Regulation of Sucrose-6-Phosphate Hydrolase Activity in Streptococcus mutans: Characterization of the scrR Gene

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Previous results have implicated an important role for the enzyme II*scr, the sucrose-specific permease, in the transport of sucrose by cariogenic Streptococcus mutans. The product of the scrB gene, sucrose-6-phosphate hydrolase (Suc-6PH), is required for the metabolism of phosphorylated sucrose. The results from the utilization of scrB::lacZ fusions in \textit{S. mutans} GS-5 have suggested that sucrose-grown cells have higher levels of \textit{scrB} gene expression than do cells grown with glucose or fructose. Northern blot analysis of \textit{scrB} transcripts has also confirmed the relative strengths of expression as sucrose>glucose>fructose. Immediately downstream from the \textit{scrB} gene, an open reading frame with homology to regulatory proteins of the GalR-LacI family as well as to ScrR proteins from several other bacteria has been identified. In addition, this gene appears to be transcribed in the same operon as \textit{scrB}. Inactivation of this gene, \textit{scrR}, did not alter the relative expression of the \textit{scrB} gene in the presence of sucrose or fructose but did increase Suc-6PH levels in the presence of glucose to that observed with sucrose. Furthermore, the \textit{S. mutans} ScrR homolog appears to bind to the \textit{scrB} promoter region as determined from the results of gel shift assays. These results suggest that the \textit{scrR} gene is involved in the regulation of \textit{scrB}, and likely \textit{scrA}, expression. However, it is not clear whether sucrose acts as an inducer of expression of these genes or, alternatively, whether glucose and fructose act as repressors.

The role of dietary sucrose and mutants streptococci, particularly \textit{Streptococcus mutans}, in the development of human dental caries has been well documented (14). Sucrose is required by these organisms for the synthesis of insoluble glucans, which play an important role in the colonization of tooth surfaces leading to dental plaque formation. However, under certain conditions, a portion of the sucrose metabolized by the mutants streptococci appears to be converted to fermentation end products such as lactic acid (32). This process likely involves the initial transport of sucrose into the cells by the sucrose phosphorylase-dependent phosphotransferase system (PTS) (28). In addition, alternate pathways for sucrose transport into \textit{S. mutans} have also been identified (19, 20) and may be prominent under certain environmental conditions as proposed earlier (14).

The sucrose PTS converts sucrose to sucrose-6-phosphate, which is then hydrolyzed to fructose and glucose-6-phosphate, the reaction being catalyzed by the product of the \textit{scrB} gene. Sucrose-6-phosphate hydrolase (Suc-6PH) (10, 15). These sugars can then be metabolized to lactic acid through the classical fermentation pathways of the homofermentative streptococci (32). However, it is likely that an alternate sucrose transport system is involved in lactic acid production under rapid growth conditions (7a). Recent results (24) have indicated that the expression of the \textit{scrA} gene encoding the enzyme II* (EnzIIScr) of the sucrose PTS is induced in the presence of sucrose rather than glucose or fructose. However, a similar analysis of the regulation of \textit{scrB} expression utilizing reporter gene constructs has not yet been carried out. Earlier results have suggested that this gene may be inducible by sucrose in \textit{S. mutans} (9, 31). However, this conclusion was based upon non-specific Suc-6PH (previously termed invertase) assays which could be confounded by the glucosyltransferase (Gtf), fructosyltransferase, and fructanase activities known to be expressed by these organisms (13). However, subsequent utilization of a more specific Suc-6PH assay did confirm that the Suc-6PH activity in the presence of sucrose was elevated relative to that in the presence of fructose and glucose by an unknown mechanism in the \textit{mutans} streptococci (30). Furthermore, in view of the demonstration that the \textit{scrA} and \textit{scrB} genes are tandemly arranged on the \textit{S. mutans} GS-5 chromosome but are transcribed from opposite DNA strands (23), it was of interest to examine the regulation of expression of the \textit{scrB} gene.

The present results with specific \textit{scrB)::lacZ} fusions demonstrate that the regulation of \textit{scrB} expression is similar to that previously demonstrated for the \textit{scrA} gene (24). In addition, a novel regulatory gene, \textit{scrR}, has been identified immediately downstream from \textit{scrB} within the same operon.

MATERIALS AND METHODS

\textbf{Bacterial strains.} \textit{S. mutans} GS-5, its spontaneous colonization-defective mutant SP2 (18), and the Gtf mutant strain SP2ΔgtfBCD (27a) were maintained and grown in Todd-Hewitt broth (THB; Gibco BRL, Grand Island, N.Y.) or TYNa (1% Bacto tryptone, 0.5% Bacto yeast extract, and 0.4% Na$_2$HPO$_4$) broth. This latter growth medium is sugar deficient and allows only minimal growth (<10% of that of sugar-supplemented cultures) in the absence of exogenous sugars (24). \textit{S. mutans} V1355 with an inactivated \textit{scrB} gene (15) was obtained from F. Macrina (Virginia Commonwealth University, Richmond). Transformants of \textit{S. mutans} were selected following growth on THB agar plates supplemented with erythromycin (10 \textmu g/ml) or tetracycline (8 \textmu g/ml).

\textbf{DNA manipulations.} DNA isolation, endonuclease restriction, ligation, and transformation of competent \textit{Escherichia coli} cells were carried out as previously described (1, 21) while transformation of \textit{S. mutans} was accomplished by procedures routinely carried out in this laboratory (18). Nucleotide sequencing and sequence analysis were carried out as indicated earlier (23).

\textbf{Construction of }\textit{scrB::lacZ} fusion plasmids. Plasmid pPV5 containing the \textit{scrB} gene has been described earlier (22). A 10-bp SalI linker was inserted into the \textit{NruI} site which is present at position 728 of the published \textit{scrB} nucleotide sequence (22) for constructing plasmid pSD10. The promoterless \textit{lacZ} SalI cartridge was excised from the pMC1871 fusion vector (Pharmacia Biotech, Piscataway, N.J.) and inserted in frame into the \textit{SalI} site of pSB10 (see Fig. 1). A \textit{scrB::lacZ} fusion fragment digested with \textit{PstI} and \textit{KpnI} from pSB10 was inserted into the pMC1871 fusion vector (Pharmacia Biotech, Piscataway, N.J.) and inserted in frame into the \textit{SalI} site of pSB10 (see Fig. 1).
treated with T4 DNA polymerase to obtain blunt ends and ligated to PfuUl-digested pResEmPvu (27) with T4 ligase. The resultant plasmid, pSDL20, was used to transform S. mutans SP2, and one selected transformant, designated SP2C4, was used for further study.

Construction of double mutant strains. Plasmid PSY5 (21a), containing the 5' and 3' ends of the scrR (previously designated dsl1 [21]) gene as well as the tetracycline (derived from Tn916) and kanamycin resistance genes (27), served as the source of the former gene. The plasmid was linearized by digestion with EcoRI or Spel and used to transform S. mutans SP2C4 such that the resultant scrR mutant (Tet-) was designated SP2CL1. In order to remove the 400-bp fragment of scrR which could express the N-terminal domain of the ScrR protein from the latter construct, an additional scrR mutant was constructed. Chromosomal DNA from strain SP2C4 was used to transform strain SP2C4 such that the resultant scrR mutant (Tet-) was designated SP2CL2.

Preparation of the labeled scrB PCR probe. For Southern and Northern blot analyses, a 1,112-bp probe containing the scrB region was prepared and labeled by PCR amplification with pVP5 as the template with primers ScrBorf-F (5'-TTCGCCCCTATCAAGATTTGAC-3'; nucleotides 258 to 276 in the scrB sequence [22]) and ScrBorf-R (5'-GGCGATCGATCAGAATTGCTCC-3'; nucleotides 1348 to 1369 in the scrB sequence) and digoxigenin-dUTP with the PCR DIG labeling kit (Roche Molecular Biochemicals, Indianapolis, Ind.). PCR conditions were as follows: denaturation for 5 min at 95°C and 30 cycles of 30 s each at 94°C, 1 min at 55°C, and 1.5 min at 72°C, followed by 7 min at 72°C in a thermal cycler (Perkin-Elmer Cetus Corp., Norwalk, Conn.). Amplified products were purified by the Wizard PCR-Prep System (Promega Corp., Madison, Wis.).

Southern blot analysis. One microgram of genomic DNA was digested with EcoRI or EcoRV, and the DNA fragments were separated by electrophoresis on 1% Tris-acetate-EDTA agarose gels, transferred to nylon membranes (Hybond-N or Hybond-ECL), and fixed by UV cross-linking. Hybridization and detection were performed in accordance with the manufacturer's protocol (Boehringer Mannheim).

Expression of RNA. Total RNA was isolated from 15 ml of log-phase cell cultures. After centrifugation, the cells were suspended with 0.3 ml of diethylpyrocarbonate-treated water. The samples were transferred to FastRNA tubes with blue caps (Bio 101, Vista, Calif.), and 0.9 ml of TRIzol reagent (Gibco BRL) was then added. Cells were broken by a FastPREP FP120 homogenizer (Bio 101) at a speed setting of 6.0 for 30 s. At 10 min after samples stood on ice for 2 min, 0.2 ml of chloroform was added and the tubes were vortexed for 1 min. The mixtures were then placed at room temperature for 2 min and centrifuged at 12,000 × g for 1 min at 4°C. 0.5 ml of supernatant was added to the supernatant, and the mixtures were vortexed and centrifuged again as described above. RNA was finally precipitated from the aqueous phase with isopropanol, and the resulting pellets were dried and resuspended in 20 ul of diethylpyrocarbonate-treated water.

Northern blot analysis. A quantity (4.5 μg) of RNA (15 μg) was mixed with 15.5 μl of sample buffer (2.0 μl of 10× MOPS [morpholinopropanesulfonic acid], 3.5 μl of 37% [vol/vol] formaldehyde, 10 μl of formamide) and denatured at 65°C for 10 min. After dye solution was added, the RNA fragments were separated by electrophoresis on a 1% Tris-acetate-EDTA gel. After the gel was washed with 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 15 min twice to remove formaldehyde, and blotting was carried out with 20× SSC overnight. The blotted membrane was washed with M 2× SSC for 30 min and fixed by UV cross-linking. Hybridization was then carried out with 5× SSC containing 0.5% sodium dodecyl sulfate–5× SSC–10% denatured human placental DNA (0.5 μg/ml)–digoxigenin-labeled PCR probe at 50°C.

Primer extension analysis. Total RNA was prepared as described above, and primer extension (11) was carried out with [γ-32P]dATP (DuPont NEN, Boston, Mass.), labeled oligonucleotide primer complementary to the 5' end of the gene (5'-TGCTTATCATGATAAGATTTGAC-3'; nucleotides 330 to 350 in the scrR sequence) and reverse transcriptase (Superscript II; Gibco BRL). Nucleotide sequencing of the region was carried out in parallel as described above with the same primer.

Gel mobility shift assays. A 215-bp DNA fragment corresponding to nucleotides 1381 to 1595 of the scrA promoter region was amplified by PCR with primers (5'-TACATCTGGTCTAGATCAGAATTGAC-3' and 5'-GACATGTTTATCTACTCTTATAA-3') and then labeled with [γ-32P]dATP and purified on 6% acrylamide gels. The mobility shift assays were carried out essentially as previously described (6) with 2 to 20 μg of DNA probe (10,000 to 20,000 cpm) and by incubation with the protein samples in 20 μl of reaction buffer (12% glycerol, 12 mM HEPES-NaOH [pH 7.9], 4 mM Tris-HCl [pH 7.9], 60 mM KC1, 1.0 mM EDTA, 1.0 mM dithiothreitol) at 30°C for 15 min. After incubation, the samples were electrophoresed on 5% polyacrylamide gels and then subjected to autoradiography. For the specificity assays, potential competitors were added on a weight basis relative to the labeled probe.

Reverse transcription-PCR (RT-PCR). RNA from S. mutans SP2 was prepared as described above, and RT-PCR was carried out with Superscript II (Gibco BRL) and Vent DNA polymerase (New England Biolabs, Beverly, Mass.) as previously described with 35 cycles (denaturation at 94°C for 1 min, annealing at 55°C for 2 min, and extension at 72°C for 2 min) (2). Four synthetic oligonucleotide primers (B-RT1, 5'-ACACAGTCTTTTCTGTTG-3'; B-RT2a, 5'-TCTGATGTTGGCTCTTATGGC-3'; B-RT1, 5'-GAAATACTCTAGGATAACAAGC-3'; and scrR1, 5'-GAAATCTTAGGATAACAGG-3') were used as three primer pairs as described in the text.

Determination of β-galactosidase activity. S. mutans scrB::lacZ constructs which were precultured overnight in TYNa broth without added sugars were grown in the same broth containing the indicated sugars (1%) into the mid-log phase. Maximal expression of the lacZ gene and scrB expression occurred at approximately 0.4% glucose or fructose levels, and therefore, 1% levels were chosen for all of the experiments described in this study. Under these conditions, little or no growth was detected in the absence of exogenous carbohydrate addition. Cultures were then centrifuged, and the cell pellets were washed and resuspended with Z buffer (60 mM Na2HPO4, 10 mM KCl, 1.0 mM MgSO4, 50 mM β-mercaptoethanol, pH 7.0) at an optical density at 600 nm of 0.93 to 0.95. β-Galactosidase activities were determined with o-nitrophenyl-β-galactoside (ONPG) as follows. The cell suspension (0.1 ml) and 0.9 ml of Z buffer were added to FastRNA tubes. After standing on ice for 30 min, the cells were disrupted by a homogenizer (FastPREP) at a speed setting of 6.0 for 30 s. The samples were then equilibrated at 28°C for 15 min, and 0.2 ml of ONPG (4 mg of ONPG/ml in Z buffer) was next added. The cells were incubated at 28°C for 30 min, and the reactions were terminated by addition of 0.5 ml of 1 M Na2CO3. After being mixed well, the samples were then centrifuged at 12,000 × g for 10 min, and the optical density at 420 nM of the supernatant was measured spectrophotometrically. All reactions were carried out in triplicate, and the data presented are the averages of the determinations. The units of activity were determined as described earlier (16).

Nucleotide sequence accession number. The nucleotide sequence of the scrR gene is available from the GenBank database under accession no. U46902.

RESULTS

Construction of scrB::lacZ fusion strains. In order to develop a system for monitoring specific scrB expression, S. mutans SP2 strains containing scrB::lacZ translational fusions were constructed. The promoterless lacZ gene derived from plasmid pMC1871 was introduced in frame into the scrB gene of plasmid pVP5 (Fig. 1). A 215-bp fragment from the resulting plasmid, pSDL10, was then introduced into the streptococcal suicide vector pResEmPvu to construct pSDL20. Transformation of S. mutans SP2 (a spontaneous GS-5 mutant which is defective in insoluble glucon synthesis, eliminating aggregation during growth in the presence of sucrose) with intact circular pSDL20 resulted in integration of the plasmid into the SP2 chromosome following a Campbell-like integration event. This resulted in the formation of two copies of the scrB gene: one containing the lacZ fusion and one intact copy of the gene. The latter allows for growth of the construct, SP2C4, in the presence of sucrose since the disaccharide is toxic to cells lacking Suc-6PH activity (15). Transformants selected on THB-erythromycin agar plates containing X-Gal (5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside) allowed for convenient isolation of the proper constructs.

Integration of pSDL20 into the SP2 chromosome was obtained following Southern blot analysis of the transformants (data not shown). Cleavage of SP2 and SP2C4 chromosomal DNA with EcoRI or EcoRV and analysis with an scrB probe yielded the predicted fragments (SP2 yielded one positive 6.6-kb fragment while the mutant SP2C4 expressed two bands, of 3.7 and 6.9 kb, following EcoRI digestion).

Regulation of scrB expression. Since previous results (4) indicated that another gene involved in sucrose transport, scrA encoding EnzIIScr, was differentially regulated by sugars, it was of interest to examine the effects of various sugars which S. mutans would be expected to encounter in the oral cavity on scrB expression. With SP2C4, it was demonstrated that maximal scrB transcription was detected when the cells were grown in the presence of sucrose (Fig. 2A).
mately twice as high in the presence of sucrose as in that of glucose and almost fivefold higher relative to growth in the presence of fructose. Growth of the cells in the presence of sucrose plus glucose or sucrose plus fructose resulted in \( \text{scrB} \) expression similar to that with growth in the presence of glucose or fructose alone, respectively (data not shown). In addition, growth of SP2C4 in the presence of either maltose or sorbitol resulted in a Suc-6PH level similar to that of cells grown in the presence of fructose (data not shown). This latter result is in contrast to previous studies indicating higher levels of \( \text{scrB} \) expression in mannitol- or sorbitol-grown cells than those in fructose-grown cells (30). Whether this difference is related to the utilization of distinct species of mutans streptococci in these two studies is unknown at present.

Since bacteria present in dental plaque are subject to fluctuations in pH influenced by the availability of nutrients (14), it was of interest to determine the effects of acidity on \( \text{scrB} \) expression. The availability of \( S. \text{mutans} \) \( \text{scrA}::\text{lacZ} \) constructs (24) also allowed for a similar analysis of this gene. The results (Fig. 3) clearly indicated that the expression of both the \( \text{scrA} \) and \( \text{scrB} \) genes was reduced under acidic conditions, pH 5.6, relative to neutral pH. Thus, two of the key enzymes involved in the major sucrose transport and metabolism system of \( S. \text{mutans} \) appear to be down regulated under acidic growth conditions.

**Identification and characterization of the \( \text{scrR} \) gene.** The apparent inducibility of the \( \text{scrB} \) gene with different sugars suggested the presence of a \( \text{scrB} \) regulatory gene on the \( S. \text{mutans} \) chromosome. Sequencing of the \( \text{scrB} \) gene (22) revealed the presence of an open reading frame (ORF) previously named \( \text{ds1} \) immediately downstream from the gene. Additional nucleotide sequencing (Fig. 4) confirmed the presence of a significant ORF in this region. This putative gene would code for a protein of approximately 35 kDa beginning at nucleotide position 61. A potential Shine-Dalgarno sequence, AGG, which was present within the 3′ end of the \( \text{scrB} \) gene, was detected 5 nucleotides upstream of the likely initiation codon. This suggested the possibility of translational coupling of the \( \text{scrB} \) gene and the ORF. In addition, no sequences similar to promoter sequences were identified in the region upstream of the \( \text{ds1} \) gene, and this suggested that both genes may be present within the same operon.

A comparison of the deduced amino acid sequence of \( \text{ds1} \) with the National Biomedical Research Foundation protein database indicated that extensive homology was observed between the \( S. \text{mutans} \) \( \text{ds1} \) protein and the N-terminal regions of the GalR-LacI family (17) of regulatory proteins (Fig. 5). However, this homology did not extend beyond the N-terminal region. Since the N-terminal region of the GalR-LacI family of regulatory proteins has been implicated in DNA binding, these results suggested that the \( S. \text{mutans} \) ORF might also be involved in such interactions. Therefore, this ORF was tentatively named \( \text{scrR} \) since it appeared to be a regulatory gene present in the same operon as \( \text{scrB} \). Moreover, the \( S. \text{mutans} \) \( \text{ScrR} \) protein exhibited homology with other DNA-binding proteins including the DegA regulatory protein of \( \text{Bacillus subtilis} \) (32% identity [4]), as well as the ScrR proteins of \( \text{Staphylococcus xylosus} \) (8) and \( \text{Klebsiella pneumoniae} \) (12), with
25 and 26% identity, respectively. In addition, the S. mutans gene is highly homologous (50.8% identity) with that for the recently identified ScrR protein from Pediococcus pentosaceus (SwissProt accession no. P43472).

**Role of ScrR in the regulation of ScrB expression.** In order to determine if ScrR plays a role as a regulatory protein in affecting scrB expression, an ScrR mutant was constructed. For this purpose, an RsrI Tet’ gene cassette was introduced into the scrR gene to produce plasmid pSYZ4 (Fig. 6). Linearization of plasmid pSYZ4 with EcoRI and transformation of strain SP2C4 resulted in the introduction of the Tet’ gene into the S. mutans chromosome following a double-crossover recombination event. Tet’ transformants were then analyzed for the predicted integration event following Southern blot analysis (data not shown). With an scrB gene probe, it was demonstrated that the predicted hybridizing fragments were observed following cleavage of the chromosomal DNA from one of the Tet’ transformants, SP2CL1, with either EcoRI or EcoRV (i.e., EcoRI produced 3.7- and 10.9-kb hybridizing bands compared to 3.7- and 6.9-kb bands for SP2C4).

Growth of strain SP2CL1 (scrB::lacZ scrR::tet) in the presence of different sugars (Fig. 2B) indicated that glucose and sucrose were equally effective in inducing scrB expression relative to that with fructose. Thus, inactivation of scrR appeared to increase the ability of glucose to elevate scrB expression relative to that with sucrose and fructose. Alternatively, if glucose is a repressor of scrB expression, the scrR mutation abolishes such repressive effects. Lactose, maltose, and sorbitol behaved like fructose in affecting scrB expression in the scrR mutant (data not shown).

**Transcriptional regulation of scrB expression.** In order to determine if the differential regulation of scrB expression by the sugars occurs at the level of transcription, Northern blot analysis was carried out (Fig. 7). Two major mRNA bands corresponding to the scrB transcript of 2.37 and 1.35 kb were identified. The smaller species may represent binding of the probe to rRNA or a degraded mRNA, although the distinct size of this band suggests that the former possibility is more likely. It is also possible that this species could represent a product of alternate transcription initiation or termination. The larger transcript is compatible in size with an mRNA containing both the scrB and the scrR genes. Maximal expression of the scrB transcript occurs in the presence of sucrose, lower levels occur for cells grown in the presence of glucose, and only trace amounts of mRNA occur in the presence of fructose. Cells cultured in the absence of exogenous sugars (Fig. 7, lane 1) or sucrose-grown cells of the scrB mutant V1355 (lane 5) displayed no detectable levels of the transcripts. These results confirm those from the translational fusion studies and further indicate that the sugars affect scrB expression at the transcriptional level. However, it is not clear whether these effects result from repression or from induction by the sugars.

Since the transcription start site for the scrB gene had not yet been identified and such a determination might provide information regarding how the gene was regulated, primer extension analysis of the scrB transcript was carried out (Fig. 8). The results clearly identified an A residue at position 198 of the previously published scrB sequence (22) as the initiation site. Based upon this transcription start site, putative −10 (TACT AT) and −35 (TTGATT) regions were identified (see reference 22 for the scrB sequence). Neither of these regions overlapped the deduced −10 and −35 regions of the divergently transcribed scrA gene (10a).

**RT-PCR analysis of transcription.** In order to confirm the coexpression of the scrB and scrR genes, RT-PCR analysis of the mRNA expressed by strain SP2 was carried out (Fig. 9). Primers B-RT1 and B-RT2a amplified a 400-bp fragment
which is part of the scrB gene. Likewise, primers R-RT1 and scrR amplified a small 140-bp fragment which is internal to the scrR gene. In addition, primers R-RT1 and B-RT2a amplified a 700-bp DNA fragment which could be synthesized only if both genes were cotranscribed on a polycistronic message. Omission of the reverse transcriptase from each reaction mixture indicated that the amplified fragments were not produced from contaminating chromosomal DNA. Interestingly, the more intense band for fragment A compared to that for fragment C might be explained by the presence of a shorter mRNA species in addition to the full-length scrB-scrR transcript. However, additional experiments will be necessary to determine if the 1.35-kb mRNA (Fig. 7) is responsible for this difference.

Gel mobility shift assays. In order to confirm that the scrR gene codes for a regulatory protein which may bind to the scrB promoter region, gel shift assays were carried out. Crude cell extracts of SP2 as well as of the scrR mutant SP2DscrR were mixed with a DNA fragment containing the promoter regions of both the scrB and the scrA genes, and the mobilities of the fragments were determined (Fig. 10). The SP2 extract produced two prominent shifted bands. However, the extract from the scrR mutant demonstrated only the less mobile of the two bands. This suggested that the more rapidly migrating shifted band represented a complex of ScrR and the promoter fragment. The identity of the other protein which binds the DNA fragment is unknown, but it could represent RNA polymerase. The specificity of binding by the ScrR protein was indicated by the inability of nonspecific DNA fragments (a fragment from the S. mutans scrK [25] promoter region, the S. mutans dgk structural gene [35], or a fragment from the Treponema denticola ATCC 35405 chromosome) to interfere with the formation of the putative ScrR-DNA complex (Fig. 11). However, the band intensities of the less mobile shifted band were decreased in the presence of the two S. mutans DNA fragments but not the T. denticola fragment. This latter result would be compatible with an S. mutans RNA polymerase-scrB promoter fragment complex in the less mobile shifted band.

Attempts were made to examine the interaction of purified

FIG. 4. Nucleotide sequence of the scrR gene and the deduced amino acid sequence. The putative Shine-Dalgarno sequence (S.D.) and restriction enzyme sites are shown.

FIG. 5. Alignment of the amino acid sequences of S. mutans scrR (top) and E. coli galR (bottom).

FIG. 6. Integration of linearized pSYZ4 into S. mutans (S.m.) SP2C4. Plasmid pSYZ4 containing the 5′ and 3′ ends of scrR was linearized with EcoRI and transformed into S. mutans SP2C4. The resultant scrR mutant was designated SP2CL1. Abbreviations for restriction endonuclease sites: EI, EcoRI; R, RsaI; H, HindIII.

FIG. 7. Omission of the reverse transcriptase from each reaction mixture indicated that the amplified fragments were not produced from contaminating chromosomal DNA. Interestingly, the more intense band for fragment A compared to that for fragment C might be explained by the presence of a shorter mRNA species in addition to the full-length scrB-scrR transcript. However, additional experiments will be necessary to determine if the 1.35-kb mRNA is responsible for this difference.

Gel mobility shift assays. In order to confirm that the scrR gene codes for a regulatory protein which may bind to the scrB promoter region, gel shift assays were carried out. Crude cell extracts of SP2 as well as of the scrR mutant SP2AscrR were mixed with a DNA fragment containing the promoter regions of both the scrB and the scrA genes, and the mobilities of the fragments were determined (Fig. 10). The SP2 extract produced two prominent shifted bands. However, the extract from the scrR mutant demonstrated only the less mobile of the two bands. This suggested that the more rapidly migrating shifted band represented a complex of ScrR and the promoter fragment. The identity of the other protein which binds the DNA fragment is unknown, but it could represent RNA polymerase. The specificity of binding by the ScrR protein was indicated by the inability of nonspecific DNA fragments (a fragment from the S. mutans scrK [25] promoter region, the S. mutans dgk structural gene [35], or a fragment from the Treponema denticola ATCC 35405 chromosome) to interfere with the formation of the putative ScrR-DNA complex (Fig. 11). However, the band intensities of the less mobile shifted band were decreased in the presence of the two S. mutans DNA fragments but not the T. denticola fragment. This latter result would be compatible with an S. mutans RNA polymerase-scrB promoter fragment complex in the less mobile shifted band.

Attempts were made to examine the interaction of purified
ScrR protein with the scrA-scrB promoter region following expression of MBP (maltose binding protein)-ScrR and transcarboxylase-ScrR fusion proteins constructed as previously described for other S. mutans genes (26, 34). Although both fusion proteins were readily expressed in E. coli and bound to the scrB promoter fragment (Fig. 10), neither fusion protein could be demonstrated to bind to the promoter fragment in a specific manner (data not shown). This suggested that the fusion of ScrR to either MBP or transcarboxylase altered the ability of the regulatory protein to specifically bind to the scrB promoter fragment. In addition, cleavage of the ScrR protein from the fusion proteins did not result in the formation of a DNA-binding protein, probably due to the propensity of DNA-binding proteins to aggregate when highly expressed in E. coli (8).

**DISCUSSION**

Because of the pivotal role of dietary sucrose in the etiology of human dental caries, the effect of this sugar on dental plaque bacterial metabolism is of considerable interest. The results of earlier studies have demonstrated that most of the sucrose metabolized by cariogenic mutants streptococci is converted to lactic acid (32). Since significant amounts of sucrose are taken up by the cells by the sucrose PTS (28), the influence of the environment of the oral cavity on this system is clearly relevant to cariogenesis. Because of the utilization of less specific assays in earlier studies of sucrose metabolism by the mutants streptococci (9, 30, 31), it was not clear how sucrose influenced sucrose transport by the cells. Nevertheless, earlier studies with different strains of the mutants streptococci did suggest that higher cell-associated invertase activities were observed for cells grown in the presence of sucrose than for those grown with glucose or fructose (9, 31). More recent results with specific gene fusion technology (24) as well as the present results with the same approach have confirmed that growth in the presence of sucrose results in enhanced expression of two key enzymes in the major transport system of this bacterium: EnzIF<sup>309</sup> and Suc-GPH. For both enzymes, the relative strengths of expression in the presence of common sugars present in the human oral cavity were demonstrated to be sucrose > glucose > fructose. This confirms similar observations made earlier with permeabilized cells of Streptococcus sobrinus 6715 (30). Thus, as previously suggested (9, 30, 31), dietary sucrose appears to alter the expression of a major sucrose transport system in S. mutans. It should also be noted that the relative strengths of expression of the scrA and scrB genes by sucrose may be greater than actually demonstrated since the multiple extracellular glycosidases of the organism likely produce both glucose and fructose during growth in the presence of the disaccharide. Since fructose appears to repress the enhancing effects of sucrose, the sucrose effects may be underestimated in these experiments. It is also of interest that sucrose also appears to induce the expression of other sucrose-metabolizing enzymes in S. mutans, such as fructosyltransferase and the Gtfs (33).

Both in vitro and in vivo studies have indicated that plaque bacteria are subjected to fluctuations in pH (14). Therefore, the present demonstration (Fig. 3) that acidity appears to modulate the expression of both scrA and scrB suggests that such a mechanism could play a role as a feedback repression system. Enhanced fermentation of sugars leading to lactic acid formation would decrease the environmental pH, leading to repression of two key enzymes involved in sucrose transport and metabolism. However, the present results do not indicate whether such effects are directly dependent upon pH or upon altered growth rates since acidic pHs decrease the growth rate of S. mutans.

It is of interest that, among the microorganisms able to transport and metabolize sucrose, a variety of scr gene organizations and regulatory mechanisms are apparent (29). However, only in the mutants streptococci (5, 23) are the scrA and scrB gene homologs transcribed from opposite DNA strands. For most bacteria, both genes are transcribed either within the same operon or independently from the same DNA strand. In addition, a variety of different regulatory gene arrangements have been demonstrated. For B. subtilis, the regulatory sacT gene involved in antitermination is found upstream of the scrA and scrB homologs (7). For the enterobacterial plasmid pUR2100 sucrose-metabolizing system, the regulatory scrR gene is located immediately downstream from the scrB gene but is expressed from its own promoter (12). Likewise, the scrR gene of Vibrio alginolyticus is transcribed from its own promoter divergently from the scrA-scrK-scrB operon (3). Therefore, the S. mutans system appears to be unique in that the scrR gene appears to be present in the same operon as one of its target genes, scrB.

The present results together with recent studies (24) indicate that both the S. mutans scrA and scrB genes are similarly regulated by different sugars. In most other bacteria, such regulation results from the presence of both genes within the
FIG. 9. RT-PCR amplification of the scrB-scrR genes. (Top) Diagrammatic representation of RT-PCR-amplified fragments. (Bottom) Agarose gel patterns of amplified fragments. Lanes: 1, C fragment (700 bp) amplified with primers B-RT2a and R-RT1; 2, B fragment (140 bp) produced with primers R-RT1 and scrR; 3, A fragment (400 bp) amplified with primers B-RT2a and B-RT1; 4, molecular size markers.

FIG. 10. Gel mobility shift assays with cell extracts of S. mutans and the end-labeled 215-bp DNA fragment containing the scrA and scrB promoter regions. Lanes: 1, no protein added; 2, 0.5 μg of MBP-ScrR fusion protein; 3 to 6, 5, 10, 20, and 40 μg, respectively, of SP2 extract; 7 to 10, 5, 10, 20, and 40 μg, respectively, of extract from SP2ΔscrR. Arrowheads: top, putative RNA polymerase-scrA scrB fragment complex; second from top, putative RNA polymerase-scrA scrB fragment complex; second from bottom, ScrR-scrA scrB fragment complex; bottom, scrA scrB fragment.

FIG. 11. Specificity of gel mobility shift assays with extracts of S. mutans. Lanes 1 to 8, crude extracts of SP2; lanes 9 to 15, crude extracts of SP2ΔscrR. Lanes: 1, no competitor; 2, 10-fold excess of unlabeled promoter fragment relative to labeled fragment; 3, 20-fold excess of promoter fragment; 4, 10-fold excess of S. mutans scrK DNA fragment; 5, 20-fold excess of scrK fragment; 6, 20-fold excess of fragment from T. denticola dBII; 7, 20-fold excess of T. denticola fragment; 8, 10-fold excess of S. mutans scrK gene fragment; 9, no competitor; 10, 10-fold excess of scrB-scrA promoter fragment; 11, 20-fold excess of scrB promoter fragment; 12, 10-fold excess of scrR fragment; 13, 20-fold excess of scrK fragment; 14, 10-fold excess of T. denticola fragment; 15, 20-fold excess of T. denticola fragment. Arrowheads: top, putative RNA polymerase-scrA scrB fragment complex; middle, ScrR-scrA scrB fragment complex; bottom, scrA scrB fragment.

enteric pUR4000 systems (12), fructose and fructose-1-phosphate appear to induce scr gene expression. By contrast, fructose appears to repress scr gene expression in S. mutans. Likewise, scr operator sequences were identified upstream of the K. pneumoniae scrB gene (12), but comparable sequences could not be detected upstream of either the S. mutans scrA or scrB gene. A direct repeating sequence was detected upstream of the scrB gene, including part of the putative Shine-Dalgarno sequence (22), but its role in the regulation of scrB expression remains to be determined.

Based upon the properties of other scrR genes (29), together with the results of inactivating the strain GS-5 scrR gene, it is likely that the scrR gene acts as a repressor of the scr operon. Likewise, the present results suggest that ScrB binds in the intergenic region between the scrA and scrB genes. According to such a model, sucrose (or one of its metabolites) inactivates the repressor better than do glucose derivatives. However, fructose metabolites apparently are not effective in inactivating the putative ScrR repressor. Since scrR mutants are still repressible with fructose, another regulatory protein together with fructose derivatives may also repress scrB expression. Alternatively, both fructose and, to a lesser extent, glucose may act as corepressors in conjunction with ScrR. Therefore, a complex system of regulation may be involved in modulating the expression of a major sucrose transport system in S. mutans.

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REFERENCES
