome and are regulated independently. Although the adhesins are functionally similar, the sspA promoter is more active than that of sspB. In this study we show an additional role for SspA in the control of sspB activity. Gel shift and DNA footprinting assays demonstrate that the SspA protein binds to the sspB promoter and protects a region 233 to 264 bp upstream of the predicted −35 promoter element. The responsiveness of the sspB promoter to SspA was investigated with a promoter-cat reporter. Expression of the sspB promoter was reduced by over 60% in an SspA-deficient mutant of *S. gordonii*. These results indicate that expression of *S. gordonii* sspB is positively regulated by the sspA gene product.

*S. gordonii* is a prominent colonizer of dental plaque, a microbial biofilm that is strongly associated with the development of caries and periodontal diseases. *S. gordonii* exhibits a wide range of adherence properties that are well characterized at the molecular level (8). SspA cell surface proteins are multifunctional adhesins that participate in binding reactions with salivary agglutinins and with other plaque bacteria such as *Porphyromonas gingivalis*, an aggressive periodontal pathogen (1, 2, 7). Such interbacterial adhesive interactions are important in the development of plaque and in its transition from a benign accumulation to a potentially pathogenic entity.

In strains of *S. gordonii* thus far examined, tandem genes encode SspA and SspB polypeptides that are highly similar with respect to structure and adhesive function (2, 6). However, the *sspA* and *sspB* genes possess individual promoter regions and are differentially regulated in response to environmental conditions (4). Further, as measured by promoter-cat reporter constructs, transcriptional activity of the *sspA* promoter is about threefold higher than that of the *sspB* promoter over a range of growth conditions (4). SspA and SspB may thus have distinct roles to play for the organism. In this study we investigated regulation of the *sspB* gene by the *sspA* gene product.

**SspA binds to the *sspB* promoter region.** To determine whether the SspA polypeptide can bind to the *sspB* upstream region, a gel mobility shift assay was performed using the BandShift kit (Amersham Pharmacia Biotech). Recombinant SspA protein was expressed from the *sspA* gene and regulatory sequences (2) in *Escherichia coli* DH5α cultured in Luria-Bertani broth. Crude periplasmic preparations were generated by osmotic shock (5). The SspA polypeptide was further purified by Sepharose 6B (Pharmacia) and DEAE-Sephadex (Sigma) chromatography. Purity was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and a single band of SspA protein was detected following silver staining. The region −347 to −17 bp from the *sspB* translational start site (3) was amplified by PCR and cloned into pCR1 (TOPO vector; Invitrogen). A 364-bp double-stranded EcoRI fragment containing the *sspB* promoter region was used for gel shift analysis. DNA (5 ng) was 5′ labeled with α-32P and purified on an Atlas Nucleospin column (Clontech). Binding reactions of SspA (4 μg of protein) and target DNA were carried out in binding buffer (40 mM Tris-HCl [pH 7.5], 200 mM NaCl, 2 mM dithiothreitol, 50% glycerol, 1% Nonidet P-40) and mixed with a synthetic competitor poly(dI-dC) in a total of 20 μl and then incubated at room temperature for 20 min. Samples were analyzed on a 5% polyacrylamide gel (run in low-ionic-strength gel buffer: 70 mM Tris-HCl [pH 7.5], 30 mM sodium acetate, 10 mM EDTA) for 90 min at 10 V/cm at 4°C. As shown in Fig. 1, purified SspA protein was capable of binding to and retarding the mobility of the *sspB* upstream region. SspA-DNA complex formation was not affected by *E. coli* DNA (0.1 mg/ml), indicating specificity of binding (Fig. 1, lane 4). In contrast, the presence of excess unlabeled target DNA abolished the SspA-mediated gel shift (Fig. 1, lane 3). Additional evidence of specificity was provided by the failure of an *E. coli* extract containing an irrelevant protein (EBNA-1) to shift the target DNA (Fig. 1, lane 5).

**SspA binding protects a 32-bp region of the *sspB* promoter.** To identify the SspA binding site upstream of the *sspB* gene, DNase I footprinting was performed. As preliminary results indicated that the protected region was at the 5′ end of the region used for gel shift analysis, a 5′ extended target was used for footprinting. A 279-bp upstream EcoRI-XmaI sspB DNA fragment (−418 to −139 from the translational start site) was amplified by PCR and mixed with purified SspA protein in binding buffer (described above) containing 10 mM MgCl2. Digestion was initiated by the addition of 2 U of DNase I (Amersham Pharmacia Biotech). The reactions were stopped by the addition of diluted DNase stop solution (768 mM sodium acetate, 128 mM EDTA, 0.56% sodium dodecyl sulfate, 256 μg of yeast RNA/ml) (Amersham Pharmacia Biotech), and the mixtures were extracted with an equal volume of phenol-chloroform (1:1). The nucleic acids were then precipitated,
dried, resuspended in loading buffer (deionized formamide containing 10 mM EDTA, 0.3% bromophenol blue, and 0.3% xylene cyanol), and electrophoresed in 8% polyacrylamide gels containing 7 M urea. The Maxam and Gilbert A+G sequencing ladder (9) was used as a size standard for the DNase I digestion experiments. The results revealed that a 32-bp region of the sspB upstream sequence is fully protected from DNase I digestion in the presence of SspA (Fig. 2). This protected sequence spans −342 to −311 bp from the translational start site of the sspB promoter and is 233 to 264 bp upstream of the predicted −35 promoter element.

**Disruption of the sspA gene affects sspB expression.** To determine whether the binding of SspA to the sspB upstream region influences transcription of sspB in *S. gordonii*, an sspB promoter-cat86 reporter fusion was introduced into the genome of an SspA-deficient strain of *S. gordonii*. *S. gordonii* strains were cultured aerobically in Trypticase peptone broth supplemented with yeast extract (5 mg/ml) and 0.5% glucose at 37°C. *S. gordonii* OB220 (kindly provided by Howard Jenkins, University of Bristol) is derived from strain DL1 and contains an insertional inactivation of the sspA gene (2). Recombinant strain HA00 was described previously (4) and is a derivative of DL1 containing a chromosomal sspB promoter-cat reporter fusion. Strain HA02 was constructed by a strategy similar to that for HA00 and is a derivative of OB220 (SspA deficient) containing a chromosomal sspB promoter-cat reporter fusion. Briefly, suicide plasmid pHA145 (4), which encodes tetracycline resistance and contains a 1.1-kb BamHI fragment with the *S. gordonii* sspB promoter-cat fusion, was isolated by Wizard Miniprep (Promega) and integrated into the chromosome of OB220. Transformants resulting from a single (Campbell) crossover were selected on brain heart infusion agar containing tetracycline (10 μg/ml).

To confirm the integration events, nylon blots of HindIII- or EcoRI-digested chromosomal DNA were probed with biotin-labeled sspB promoter or the cat gene (not shown). In addition, the inserted fragment was amplified by PCR and sequenced directly using a cat defined primer (5'-CAGGAGTCGAAAT ACCAGAGAAT-3'). Hence, the recombinant strains HA00 (sspB_cat) and HA02 (SspA-deficient sspB_cat) contain two copies of the sspB promoter, one driving expression of CAT (chloramphenicol acetyltransferase) and the other driving expression of the structural SspB protein. Strains HA00 and HA02 were grown in batch culture, and CAT activity was measured over time. Cells were harvested by centrifugation (6,000 × g, 10 min, 4°C) and washed once in TPE buffer (100 mM Tris-HCl [pH 7.8], 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride). Cells were then suspended in spheroplasting buffer (20 mM Tris-HCl [pH 6.8], 10 mM MgCl2, 1 mM phenylmethylsulfonyl fluoride, 2% raffinose, 500 U of mutanolysin/ml) and incubated at 37°C for 30 min. To disrupt the cells, 0.1-ml glass beads (0.10 to 0.11 mm in diameter; Braun Mel- sugen AG) and 0.4 ml of ice-cold TPE buffer were added and the cells were vortex mixed twice for 30 s. Suspensions were then centrifuged (12,000 × g, 20 min, 4°C) to pellet beads and cell debris, and supernatants were removed for enzyme assays.

Protein concentrations were determined by a Bio-Rad protein assay kit with bovine gamma globulin as the standard. CAT enzyme activity was assayed by the spectrophotometric method of Shaw (10), utilizing a Beckman DU-70 recording spectrophotometer with a temperature-controlled cuvette chamber. Briefly, 0.05 ml of streptococcal cell extract (containing 40 to 60 μg of protein) was added to a 0.5-ml reaction mixture (100 mM Tris-HCl [pH 7.8], 0.1 mM acetyl coenzyme A [Sigma], 1 mM 5,5'-dithiobis-2-nitrobenzoic acid) in 1-ml glass cuvettes. The reaction mixture was warmed at 37°C for 2 min, and the background change in absorbance at 412 nm was recorded for a further 2 min. Chloramphenicol (16 μl, 0.1 mM) was added, and the change in absorbance was recorded for 2 min. The reaction rate was determined from the linear portion of the graph, corrected for the background change in A412, and divided by 0.0136 to yield CAT activity expressed as nanomoles of chloramphenicol acetylated per minute at 37°C.

As shown in Fig. 3, sspB promoter activity, as determined by CAT specific activity, was reduced by 60 to 95% over the growth curve in strain HA02 (SspA deficient) compared to
HA00 (SspA<sup>+</sup>). In both strains, sspB promoter activity was lowest in the lag phase and increased 10-fold in late-log-phase cells, as observed previously (4), indicating that growth phase-dependent regulation of sspB is independent of SspA and was not disrupted by the chromosomal insertions. Growth curves of HA00 and of HA02 were similar, although HA02 grew slower and to a slightly lower final density. However, previous results (4) demonstrated that sspB promoter-driven cat expression is not affected by doubling time. Thus, the reduction in sspB expression in HA02 is unlikely to be a reflection of the reduced growth rate of the organism. The results are therefore consistent with positive regulation of sspB by the sspA gene product. It is possible that the presence of ermAM, which was used to disrupt sspA (2), affects transcription of sspB. However, sspA and sspB are independently transcribed, and a stem-loop structure that resembles a transcription terminator exists just downstream of sspA (3, 4, 7), thus arguing against this situation. Furthermore, expression of SspB occurs in the sspA mutant strain (2) and other control pathways are not disrupted in HA02, as evidenced by the response of the sspB promoter to the growth phase of the organism.

The advantages to Streptococcus gordonii conferred by the production of two structurally and functionally related adhesins (SspA and SspB) can be hypothesized to include more avid attachment, diversity of substrate recognition, and immune avoidance. This arrangement, however, may necessitate tightly controlled expression of the genes. Our previous studies have shown that the sspA and sspB genes each possess functional promoters with differing activities that respond independently to environmental cues (4). The results of this study indicate that in addition to its role as a surface adhesin, intracellular SspA can positively regulate transcription of the sspB gene through binding to a region 233 to 264 bp upstream of the sspB -35 site. Thus, although the transcriptional activities of sspA and sspB differ according to prevailing conditions in the oral cavity, expression of SspA and SspB is linked through the activity of the SspA protein.

We thank Howard Jenkinson for provision of strains and helpful discussions.

This work was supported by NIDCR grants DE12505, DE13061, and DE07023.

REFERENCES