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Genetic Regulation of Fructosyltransferase in *Streptococcus mutans*

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Streptococcus mutans **possesses several extracellular sucrose-metabolizing enzymes which have been implicated as important virulence factors in dental caries. This study was initiated to investigate the genetic regulation of one of these enzymes, the extracellular fructosyltransferase (Ftf). Fusions were constructed with the region upstream of the *S. mutans* GS5 Ftf gene (*ftf*) and a promoterless chloramphenicol acetyltransferase (CAT) gene. The fusions were integrated at a remote site in the chromosome, and transcriptional activity in response to the addition of various carbohydrates to the growth medium was measured. A significant increase in CAT activity was observed when glucose-grown cells were shifted to sucrose-containing medium. Sucrose-induced expression was repressed immediately upon addition of phosphoenolpyruvate phosphotransferase system sugars to the growth media. Deletion analysis of the *ftf* upstream region revealed that an inverted repeat structure was involved in the control of *ftf* expression in response to carbohydrate. However, the control of the level of *ftf* transcription appeared to involve a region distinct from that mediating carbohydrate regulation. CAT gene fusions also were constructed with the *ftf* upstream region from *S. mutans* V403, a fructan-hyperproducing strain which synthesizes increased levels of Ftf. Sequence analysis of the upstream *ftf* region in this strain revealed several nucleotide sequence changes which were associated with high-level *ftf* expression. Comparison of the GS5 and V403 *ftf* expression patterns suggested the presence of a *trans*-acting factor(s) involved in modulation of *ftf* expression in response to carbohydrate. This factor(s) was either absent or altered in V403, resulting in the inability of this organism to respond to the presence of carbohydrate. The sequences of the *ftf* regions from three additional fructan-hyperproducing strains were determined and compared with that of V403. Only one strain displayed nucleotide changes similar to those of V403. Two additional strains did not have these changes, suggesting that several mechanisms for up-regulation of *ftf* expression exist.**

Streptococcus mutans produces a variety of extracellular sucrose-metabolizing enzymes which contribute to the pathogenicity of this organism (9, 21, 26, 42). These enzymes include three glucosyltransferases (GtfB, GtfC, and GtfD) and a fructosyltransferase (Ftf), all of which utilize sucrose to synthesize extracellular polymers (1, 10, 11, 13, 31, 45, 52). Gtfs split sucrose and polymerize the glucose moiety into both water-soluble and water-insoluble glucan polymers with the release of free fructose. Water-insoluble glucan has been shown to mediate the tenacious attachment of *S. mutans* to the tooth surface (9, 21). Ftf utilizes the fructose moiety of sucrose to form a high-molecular-weight, relatively water-insoluble fructan polymer with the release of free glucose (2, 6, 37, 44). Fructan is thought to serve as an extracellular carbohydrate reserve which can be degraded during periods of nutrient deprivation to provide the cell with metabolizable carbohydrate (8). Inactivation of the *gtfB*, *gtfC*, or *ftf* gene results in reduced ability of *S. mutans* to elicit smooth-surface dental caries in the gnotobiotic rat model (26, 42). Because these enzymes are needed for pathogenicity, an understanding of the mechanisms involved in the regulation of these determinants will help define a clearer picture of the regulatory network which operates to optimize the virulence potential of *S. mutans* in the oral cavity.

Expression of the *gtfBC* and *ftf* genes has been measured under a variety of environmental conditions by utilizing chloramphenicol acetyltransferase (CAT) operon fusions (14, 15, 54). Results of these studies have demonstrated that the enzymes are expressed at a constant level during growth in glucose or fructose; however, environmental pH and growth rate can dramatically influence this steady-state level of expression. Growth in the presence of sucrose, the substrate of these enzymes, results in two- to threefold induction of expression. Although the expression of these enzymes is regulated by multiple environmental variables, the specific mechanisms governing this regulation have not been elucidated. It was the goal of this study to determine the involvement of *cis*-acting promoter elements and *trans*-acting factors in the regulation of *ftf* expression. CAT gene fusions were constructed with the region upstream of the *ftf* gene from *S. mutans* GS5 to measure transcriptional activity in the presence of various carbohydrates. Deletion derivatives of the upstream region were constructed to define *cis*-acting regulatory elements involved in carbohydrate response and transcriptional level control. In addition, CAT gene fusions were created with the *ftf* region of *S. mutans* V403, a fructan-hyperproducing strain which synthesizes increased levels of Ftf. Comparison of *ftf* expression in GS5 and V403 suggested factors important in *ftf* regulation.

MATERIALS AND METHODS

Chemicals. All of the chemicals used in this study were from Sigma Chemical Company (St. Louis, Mo.) unless otherwise specified.

Bacterial strains and media. The *S. mutans* strains used in this study are described in Table 1. These strains were cultured anaerobically at 37°C on brain heart infusion agar plates (Difco Laboratories, Detroit, Mich.). Transformants of *S. mutans* which had acquired antibiotic resistance genes were selected by growth on brain heart infusion agar plates containing erythromycin (10 μg/ml) or tetracycline (5 μg/ml). Mitis

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TABLE 1. *S. mutans* strains used in regulation studies

Strain	Relevant characteristics[a]	Source
GS5	Serotype c	H. Kuramitsu
V403	Fructan-hyperproducing strain (serotype c)	R. Facklam
V2226	Tc^r; GS5 containing GS5 *ftf*-CAT gene fusion integrated into chromosome	This study
V2227	Tc^r; GS5 containing 403 *ftf*-CAT gene fusion integrated into chromosome	This study
V2224	Tc^r; V403 containing 403 *ftf*-CAT gene fusion integrated into chromosome	This study
V2225	Tc^r; V403 containing GS5 *ftf*-CAT gene fusion integrated into chromosome	This study
V2139	Tc^r; GS5 containing promoterless CAT gene fusion integrated into chromosome	This study

[a] Tc^r, tetracycline resistance.

salivarius agar (Difco) was used to examine colonial morphology resulting from polymer production. *S. mutans* was grown in Todd-Hewitt broth (Difco) for preparation of total cellular DNA. Preparation of genetically competent cells involved growth in Todd-Hewitt broth containing 10% heat-inactivated horse serum (GIBCO, Gaithersburg, Md.). Brain heart infusion broth supplemented with 20 mM DL-threonine and 20 mM DL-alanine was used for small-scale preparation of chromosomal DNA. *S. mutans* cells used in CAT assays were grown in FMC, a chemically defined medium (50) commercially prepared by JRH Biosciences (Lenexa, Kans.) and supplemented with either 1% glucose or 1% sucrose. *Escherichia coli* HB101 (F$^-$ *hsd-20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44* λ^-) and DB11 (hypersensitive to erythromycin [25]) were maintained on Lennox broth agar plates (GIBCO) aerobically at 37°C. For selection of *E. coli* antibiotic-resistant transformants, erythromycin (50 μg/ml), tetracycline (5 or 15 μg/ml), carbenicillin (30 μg/ml), or chloramphenicol (20 μg/ml) was added to Lennox broth agar plates. For plasmid DNA isolation and preparation of competent cells, *E. coli* was grown aerobically at 37°C in Lennox broth.

DNA isolation and characterization. Large-scale plasmid DNA isolation from *E. coli* was performed with Qiagen anion-exchange columns as recommended by the manufacturer (Qiagen Inc., Chatsworth, Calif.). Small-scale plasmid preparations were performed by the method of Birnboim and Doly (3). The plasmids used in this study are listed in Table 2. *S. mutans* chromosomal DNA was isolated by the method of Marmur with the modifications described by Schroeder et al. (42). Restriction endonuclease digestion of plasmid and chromosomal DNAs was carried out as recommended by the manufacturer (Bethesda Research Laboratories, Inc., [BRL], Gaithersburg, Md.). Restriction endonuclease digests of chromosomal DNA were transferred to 0.45-μm-pore-size reinforced nitrocellulose (Schleicher & Schuell, Keene, N.H.) as described by Smith and Summers (47) and were analyzed by Southern hybridization (40). Colony hybridization was done as described by Maniatis et al. (40), with 82-mm-diameter circular nitrocellulose filters (Schleicher & Schuell). The DNA was immobilized on the nitrocellulose filters with the Stratalinker 2400 UV cross-linker (Stratagene, La Jolla, Calif.). Probe DNA was labeled with 50 μCi of [^{32}P]dCTP (3,000 Ci/mmol, ICN, Costa Mesa, Calif.) by a commercially available nick translation system (BRL). When needed, DNA size standards were labeled with 50 μCi of [^{32}P]ATP (4,500 Ci/mmol; ICN) by a commercially available 5′ terminus labeling system (BRL). Unincorporated radioactive nucleotides were removed by Sephadex G-50 Mini-Spin columns as recommended by the manufacturer (Worthington Biochemical, Freehold, N.J.). DNA restriction fragments were extracted from agarose gels with phenol and purified by the Magic DNA Clean-Up system (Promega, Madison, Wis.) as recommended by the manufacturer.

DNA enzymology. Klenow fragment of *E. coli* DNA polymerase I (BRL) was used to fill recessed 3′ ends of restriction endonuclease-digested plasmid DNA as described by Maniatis et al. (40). DNA ligation reactions were performed with T4 DNA ligase (BRL) and buffer supplied by the manufacturer. PCR products were phosphorylated as described by Maniatis et al. (40), with T4 polynucleotide kinase (BRL).

DNA sequencing. The nucleotide sequences of PCR products were determined with the Sequenase DNA sequencing kit (Bio-Rad, Richmond, Calif.) and instructions provided by the manufacturer. The sequence of the V403 *ftf* promoter region was determined with the DyeDeoxy terminator cycle sequencing kit (Applied Biosystems, Foster City, Calif.) and instructions provided by the manufacturer. Cycle sequencing reac-

TABLE 2. Plasmids used in this study

Plasmid	Relevant characteristics[a]	Source or reference
pResAmpHind	Ap^r; contains 4.3-kb *Hin*dIII fragment of GS5 chromosomal DNA which includes entire *ftf* gene and flanking open reading frames	H. Kuramitsu
pVA2137	Ap^r; pSP64 containing 4.3-kb *Hin*dIII fragment of V403 chromosomal DNA which includes entire *ftf* gene and flanking open reading frames	This study
pYA580	Cm^r; pACYC184 containing 3.28-kb *Hin*dIII fragment of *lac* operon of *S. mutans*	18
pSP64	Ap^r; SP6 expression vector derivative of pUC13	Promega
pMH109	Tc^r; contains promoterless gram-positive CAT gene preceded by multiple cloning site of pUC13	16
pVA981	Tc^r; pBR325 containing 4-kb *Hin*cII fragment which carries Tc^r marker from *S. mutans*	51
pVA1414	Em^r; pACYC184 containing *ermAM* gene on 2-kb *Hin*dIII fragment	24
pVA1404	Tc^r; pACYC184 containing 6.3-kb *Eco*RI fragment which carries *scrA* and *scrB* genes from V403	23
pSS22	Tc^r; pACYC184 containing 3.5-kb *Eco*RI fragment which carries most of *S. mutans* GS5 *ftf* gene	45
pVA2228	Ap^r; pSP64 containing 3.28-kb *Hin*dIII fragment of pYA580 ligated into *Hin*dIII and *Pst*I sites of multiple cloning site	This study
pVA2215	Ap^r; pSP64 containing *Bam*HI-*Pst*I-*Sst*I-*Eco*RI linker	This study
pVA2229	Ap^r; pVA2215 containing 4.2-kb *Hin*cII fragment of pVA981 which carries *tet* gene	This study
pVA2216	Ap^r; pVA2228 containing 4.2-kb *Pst*I fragment of pVA2229 which carries *tet* gene	This study
pVA2154	Em^r; pVA1404 containing *Hin*dIII fragment of pVA1414, which carries *erm* gene ligated into *Pst*I site	This study

[a] Tc^r, tetracycline resistance; Em^r, erythromycin resistance; Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance.

TABLE 3. Oligonucleotide primers used in this study

Designation	Sequence
419	5′ TTG TTA GCT CGC TTT ACC 3′
484	5′ GTT TTT TGG TTC AAG AGA 3′
545	5′ TGT CAT GAA AAT GAG ATG 3′
610	5′ AAC ATA AAT GAT ATC GTG 3′
831	5′ TTC TTG AAC CTG ACT TCT 3′
729	5′ GTC GTG ATG GTG GCT AAC AC 3′
ORF	5′ GCG TCG GAC ACT TCA GAC AG 3′

tions were analyzed on an Applied Biosystems 373A DNA Sequencer. The *ftf* promoter sequences of LM7, KPKS2, and MT6861 were determined with the CircumVent thermal cycle sequencing kit and instructions provided by the manufacturer (New England BioLabs, Beverly, Mass.).

Bacterial transformation. *E. coli* HB101 and DB11 were transformed by the $CaCl_2$ method (40) with mid-log-phase cells harvested at an optical density at 660 nm of 0.2 to 0.3. Genetic transformation of *S. mutans* was performed as described by Lindler and Macrina (20). Antibiotic-resistant transformants were selected by growth on solid medium containing the appropriate antibiotic(s).

PCR. PCRs were performed as described by Maniatis et al. (40), in a 480 DNA Thermal Cycler (Perkin Elmer-Cetus, Emeryville, Calif.) with 700 ng of each primer and 70 ng of *S. mutans* chromosomal DNA. Primers were obtained from Oligos Etc (Guilford, Conn.) and are listed in Table 3. Amplification conditions consisted of 28 cycles of denaturation at 94°C for 1 min, annealing at 37°C for 2 min, and polymerization at 72°C for 3 min. PCR products were prepared for blunt-end DNA ligation by treatment with the Klenow fragment of *E. coli* DNA polymerase I and phosphorylation with T4 polynucleotide kinase.

Preparation of cell lysates for CAT assays. Growth conditions for *S. mutans* strains containing chromosomally integrated *ftf*-CAT gene fusions were as described by Hudson and Curtiss (14), with minor modification. Strains were grown anaerobically for 18 h at 37°C in 10 ml of FMC medium supplemented with 1% glucose. The cultures were diluted to an optical density at 660 nm of 0.100 in 30 ml of warmed FMC–1% glucose and incubated anaerobically for 2 h at 37°C. The cells were harvested by centrifugation at 4,000 × *g* for 15 min at 25°C, and the pellets were suspended in 18.5 ml of warmed FMC. One-milliliter samples of cells were inoculated into 9 ml of either FMC–1% glucose or FMC supplemented with 1% sucrose. The cultures were incubated anaerobically at 37°C for 1 to 8 h. Cultures were removed hourly and centrifuged at 4,000 × *g* for 15 min at 4°C. The cells were washed with 0.25 M Tris HCl (pH 8.0), suspended in the same buffer to a final volume of 1 ml, and transferred to microcentrifuge tubes containing 0.5 volume of 0.1-mm-diameter zirconium beads (Biospec Products, Bartlesville, Okla.). The cells were lysed in a Mini-Bead Beater homogenizer (Biospec Products) for 3 min. Beads and cellular debris were removed by centrifugation at 12,000 × *g* for 5 min to obtain a clear lysate. The lysates were kept at 0°C until assays for total protein and CAT activity could be performed. Protein determination was done by the method of Lowry et al. (22), with bovine serum albumin as the standard. Assays for CAT activity were performed by a spectrophotometric method as described below.

CAT assay. CAT activity of cell lysates was determined by the method of Shaw (43). The assay is based on the liberation of a free coenzyme A sulfhydryl group from acetyl coenzyme A by the action of CAT. The reduced coenzyme A reacts with 5,5′-dithiobis-2-nitrobenzoic acid to yield a colored product with a molar extinction coefficient of 13,600 at 412 nm. The assay was carried out in a DU-65 recording spectrophotometer (Beckman Instruments, Fullerton, Calif.) equipped with kinetics software and a temperature-controlled cuvette chamber. The cuvette chamber was equilibrated to 37°C prior to the start of the assay. Twenty microliters of cell extract was added to 500 μl of a warm (37°C) reaction mixture containing 100 mM Tris-HCl (pH 8.0), 0.1 mM acetyl coenzyme A (Pharmacia), and 1 mM 5,5′-dithiobis-2-nitrobenzoic acid in 2.9-ml polystyrene cuvettes. The reaction mixture was placed in the spectrophotometer, and the background slope was recorded for 1 min at 412 nm. Ten microliters of 0.1 mM chloramphenicol (diluted in distilled H_2O from a 50 mM stock in 95% ethanol) was then added, and the slope was recorded for an additional 3 min. The reaction rate was determined from the linear portion of the graph. A blank, without extract, was run in parallel with each set of reactions. The background absorption of the blank was taken into account by the kinetics program in the calculation of the reaction rate. CAT activity was expressed as nanomoles of chloramphenicol acetylated per minute per milligram of protein.

RESULTS

Construction of *ftf*-CAT transcriptional fusions. Deletion analysis of the region upstream of the *ftf* gene was undertaken to define sequences involved in sucrose induction and regulation of expression. Two inverted repeat regions present upstream of the *ftf* gene were defined by sequence analysis. The repeat centered at nucleotide position 579 was defined by Shiroza and Kuramitsu (45) and consisted of perfectly matched 13-bp repeats separated by three nucleotides. Further analysis revealed perfectly matched 14-bp inverted repeats separated by 10 nucleotides centered at position 490. Also, a promoter-like sequence, ATGATA-N_{17}-TAGGAT, has been found between positions 617 and 645 (45). These regions may be involved in regulation of the *ftf* gene and therefore were targeted for examination by deletion analysis with the PCR. Since the entire sequence of the GS5 *ftf* gene and the upstream region was known, PCRs were performed with *S. mutans* GS5 chromosomal DNA (45). Primers which would allow amplification of successively smaller fragments of the upstream region were chosen. To accomplish this, four primers, designated 419, 484, 545, and 610, were chosen within the upstream region while the primer on the opposing strand (primer 831) was held constant for each of the four reactions (Table 3). The PCR products and their sizes are depicted in Fig. 1. These products were treated with Klenow fragment, phosphorylated, and ligated into the *Sma*I site of pMH109, upstream of the promoterless CAT gene present on this plasmid (Fig. 2) (16). Tetracycline-resistant transformants of *E. coli* HB101 were selected and screened for chloramphenicol resistance. Properly oriented promoter fragments were able to direct CAT expression in *E. coli*. The orientation of the fragments was confirmed by using an internal *Eco*RV site present in all of the PCR products. The nucleotide sequence of each of the synthetic promoter fragments was determined to ensure that no mistakes were made in the PCR amplification process.

Construction of an *S. mutans* integration vector. A vector which would allow the *ftf*-CAT gene fusions to be integrated into the *S. mutans* chromosome at a remote site was constructed (Fig. 3). A previous vector constructed to transfer *ftf*-CAT gene fusions into the chromosome relied on Campbell-type integration into the *ftf* locus (14). This type of vector would not be useful when integrating deletion derivatives of

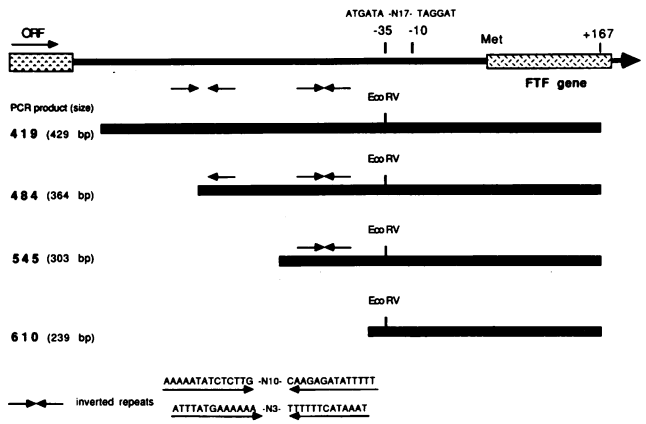

FIG. 1. PCR amplification products of the *ftf* upstream region. The region upstream of the *ftf* gene is depicted as a solid black line at the top. The sequence of the proposed −35 and −10 regions is shown. Inverted repeats are shown as arrows, and their sequences are given at the bottom. The PCR products and their sizes are shown below the *ftf* gene diagram. ORF, open reading frame.

the promoter region, since integration could regenerate a full promoter construct.

The multiple cloning site of pSP64 was modified by digestion with *Bam*HI-*Eco*RI and insertion of a linker containing an additional *Pst*I site needed in later cloning steps (Table 2, pVA2215). The 4-kb *Hin*cII fragment of pVA981 (51), containing a streptococcal tetracycline resistance marker, was ligated into the *Hin*cII site of pVA2215 to provide a selectable marker for *S. mutans* transformations. The tetracycline resistance marker and adjacent cloning sites were removed from pVA2215 by digestion with *Pst*I and ligated into the *Pst*I sites of pVA2228, which contained a subcloned region of the *S. mutans lac* operon from pYA580 (18). The resultant plasmid, pVA2216, contained a *Bam*HI site adjacent to the tetracycline resistance marker, which facilitated cloning of the *ftf*-CAT gene fusions removed from pMH109 by digestion with *Bam*HI (Fig. 2). The placement of the tetracycline resistance determinant and the CAT gene fusions between the *lac* operon sequences allowed recombination of the fusions into the *S. mutans* chromosome via allelic exchange (Fig. 4). DNA from the *lac* operon was chosen for this purpose because of its noninvolvement in sucrose metabolism. This was important because integration of the CAT gene fusions into the chromosome would inactivate any genes within the region of integration. The integration vectors were linearized at the unique *Pvu*I site and were used to transform *S. mutans* GS5. Total cellular DNA from tetracycline-resistant transformants was digested with *Bam*HI and subjected to Southern blot analysis with ^{32}P-labeled pVA2228, which contained the *lac* operon region. Digestion of the parental genomes with *Bam*HI released a single 5.1-kb fragment which hybridized with the probe. Integration of the *ftf*-CAT gene fusions into the *lac* operon resulted in the presence of two additional *Bam*HI sites, thereby generating two fragments of 6.5 and 1.8 kb which hybridized with the probe (Fig. 4). The orientation of the integrated *ftf*-CAT gene fusions prevented interference from *tet* transcription, as well as transcription originating in the chromosome. A promoterless CAT gene was integrated into the chromosome to serve as a control to confirm the lack of transcriptional activity in the absence of cloned *ftf* promoter fragments.

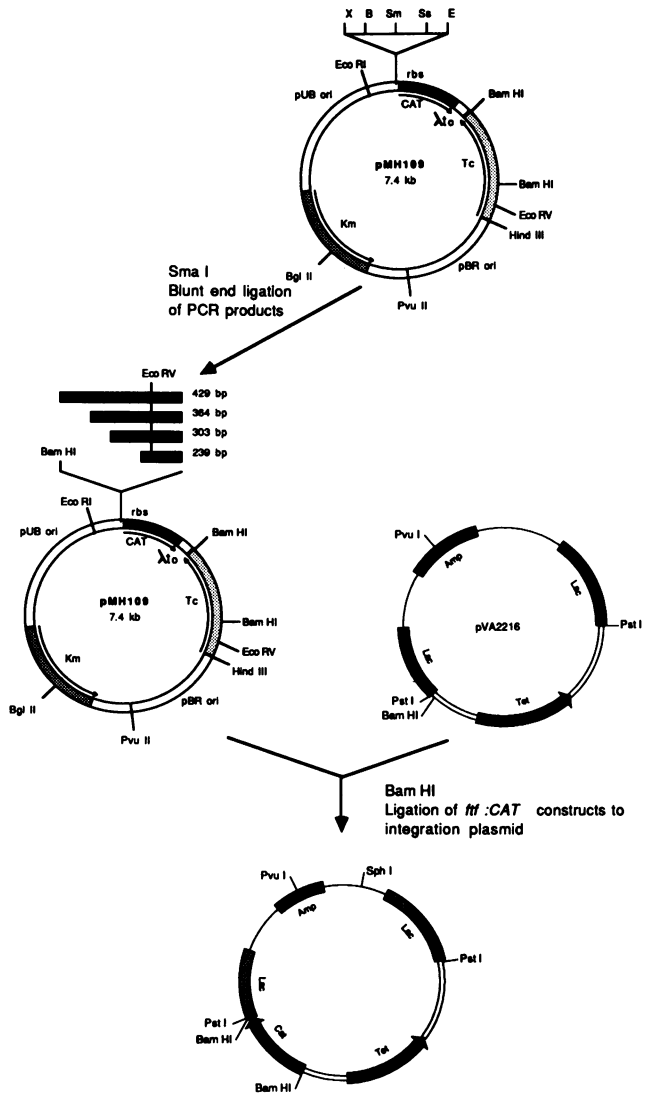

FIG. 2. Construction of *ftf*-CAT gene fusions. pMH109 contained a promoterless CAT gene positioned downstream of a multiple cloning site. Fragments of the *ftf* upstream region created by PCR were treated with Klenow and cloned into the *Sma*I site of pMH109. The *ftf*-CAT gene fusions were then removed as *Bam*HI fragments and cloned into the *Bam*HI site of integration vector pVA2216. The resultant plasmids were linearized with *Pvu*I and transformed into *S. mutans* GS5.

Sucrose induction and transcription level of *ftf*-CAT gene fusions. CAT specific activity was used to monitor *ftf* expression in strains containing the CAT gene fusions. Cells were grown in medium with either glucose or sucrose as the carbon source for 5 h. The results for each PCR *ftf*-CAT gene fusion in strain GS5 are shown in Fig. 5. The 419 PCR fusion represented most of the region upstream of the *ftf* gene. This fusion was inducible by sucrose, and the transcription level was defined as 100%. Disruption of the first inverted repeat (primer 484) had no effect on either sucrose induction or transcription level. Deletion of the region between the two inverted repeats (primer 545) resulted in a 40% drop in the level of transcription; however, the fusion was still inducible by sucrose. These results suggested that sequences within this region were involved in control of the level of *ftf* transcription.

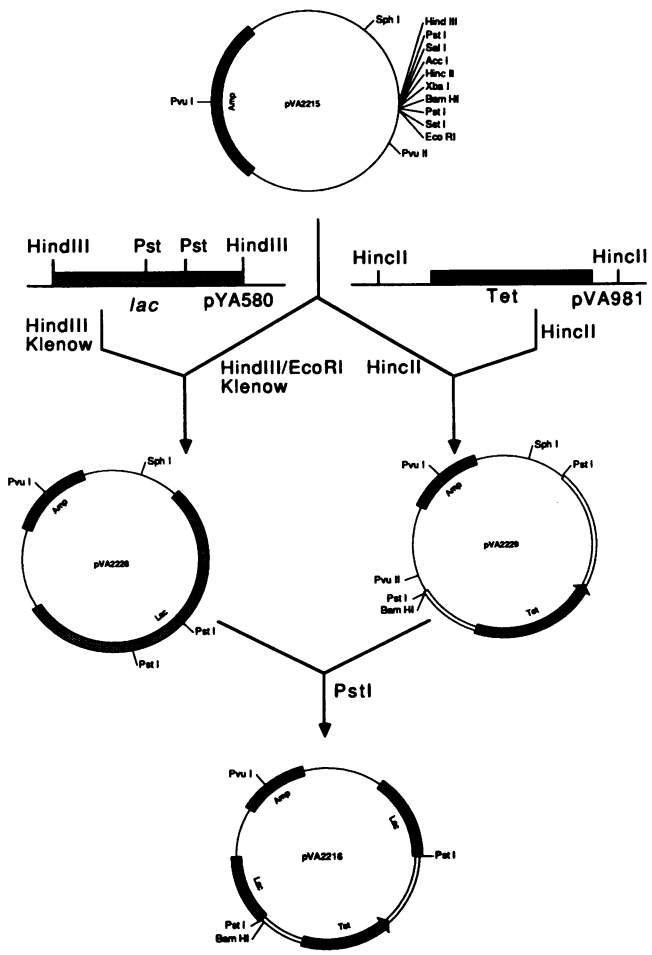

FIG. 3. Construction of pVA2216. A 3.3-kb region of the *S. mutans* *lac* operon from pYA580 was cloned as a blunt *Hin*dIII fragment into *Hin*dIII-*Eco*RI-digested, Klenow-treated pVA2215 to obtain pVA2228. A streptococcal tetracycline resistance gene from pVA981 was cloned as a 4-kb *Hin*cII fragment into *Hin*cII-digested pVA2215 to obtain pVA2229. pVA2228 and pVA2229 were digested with *Pst*I to allow insertion of the *tet* gene into the *lac* operon sequence (pVA2216).

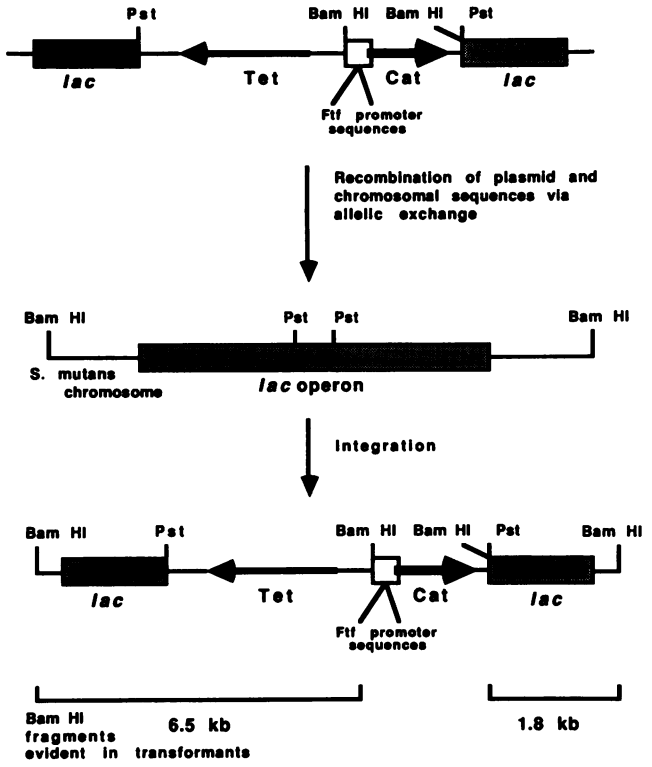

FIG. 4. Chromosomal integration of *ftf*-CAT gene fusions. Linearized pVA2216 containing an *ftf*-CAT gene fusion is shown at the top. Recombination of the *lac* sequences on the plasmid with the homologous region in the chromosome resulted in chromosomal integration of the fusions as depicted at the bottom. Integration was selected by resistance to tetracycline.

Deletion of the second inverted repeat in the 610 construct resulted in no detectable CAT activity in either sucrose- or glucose-grown cells, even though the proposed −35 and −10 regions were present.

Effect of *scrA* inactivation on *ftf* expression. In *B. subtilis*, the levansucrase gene (*sacB*) is inducible by sucrose. This induction involves an antitermination mechanism controlled by the phosphorylated state of components of a minor sucrose phosphotransferase system (PTS) (4). To determine whether the *S. mutans* GS5 sucrose PTS was involved in the regulation of *ftf* expression, the structural gene for EII^{scr} (*scrA*) was inactivated by allelic exchange (41). pVA1404 (23), which contains the *scrA* gene, was digested with *Pst*I and treated with Klenow to remove a 250-bp fragment internal to *scrA*. The 2-kb streptococcal erythromycin resistance determinant of pVA1414 (24) was removed by digestion with *Hin*dIII, treated with Klenow, and ligated into Klenow-treated pVA1404. The resulting plasmid (pVA2154) was linearized with *Sal*I and used to transform the GS5 strain containing the 419 *ftf*-CAT gene fusion. DNA from erythromycin-resistant transformants was digested with *Eco*RI and analyzed by Southern blot analysis with ^{32}P-labeled pVA1404. The probe hybridized with a fragment in the transformant strains which was 2 kb larger than the fragment evident in the parental strain, indicating insertion of the 2-kb erythromycin resistance marker into the *scrA* gene. MudE transposon insertions into this same region of the *scrA* gene abolish sucrose PTS activity (41). Although sucrose uptake was impaired in the mutant strain, results of CAT assays revealed

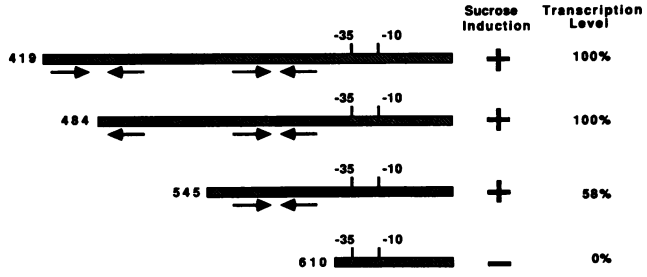

FIG. 5. Sucrose induction and level of transcription of *ftf*-CAT gene fusions in GS5. The bars represent PCR *ftf*-CAT gene fusions. Cells containing these integrated fusions were grown in either glucose- or sucrose-containing medium for 5 h as described in Materials and Methods. CAT activity was determined by measuring the rate of chloramphenicol acetylation with a spectrophotometric assay. The arrows represent inverted repeat regions.

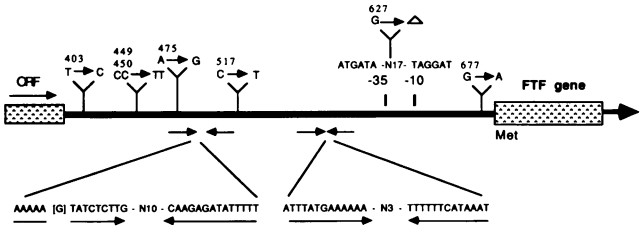

FIG. 6. Nucleotide sequence changes in the region upstream of the V403 *ftf* gene. The solid black line represents the *ftf* upstream region of *S. mutans* V403. Nucleotide sequence changes are shown above the line, and the nucleotide position numbers are given relative to the GS5 sequence. The symbol Δ represents a nucleotide deletion. The opposing arrows below the line represent inverted repeat sequences. ORF, open reading frames.

that sucrose induction of *ftf* expression was not affected (data not shown).

Analysis of *S. mutans* V403. *S. mutans* V403, a fructan hyperproducer, was chosen for analysis of *ftf* expression because of the obvious alteration in the levels of Ftf produced by this strain. On mitis salivarius agar, V403 exhibited the large, mucoid colonial morphology associated with increased fructan production (42). This morphology was the result of increased levels of extracellular Ftf, which could be demonstrated by sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis. The prominent 88-kDa Ftf band in the V403 extracellular protein preparations was in stark contrast to the correspondingly faint band observed with GS5 (data not shown).

To examine *ftf* expression in a fructan-hyperproducing strain, the region upstream of the V403 *ftf* gene was cloned and sequenced. Previously, Shiroza and Kuramitsu (45) isolated from strain GS5 a 4.3-kb *Hin*dIII chromosomal fragment which contained the intact *ftf* gene and promoter. Therefore, we reasoned that the *ftf* region could be isolated in a similar manner from V403. Total cellular DNA from V403 was digested to completion with *Hin*dIII, and the fragments were cloned into *Hin*dIII-digested pSP64. Ampicillin-resistant transformants of *E. coli* HB101 were screened for the presence of the *ftf* gene by colony blot hybridization with ^{32}P-labeled pSS22, a plasmid which contained the *ftf* region of GS5 (45). One positive clone (V2137) which contained the predicted 4.3-kb *Hin*dIII fragment was detected. Additional confirmation was obtained by cleavage of the plasmid with *Eco*RI, which produced fragments of the correct sizes as determined from the published GS5 *ftf* sequence (45). The nucleotide sequence of the region upstream of the *ftf* gene was determined for both strands by using primers 729 and ORF. This sequence was compared with the published GS5 sequence. A number of base changes, several of which may have important regulatory implications, were evident (Fig. 6). These included an A-G transition (nucleotide 475) within an upstream inverted repeat which disrupted the twofold symmetry of this region. In addition, a deletion of a guanosine nucleotide (nucleotide 627) occurred between the proposed −35 and −10 sequences. This would serve to decrease the distance between the predicted RNA polymerase contact sites. Finally, a nucleotide change (nucleotide 517) was observed in the region which may be involved in transcriptional level control occurring between the two inverted repeats.

Comparison of *ftf* expression in V403 and GS5. Similar *ftf*-CAT gene fusions were constructed to compare the regulation of *ftf* expression in strains V403 and GS5. The upstream regions of GS5 and V403 were isolated as 885-bp *Hin*dIII-*Dde*I fragments from pResAmpHind and pVA2137, respectively. The fragments were treated with Klenow fragment and cloned into the *Sma*I site of pMH109. Correct fusions were cloned as *Bam*HI fragments into integration vector pVA2216. The fusions were integrated into the chromosomes of V403 and GS5 via allelic exchange as described previously. Correct integrations were confirmed by Southern blot analysis (data not shown).

The strains carrying the *ftf*-CAT gene fusions were grown for 8 h in either glucose- or sucrose-containing medium. Expression of *ftf* was monitored by measuring CAT activity. V2226 (GS5 containing the GS5 *ftf*-CAT gene fusion) exhibited a low, steady-state level of CAT expression in glucose-containing medium (Fig. 7A). Induction of expression was observed when cells were grown in the presence of sucrose. This induction peaked at 4 h (900 U) and then steadily declined over the next several hours. These results were similar to those obtained with the 419 PCR *ftf*-CAT gene fusion. A very different pattern of regulation was observed with V2224 (V403 containing the V403 *ftf*-CAT gene fusion) (Fig. 7B). Expression patterns in both glucose- and sucrose-containing media were similar in that CAT activity increased exponentially over the 8-h time course of the assay, from 1,200 U to a peak value of 12,900 U, more than 12 times greater than the highest activity observed in sucrose-grown V2226. If the sequence changes in the V403 upstream region were solely responsible for this pattern of regulation, then placing the V403 *ftf*-CAT gene fusion into GS5 should result in a similar expression profile (Fig. 7C). As was expected, V2227 demonstrated elevated CAT expression in both glucose- and sucrose-containing media; however, the expression appeared to be regulated in a manner similar to that seen with V2226. Specifically, expression was relatively constant in glucose-containing medium but could be induced in the presence of sucrose. Unlike the results obtained with V2226, the expression seen in sucrose-grown V2227 cells did not decline over time, although at several time points the expression did fluctuate. These results suggested that two regulatory mechanisms were operating to control *ftf* expression. The first involved the level of transcription, which was determined by sequences upstream of the *ftf* gene. The second mechanism involved transcriptional control in response to carbohydrate, which was mediated by a *trans*-acting factor(s). The presence of this factor(s) in GS5 could control expression of the V403 *ftf*-CAT gene fusion. Next, the GS5 *ftf*-CAT gene fusion was placed in V403 to create V2225 (Fig. 7D). As would be expected for the V403 background, there was not much difference in expression in either glucose- or sucrose-grown cells. Also, the low expression level was similar to that observed with V2226. These results confirmed the importance of sequences upstream of the *ftf* gene in controlling the level of transcription.

Effects of various carbohydrates on sucrose induction. Sucrose-induced expression in GS5 gradually declined over time, although excess inducer was present in the media. This decline was not due to fructan polymer accumulation in the medium, since Hudson and Curtiss have shown that addition of 0.5% fructan to sucrose-grown cells had little effect on expression (14). When cells were grown in sucrose, no other metabolizable carbohydrate was initially present; however, continued action of the extracellular Gtf and Ftf enzymes produced increasing concentrations of both fructose and glucose, respectively. Therefore glucose and fructose, as well as several additional PTS sugars, were examined for the ability to repress sucrose-induced *ftf* expression. V2226 cells were pulsed with a

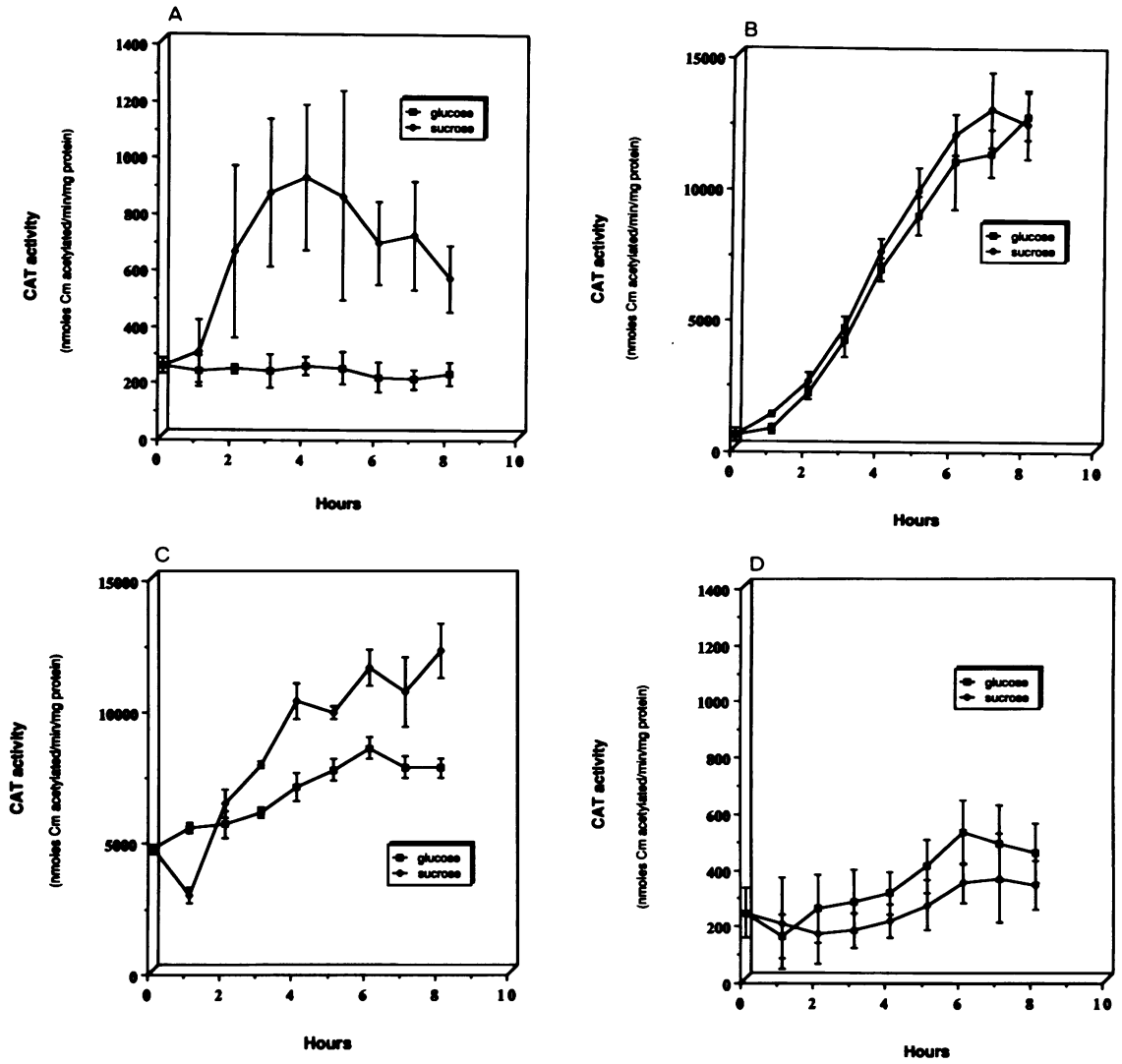

FIG. 7. CAT activity of *S. mutans* GS5 and V403 containing integrated CAT gene fusions. *S. mutans* strains containing CAT gene fusions were grown in either glucose- or sucrose-containing medium for 8 h as described in Materials and Methods. CAT activity was determined in triplicate for each time point by measuring the rate of chloramphenicol (Cm) acetylation with a spectrophotometric assay. Each point represents the mean of three independent experiments. The error bars indicate standard deviations. Panels: A, *S. mutans* V2226 (GS5 containing the GS5 *ftf*-CAT gene fusion); B, *S. mutans* V2224 (V403 containing the V403 *ftf*-CAT gene fusion); C, *S. mutans* V2227 (GS5 containing the V403 *ftf*-CAT gene fusion); D, *S. mutans* V2225 (V403 containing the GS5 *ftf*-CAT gene fusion).

0.1% concentration of each PTS carbohydrate after 2 h in either glucose- or sucrose-containing medium. Addition of glucose, fructose, mannose, trehalose, and maltose effectively repressed sucrose-induced expression to levels found in glucose-grown cells (Fig. 8). Addition of glucose or mannose did not have an effect on *ftf* expression in glucose-grown cells. However, addition of fructose, trehalose, and maltose did appear to affect expression in glucose. In the case of fructose and maltose, there was an immediate drop in the level of expression which lasted approximately 2 h. Expression then began to rise to levels higher than those observed during the initial hours of the assay. Addition of trehalose also decreased expression in glucose-grown cells; however, the decrease was more gradual and remained at a lower steady-state level for the duration of the assay.

Construction of a hybrid *ftf* promoter. High-level *ftf* expression of V403 was associated with sequence changes upstream of the V403 *ftf* gene. A fusion between *ftf* promoter sequences from V403 and GS5 was created to determine whether the nucleotide deletion between the −35 and −10 sequences of the V403 *ftf* promoter had an effect on the level of *ftf* expression. Such a deletion would serve to decrease the distance between the putative RNA polymerase contact sites and consequently could have an effect on the level of transcription. An *Eco*RV site within the −35 sequence was used to join the upstream sequences of GS5 to the V403 sequences downstream of the −35 region which included the deleted guanosine nucleotide. The hybrid promoter was cloned as an 885-bp Klenow-treated *Hin*dIII-*Dde*I fragment upstream of the CAT gene, and the *ftf*-CAT gene fusion was integrated into the chromosome of GS5 as described previously. Cells containing the integrated fusion were grown in glucose- or sucrose-containing medium for 8 h, and *ftf* expression was monitored by measuring CAT activity. The level of CAT activity obtained

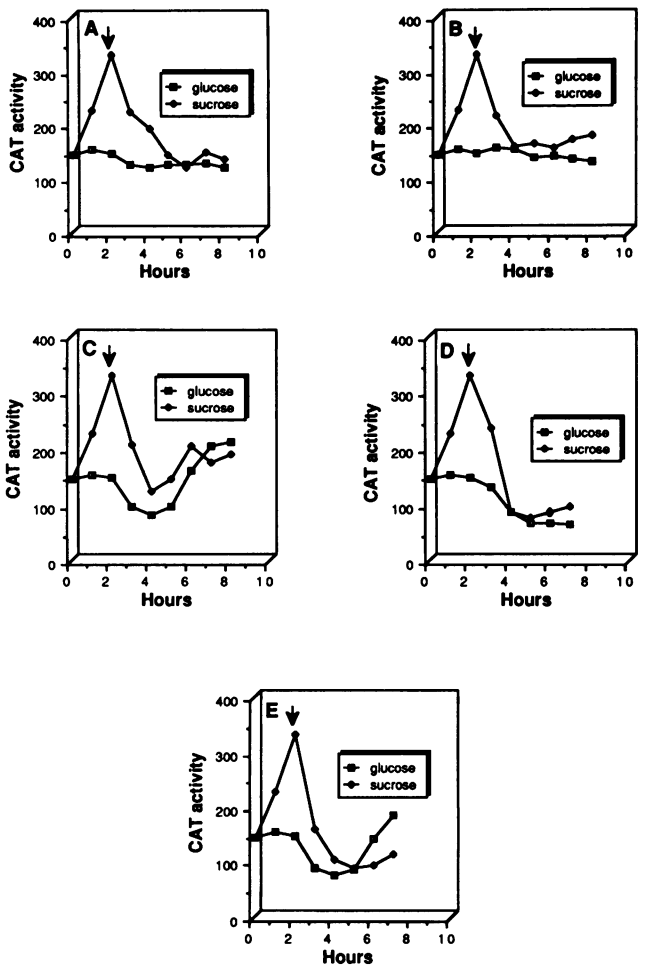

FIG. 8. Effect of carbohydrate on sucrose-induced *ftf* expression in V2226. *S. mutans* V2226 was grown in either glucose- or sucrose-containing medium for 7 to 8 h as described in Materials and Methods. At 2 h, a 0.1% concentration of a particular carbohydrate was added to the cells (indicated by the arrow). CAT activity was determined in triplicate for each time point by measuring the rate of chloramphenicol acetylation with a spectrophotometric assay. Each point represents the mean of three independent experiments. CAT activity is expressed as nanomoles of chloramphenicol acetylated per minute per milligram of protein. Panels: A, addition of glucose; B, addition of mannose; C, addition of fructose; D, addition of trehalose; E, addition of maltose.

was comparable to that observed with the native GS5 promoter (data not shown), indicating that the deleted guanosine nucleotide, at least in this specific construct, had no effect on the level of *ftf* expression.

Sequence analysis of *ftf* upstream regions from fructan-hyperproducing strains. The sequence of the *ftf* upstream region was determined from three additional, independently isolated, fructan-hyperproducing strains (*S. mutans* LM7, KPKS2, and MT6861) to determine the presence of common nucleotide changes which might mediate high-level *ftf* expression. Primers 419 and 831 were used to amplify the *ftf* regions from each strain by PCR. The sequences of the purified PCR products were determined and compared with the GS5 *ftf* sequence. V403 and LM7 displayed similar nucleotide changes, whereas KPKS2 exhibited two nucleotide changes, only one of which was evident in V403. Two nucleotide changes in the MT6861 *ftf* upstream region were not present in V403. Therefore, a different mechanism of *ftf* up-regulation may be operative in KPKS2 and MT6861 since nucleotide changes similar to V403 were not observed.

DISCUSSION

The extracellular Ftf of *S. mutans* produces high-molecular-weight fructan polymers from sucrose. These polymers are believed to function as extracellular storage polysaccharides which can provide the organism with a metabolizable carbon source during periods of nutrient deprivation (8). Inactivation of the *ftf* gene results in reduced virulence of *S. mutans* in the rat dental caries model (26, 42), implicating Ftf as an important virulence factor. Understanding the mechanisms involved in the expression of *ftf*, as well as additional virulence genes, will provide insight into how this organism persists and initiates disease in the constantly changing environment of the oral cavity. Results of previous studies on the regulation of *ftf* expression have shown that production of Ftf is markedly influenced by environmental factors (14, 53, 54). The specific growth rate of the organism and the medium pH are able to alter *ftf* expression dramatically. The mechanisms by which this control is exerted are not understood. Therefore, it was the goal of this study to define *cis*-acting promoter elements and *trans*-acting factors involved in the regulation of *ftf* expression, principally in response to carbohydrate.

Fusions were constructed with the *ftf* upstream region and a promoterless CAT gene. These fusions were integrated, via allelic exchange, into a remote site of the *S. mutans* chromosome. The integration occurred within the *lac* operon, which is greater than 500 kb from the *ftf* locus and is not adjacent to any genes known to be involved in sucrose metabolism (12). This strategy had three major advantages over the Campbell-type integration used previously in *ftf* expression studies (14). (i) Since integration occurred at a site distant from the native *ftf* gene and promoter region, it ensured that *ftf* expression would not be altered. (ii) Integration of deletion derivatives would not be possible with a Campbell integration vector, since integration could regenerate the entire upstream promoter region. (iii) Analysis of promoter regions with significant sequence changes, such as occurred with V403, could produce aberrant results, depending upon the source of the homologous promoter region used for integration. The Campbell integration vector previously constructed to monitor *ftf* expression utilized the *ftf* upstream region from *S. mutans* GS5 (14). Use of this vector in this particular study would have created a hybrid *ftf* upstream region when integrated into V403. This would not have given a true picture of the unique expression pattern of this strain.

Results of deletion analysis of the region upstream of the *ftf* gene in GS5 revealed that the region involved in transcriptional level control was distinct from that mediating the response to carbohydrate. Deletion of sequences between the two inverted repeats resulted in a 40% drop in *ftf* expression, suggesting that this region was involved in control of the level of *ftf* transcription. The high level of Ftf produced by V403 was due to increased transcription associated with nucleotide sequence changes upstream of the *ftf* gene. Results of CAT assays with a hybrid promoter constructed with GS5 and V403 suggested that sequence changes downstream of the V403 −35 region were not responsible for elevating transcription. A C-T nucleotide change, occurring between the two inverted repeats, was the most likely upstream change which could be involved in affecting transcription. Since this change occurred in the region which appeared to control the transcription level

in GS5, it may be important in elevating *ftf* expression in V403. Site-directed mutagenesis will have to be done to determine the importance of nucleotide changes in this region. Sequence changes similar to those observed in the upstream region of V403 were found in only one additional fructan-hyperproducing strain, LM7. The absence of these changes in KPKS2 and MT6861 suggested that the sequence of the upstream region in these strains did not mediate the high level of *ftf* expression. It is possible that these strains possess an alteration in a regulatory factor which controls the level of *ftf* transcription.

The region involved in carbohydrate regulation was localized to within 135 bp of the ATG initiation codon and encompassed an inverted repeat. The sequence of this repeat was conserved in all of the fructan-hyperproducing strains examined by sequence analysis. Inverted repeat sequences in gram-positive bacteria appear to be targets for regulatory proteins involved in catabolite repression (48). These *cis* elements have been found in the lactose operons of *S. aureus* (28) and *S. mutans* (39) and the *citB* operon of *B. subtilis* (7). Inverted and direct repeats upstream of the *S. aureus* lactose operon were found to be important for repressor (LacR) binding, as well as binding of a catabolite repressor (28). These transcriptional regulators appeared to have overlapping target sites; however, their effects on transcription were clearly distinct. Mutants unable to synthesize the LacR repressor exhibited high-level constitutive expression of the *lac* operon; however, expression was still inducible by galactose and subject to catabolite repression by glucose (27, 28). This was reminiscent of the expression of the V403 *ftf*-CAT gene fusion in GS5 (V2227). Although *ftf* expression was elevated, it was still inducible by sucrose and appeared to be repressed by glucose. In contrast to that in the *S. aureus* LacR mutants, the high level of *ftf* expression in V403 appears to be due to sequence changes upstream of the *ftf* gene rather than an alteration in a transcriptional regulator. As was suggested previously, the opposite may be true for fructan-hyperproducing strains KPKS2 and MT6861 because of the absence of sequence changes similar to those observed in the V403 upstream region. LacR appears to function mainly as a negative regulator of the level of *lac* transcription. However, *ftf* transcription in *S. mutans* may involve activation since deletion of the region between the inverted repeats results in decreased expression. This region was distinct from that involved in the response to carbohydrate, suggesting that as in *S. aureus lac* operon expression, *ftf* expression involves more than one regulatory mechanism.

S. mutans ftf expression is not catabolite repressed in the true sense, in that glucose does not completely abolish expression. However, it is clear that glucose and other rapidly metabolizable PTS sugars have a repressive effect on expression. This repressive mechanism did not appear to be operating in V403, since this strain exhibited uncontrolled *ftf* expression in the presence of glucose. The fact that the V403 *ftf*-CAT gene fusion in GS5 (V2227) was regulated by the presence of carbohydrate indicated that this regulation was mediated by a *trans*-acting factor(s). It is tempting to speculate that the inverted repeat region is the binding site for a dimeric regulatory protein involved in the modulation of *ftf* expression in response to carbohydrate. This *trans*-acting factor(s) may be altered in V403, resulting in the inability of this organism to regulate *ftf* expression.

Deletion of the inverted repeat in the 610 PCR fusion abolished expression, suggesting that the proposed -35 and -10 sequences were not sufficient for *ftf* expression in *S. mutans*. These sequences may not represent the true promoter, or the inverted repeat, which was deleted in this construct, was necessary for efficient expression. This fusion was able to function in *E. coli*, since this region could direct CAT expression when placed upstream of the CAT gene in pMH109. It is possible that the expression necessary to generate chloramphenicol resistance in *E. coli* was less than that needed to detect CAT activity by an enzymatic assay in *S. mutans*. In addition, the CAT gene was present in multiple copies in *E. coli*, whereas only a single copy was present in the *S. mutans* genome. S1 nuclease fragment protection studies are necessary to define the actual *ftf* promoter and start site of transcription. Difficulties associated with lysis of viridans group streptococci have made *S. mutans* mRNA isolation and analysis difficult.

Carbohydrate regulation of gene expression has been shown to occur through the PTS. The phosphorylated state of the EIIA component of the gram-negative glucose PTS is able to regulate gene expression via inducer exclusion and its effect on cyclic AMP levels (29, 38). Although cyclic AMP has not been found in *S. mutans*, a type of inducer exclusion has been shown to occur (5, 17, 19, 46). Glucose is able to inhibit the uptake of various PTS sugars, which effectively prevents induction of the genes associated with the catabolism of these sugars. This preferential translocation of one sugar over another may involve differential rates of histidine phosphorylation of HPr, a PTS phosphocarrier protein (35, 36). There is also evidence of competition between EII proteins for a common EIII phosphoryl donor, as occurs with EII^{glc} and EII^{scr} in *B. subtilis* (49). Competition for a common phosphoryl donor appears to be important for PTS regulation in gram-positive organisms. The decline in sucrose-induced expression over time seen with GS5 was interesting in that excess sucrose inducer was present throughout the time course of the assay. Cells grown with sucrose as the sole carbohydrate eventually encounter both glucose and fructose in the medium as the result of extracellular Gtf and Ftf activities. These carbohydrates, as well as several additional PTS sugars, had a repressive effect on sucrose-induced *ftf* expression. This effect was concomitant with the utilization of the added sugars, as was evidenced by a growth rate increase. It is possible that the proteins involved in sucrose transport cannot compete as effectively for the low levels of HPr (His-P) in cells metabolizing a second PTS carbohydrate, and this results in inhibition of the uptake of inducer (sucrose). To determine whether the uptake of sucrose affected *ftf* expression, the *scrA* gene, which encodes the EII of the sucrose PTS, was inactivated. *scrA* mutants were still able to induce *ftf* expression in the presence of sucrose. This result indicates the lack of involvement of the major sucrose uptake pathway in the control of *ftf* expression, at least under the growth conditions used in this study. However, it does not rule out the possibility of regulation via sucrose uptake, since non-PTS transport mechanisms exist, as well as low-affinity PTS-mediated uptake through the trehalose PTS (30, 46). The results suggest a model of the regulatory control of *ftf* expression. Growth of cells in the presence of glucose results in a low, constant level of *ftf* expression. This low level of expression is needed in the environment of the oral cavity to maintain a level of extracellular Ftf which can immediately begin fructan polymer synthesis as soon as sucrose is available. Since the supply of sucrose in the oral cavity is limited and unpredictable, an immediate response, in terms of polymer production, would result in increased virulence. It is advantageous, from a pathogenesis standpoint, that the presence of sucrose would also induce virulence genes, such as *ftf*, which require sucrose as a substrate. Induction of *ftf* by sucrose occurs for a period of time and then gradually declines as extracellular glucose and fructose accumulate through the action of both Gtf and Ftf.

This provides an effective means of decreasing *ftf* expression once a sufficient amount of the enzyme is produced.

In addition to the repressive effects on sucrose-induced *ftf* expression, certain PTS carbohydrates were able to affect expression in glucose-grown cells as well. A possible explanation for these results involves PTS specificity for these particular carbohydrates. Glucose and mannose share a common PTS, so addition of these sugars to cells growing in glucose would probably not increase the amount of sugar taken into the cells, since the amount of glucose already present is at a saturating level. Maltose, trehalose, and fructose do not share a PTS with glucose; therefore, addition of these carbohydrates to glucose-grown cells results in an increase in the overall amount of sugar entering the cells. This may cause the decrease in *ftf* expression seen in glucose-grown cells upon addition of these particular carbohydrates.

A unique mechanism of transcriptional regulation associated with catabolite repression has been documented in gram-positive organisms. This regulation involves the phosphorylation of HPr on a serine residue through the action of HPr serine kinase, an enzyme found only in gram-positive bacteria (32). Recent evidence suggests that HPr (Ser-P) is involved in regulation of gene expression, since *B. subtilis* HPr mutants, unable to undergo serine phosphorylation, do not exhibit catabolite repression of the gluconate operon (34). Also, heterofermentative lactobacilli, which lack a functional PTS, still possess HPr and HPr serine kinase, suggesting that HPr serves a function other than sugar transport (33). In *S. mutans*, repression of *ftf* expression did not appear to be specific for carbohydrates transported via a particular PTS. All of the PTS carbohydrates used in this study were able to inhibit sucrose-induced expression. These results suggest that a protein(s) common to all of the PTSs, such as HPr, is involved in catabolite repression of the *ftf* gene. Although *ftf* expression in V403 is not repressed by PTS carbohydrates, catabolite repression still occurs in this strain. V403 exhibits diauxic growth in mixtures of glucose and lactose, suggesting that glucose represses expression of the *lac* operon (unpublished observation). The general mechanism of catabolite repression may be operative in V403, but the specific *ftf* catabolite repressor protein may not be functional. As Stewart (48) has suggested, the variety of *cis*-element sequences involved in catabolite repression indicates that each operon or gene has its own specific catabolite repressor protein. A global mechanism may exist for the generation of a catabolite-repressing signal which is then transmitted to a variety of specific repressor proteins. The *gtfB* gene of *S. mutans* is regulated by carbohydrate in a manner similar to that of *ftf* (14, 15). However, an inverted repeat analogous to that involved in *ftf* expression was not observed upstream of *gtfB*. Although *ftf* and *gtfB* may respond similarly to carbohydrate, the regulatory protein(s) involved may be specific for each gene.

This study has demonstrated the complexity of *ftf* gene regulation in *S. mutans*. Both *cis*-acting promoter elements and a *trans*-acting factor(s) appear to play a role in modulating expression of this gene. Carbohydrates appear to serve as the major regulatory signals for control of *ftf* transcription; therefore, involvement of the PTS in this regulation would not be surprising. The PTS provides a means of detecting the types and concentrations of various carbohydrates in the external environment. For *S. mutans*, this system would enable it to respond to carbohydrate fluctuations in the oral cavity and adjust the expression of its virulence-associated genes accordingly, to optimize its virulence potential.

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