Cloning of a *Streptococcus mutans* Glucosyltransferase Gene Coding for Insoluble Glucan Synthesis

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The gtfB gene coding for a glucosyltransferase (GTF) activity of *Streptococcus mutans* GS-5 was isolated on a 15.4-kilobase DNA fragment by using a λL47.1 gene library. The activity was catalyzed by gene products of 150 and 145 kilodaltons which reacted with antibodies directed against both soluble and insoluble glucan-synthesizing GTFs. The enzyme present in crude *Escherichia coli* extracts synthesized both soluble and insoluble glucans. The enzyme was partially purified from lysates of the ADS-76 clone and synthesized both types of glucans in a primer-independent fashion. In addition, the purified enzyme exhibited a pI of approximately 5.0. Southern blot analysis indicated that the cloned GTF gene represented a contiguous nucleotide sequence on the strain GS-5 chromosome. Furthermore, evidence for the existence of a distinct gene sharing partial homology with gtfB was also obtained. The gtfB gene was subcloned into plasmid pACYC184 into *E. coli* and exhibited GTF activity when carried on GS-5 inserts as small as 5 kilobases. The approximate location of the GTF promoter and the direction of gene transcription were also determined. The cloned enzyme was not secreted through the cytoplasmic membrane of *E. coli*, since most of the activity was found in the cytoplasm and, in lesser amounts, associated with the cytoplasmic membrane. The gtfB gene was insertional inactivated by introducing a gene fragment coding for erythromycin resistance into the GTF coding region. After transformation of strain GS-5 with the altered gene, transformants defective in insoluble glucan synthesis were identified. These results indicate that the gtfB gene codes for a GTF involved in insoluble glucan synthesis in strain GS-5.

The conversion of dietary sucrose to glucan polymers is an important cariogenic property of *Streptococcus mutans* (5). In particular, insoluble glucan production has been shown to greatly increase the ability of these organisms to colonize tooth surfaces. The results from several laboratories have suggested that multiple glucosyltransferases (GTFs) are involved in the synthesis of these polymers (3, 4, 14). Two separate GTFs have been identified in *S. mutans* serotype d (4, 10) and c strains (9, 14, 20), and three enzymes have been characterized from a serotype g strain (30). In addition, recent results have suggested that a lower-molecular-weight GTF, gtfA (26), exists in many strains of these organisms and may also play a role in glucan synthesis (25). Taken together, the results from these investigations suggested that one of the GTFs, termed GTF-S or dextranucrase, may be responsible for synthesizing the primer required for another enzyme, GTF-I or mutansynthetase, to produce an insoluble glucan polymer.

Serotype c strains, which are the predominant *S. mutans* serotype isolated from the human oral cavity of many populations (1), may synthesize insoluble glucan by two related mechanisms. Like other serotypes, these organisms can produce insoluble glucan by the combined action of the previously characterized GTF-S and GTF-I (9, 14, 20). In addition, under conditions of aggregation (14, 21), the GTF-S of these organisms can also catalyze insoluble glucan synthesis. However, the relative contributions of these two mechanisms to insoluble glucan synthesis under conditions existing in the human oral cavity is still unclear. One approach toward resolving this question would be to produce specific mutants in the GTF-S and GTF-I genes of these organisms. Although mutants defective in the latter activity were previously isolated (13, 24), specific GTF-S mutants were not. In addition, the former mutants have not been extensively characterized to determine whether or not single mutations are responsible for the observed phenotypic traits. One strategy to produce such mutants involved initially isolating the genes for the respective enzymes by recombinant DNA techniques for subsequent in vitro mutagenesis, followed by reintroduction of the altered genes back into serotype c strains by transformation (22). Recently, several laboratories have reported the isolation of *S. mutans* genes which express GTF activities (6; Y. Abiko, and H. Takiguchi, J. Dent. Res. 64:737, 1985; H. Aoki, and H. K. Kuramitsu, J. Dent. Res. 64[special issue]:258, 1985). This report describes the isolation and characterization of a GTF gene (designated gtfB) isolated from a serotype c strain GS-5.

**MATERIALS AND METHODS**

**Microorganisms.** *S. mutans* GS-5 was maintained and grown routinely as previously described (13). *Escherichia coli* C600 and C600(P2) were kindly provided by R. Russell, Royal College of Surgeons, Downe, England, and strain WL66 was obtained from Amersham Corp., Arlington Hts., Ill. L47.1 DNA was also obtained from Amersham Corp. *E. coli* strains were grown in L broth (10 g of tryptone [Difco Laboratories, Detroit, Mich.], 5 g of yeast extract (Difco), 5 g of NaCl per liter) with the appropriate antibiotics. Phage stocks were prepared on LB agar plates in soft agar overlays (16).

**DNA manipulations.** Chromosomal DNA from *S. mutans* GS-5 was purified essentially as previously described (8: Barletta and Curtiss, personal communication), extracted twice with phenol, precipitated with ethanol (2x), and dialyzed against TE buffer (10 mM Tris hydrochloride, 1.0 mM EDTA).
FIG. 1. Isolation of the gtfB gene inactivated with an Em+ gene. - Plasmid vectors; B, GS-5 chromosomal DNA; pVA891. The relevant restriction sites are B (BamHI), E (EcoRI), H (HindIII), and S (Sall).

mM EDTA (pH 8.0)). Phage DNA was purified from individual clones as previously described (16). Plasmid DNA was isolated by the alkaline-sodium dodecyl sulfate procedure (16). Restriction digestion by endonucleases and ligations was done by following the directions of the suppliers (Bethesda Research Laboratories, Inc., Gaithersburg, Md., and Amersham Corp.).

Construction of the S. mutans clone bank. Strain GS-5 DNA was partially digested with Sau3AI and mixed with λA47.1 DNA which was completely digested with BamHI in a 1:1 ratio (total DNA concentration was 300 μg/ml). The DNA mixtures were phenol extracted, washed with ether, and ethanol precipitated. The precipitates were dissolved with TE buffer and ligated at 12°C for 16 h in the presence of T4 DNA ligase. After ligation, the mixtures were extracted with five cycles of phenol extraction, washed with ether, and precipitated with ethanol.

In vitro packaging of the ligation mixtures was done with a packaging (Promega, Madison, Wis.) system by the directions of the supplier. Samples of the packaging mixture were then incubated with E. coli C600(P2) cells for 20 min at 37°C in 10 mM MgSO4. The samples were mixed with 3.0 ml of LB top agar and plated on LB agar plates at 37°C for 8 to 16 h. The resultant plaques were harvested by scraping each plate with 3.0 ml of dilution buffer (10 mM Tris, 10 mM MgSO4 [pH 7.5]). The clone bank was stored at 4°C and was used to infect C600 cells for subsequent screening.

Screening of the GS-5 clone bank for GTF clones. The clone bank was initially screened for GTF clones by examining plaques for sucrose-hydrolyzing (sucrase) activity essentially as previously described (6). Samples of the clone bank (10 μl) were mixed with 0.10 ml of mid-log-phase C600 cells and incubated at 37°C for 15 min in the presence of 10 mM MgSO4. After the addition of 3.0 ml of LB top agar containing 0.1% triphenyl tetrazolium chloride, the infected cells were overlayed onto LB-1% sucrose agar plates. After incubation for 16 h, sucrase-positive clones could be readily detected by the appearance of a red zone surrounding the positive plaques.

The clone bank was also screened for plaques that expressed antigens which react with antibody directed against a partially purified GS-5 GTF fraction, GTF-A (11). Plaques developed on LB agar plates were blotted to nitrocellulose disks, incubated with anti-GTF-A, washed, and developed with the peroxidase-chloronaphthol system (28).

Transformation of E. coli cells. Plasmids and λA47.1 DNA containing the GTF inserts were cleaved with the appropriate restriction enzyme, ligated, and transformed into C600 cells as previously described (16). Transformants were selected on LB agar plates containing the appropriate antibiotic: chloramphenicol (30 μg/ml), tetracycline, (12.5 μg/ml), or erythromycin (Em) (200 μg/ml).

Transformation of S. mutans. Transformation of strain GS-5 was carried out as previously described (24) by using the appropriate plasmid constructs (2.5 μg/ml). The transformants were isolated on mitis salivarius-em (10 μg/ml) agar plates.

Enzyme assays. E. coli extracts were prepared and fractionated for isolation of the cytoplasmic, cytoplasmic membrane, and periplasmic fractions as previously described (7).

Sucrase activity was determined as previously described (17) in a standard incubation mixture containing 100 mM potassium phosphate buffer (PB) (pH 6.0), 2% sucrose, 0.2% saline-NaNO3, enzyme, and water in a total volume of 0.1 ml. The reaction mixtures were incubated for 30 min at 37°C and analyzed for reducing sugar formation.

GTF activity was determined as previously described (12) by using sucrose [U-14C]glucose, except that the reaction mixtures were incubated for 1 to 16 h at 37°C. Longer incubation periods (16 h) were necessary for some fractions to maximize activity (14). Insoluble, soluble, and total glucan formation were also determined as previously reported (14).

Gel electrophoresis. DNA was analyzed after electrophoresis on horizontal 0.7% agarose gels with TBE buffer (89 mM Tris hydrochloride, 89 mM boric acid, 2.5 mM EDTA [pH 8.3]). The gels were stained in ethidium bromide, washed, and photographed as recently described (29).

Proteins were analyzed on 8% polyacrylamide gels containing sodium dodecyl sulfate as described earlier (32). The gels were either stained with Coomassie blue for detecting

FIG. 2. SDS-PAGE analysis of λDS-76 lysates. (A) Coomassie blue staining. (B) Western blotting with anti-GTF-S. (C) Activity displayed after incubation with sucrose. Lane 1, Concentrated chromatofocused GTF fraction; lane 2, λDS-76 crude lysate; lane 3, crude λA47.1 lysate.
proteins and immunoblotted to nitrocellulose followed by reaction with antibody and peroxidase-chloronaphthol (28), or analyzed for GTF activity as previously described by Russell (27), with subsequent periodate Schiff base used to stain both soluble and insoluble glucan synthesizing activities (19).

**Southern blot analysis.** Fragment transfer to nitrocellulose paper, hybridization with 32P-labeled probes, washing, and film development were carried out as previously described (16) except that 50% formamide buffers were utilized and the filters were washed at 37°C (approximately 15°C below the melting temperature for bacterial DNA [18]). 32P-labeled probes were constructed after nick translation with the Klenow fragment (Pharmacia, Inc., Piscataway, N.J.) using [α-32P]dCTP (Amersham Corp.) and [α-32P]dCTP (Amersham Corp., Piscataway, N.J.) using [α-32P]dCTP (Amersham Corp.) and [α-32P]dCTP (Amersham Corp.) as recommended by the supplier. The gtfB fragments were subcloned into vector pUC9, and the resultant plasmids were used as probes.

**In vitro inactivation of the gtfB gene.** The gtfB gene contained on a 4.5-kilobase (kb) PstI-SalI fragment in vector pUC9 (designated pTS20) was digested with BamHI, and the internal 1.6-kb BamHI fragment was isolated after agarose gel electrophoresis (Fig. 1). The fragment was ligated to BamHI-cleaved pUC9dH (pUC9 with the HindIII site removed after HindIII cleavage, blunt ending with the Klenow fragment, then ligation) to produce plasmid pTS60.

The 1.8-kb fragment coding for erythromycin resistance (Em') was isolated from agarose gels after BamHI cleavage of plasmid pTS19E. This latter plasmid was constructed from the transfer of the blunt-ended Em' fragment derived from pVAB91 (15) into pUC19. The Em' fragment was filled in with the Klenow fragment and ligated into the compatible site produced in plasmid pTS60 after HindIII cleavage and subsequent treatment with the Klenow fragment. The resultant Ap' Em' plasmid pTS61 was used as the source of the in vitro-inactivated gtfB gene. The linear 3.4-kb gtfB gene fragment with an Em' insert was isolated from agarose gels after complete digestion of pTS61 with BamHI.

**Purification of cloned GTF activity.** Lysates (1.5 liters) of C600 cells infected with XDS-76 were prepared as described recently (28). After lysis, cellular debris was removed by centrifugation at 6,000 × g for 10 min at 4°C. The supernatant fluids were then treated with ammonium sulfate (516 g/liter) and allowed to stand for 18 h at 4°C. The resultant precipitate was collected by centrifugation at 6,000 × g for 10 min and dissolved in 20 mM PB buffer. The dissolved sample was dialyzed against PB buffer and applied to a hydroxylapatite (Bio-Gel HTP hydroxylapatite; Bio-Rad Laboratories, Richmond, Calif.) column previously equilibrated with PB buffer and eluted with a linear 20 mM–1.0 M PB buffer gradient and assayed for sucrase and GTF activities (12). The fractions containing significant activity were pooled, concentrated through an Amicon UM-10 ultrafilter (Amicon Corp., Lexington, Mass.), and dialyzed against 20 mM PB buffer.

The concentrated enzyme sample was next resolved on a DEAE-Bio-Gel A (Bio-Rad) column with a linear gradient consisting of PB buffer–1.0 M NaCl–PB buffer. The fractions containing GTF activity were pooled, concentrated through an ultrafilter, dialyzed against 25 mM imidazole-hydrochloride buffer (pH 7.4), and applied to a chromatofocusing column (Pharmacia). The resin, previously equilibrated with imidazole-hydrochloride buffer, was washed with a 180-ml Polybuffer 74 linear gradient as recommended by the manufacturer. The fractions containing GTF activity were pooled, concentrated, and dialyzed against 20 mM PB buffer.

### RESULTS

**Isolation of a GTF clone.** The XL47.1 clone bank was initially screened for the presence of sucrase-positive clones by growing C600 infected cells in soft agar overlays containing sucrose and triphenyl tetrazolium chloride. In addition, the clone bank was propagated in C600 cells grown in minimal medium containing sucrose and low levels of glucose to detect sucrase-positive clones (6). By both screening tests, a large number of sucrase-positive clones were isolated. Most of these appeared to express sucrase-6-

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**TABLE 1. Properties of cloned GTF activity**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>GTF activity* (cpm/16 h per ml of enzyme)</th>
<th>Soluble glucan</th>
<th>Insoluble glucan</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without dextran</td>
<td>With dextran</td>
<td>Without dextran</td>
</tr>
<tr>
<td>Crude XDS-76 lysate</td>
<td>2.870</td>
<td>4.890</td>
<td>2.370</td>
</tr>
<tr>
<td>Crude MH76 extract</td>
<td>3.500</td>
<td>38.000</td>
<td>9.050</td>
</tr>
<tr>
<td>Purified enzymeb</td>
<td>2.500</td>
<td>8.900</td>
<td>2.300</td>
</tr>
</tbody>
</table>

* GTF activity was measured by using sucrose-[U-14C] glucose.  
b The pooled concentrated chromatofocused enzyme was used as the purified enzyme.
phosphate hydrolase activity, and one has been further characterized (M. Hayakawa, H. Aoki, and H. K. Kuramitsu, submitted for publication). Several also coded for gtfA activity (26) and were not further investigated. In addition, two clones expressed fructosyltransferase activity and have been recently characterized (29). However, none of the sucrase-positive clones expressed GTF activity when assayed by the standard radioactive procedure.

Since it was possible that a GTF-positive clone would express only weak activity which might not be detectable by the sucrose screening assay, an immunoblot screening assay was used to screen the clone bank. Therefore, antibody against a partially purified GTF fraction (11) which synthesizes both soluble and insoluble glucans was used to screen the clone bank. Approximately 20,000 plaques were screened with anti-GTF-A, and 193 positive clones were identified. Lysates of each positive clone were assayed directly for GTF activity. Only one active clone, ADS-76, was identified.

Characterization of ADS-76. When sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels was used to observe GTF activity, two white insoluble glucan bands were present in ADS-76 lysates and none were observed in control λL47.1 lysates (Fig. 2C). Since antibody against purified GTF-S preparations from strain GS-5 was available (11), it seemed possible to conveniently discriminate between GTF-S and GTF-I by ADS-76 DNA. The antibody reacted with a single major protein band of 155 kilodaltons (kDa) after Western blot analysis of crude GS-5 culture fluids (data not shown). After SDS-PAGE and Western blotting, it was possible to demonstrate that ADS-76 protein bands of molecular weights 150 and 145 kDa reacted with anti-GTF-S sera and that comparable bands were not present in crude lysates of λL47.1-infected cells (Fig. 2). However, antisera directed against a purified GTF-I from another serotype c strain Ingbritt (kindly provided by H. Mukasa, National Defense Medical College, Tokorozawa, Japan) also reacted with the same GTF bands from ADS-76 and the GS-5 culture fluids (data not shown).

When the ADS-76 lysate was assayed for GTF activity, it was observed that both soluble and insoluble glucans were synthesized (Table 1). As previously determined (12), the soluble glucan was degraded by dextranase, whereas the insoluble product was quite resistant to the hydrolase (data not shown). Neither soluble nor insoluble glucan synthesis required the addition of exogenous dextran.

Purification and characterization of the GTF activity. Crude lysates of ADS-76 were subjected to protein purification after ammonium sulfate precipitation, hydroxylapatite chromatography, and DEAE-Bio-Gel A anion exchange chromatography followed by chromatofocusing. The resultant GTF activity was resolved from most of the contaminant proteins, but several lower-molecular-weight proteins were still present in the final preparation (Fig. 2A). This latter fraction represented an approximate 25-fold increase in the specific activity relative to the crude lysate. It was of interest that the apparent pi of the purified activity was approximately 5.0, whereas a minor fraction eluted through the column with an apparent neutral or alkaline pi (Fig. 3). Like the crude GTF preparations, the purified GTF fraction synthesized primer-independent soluble and insoluble glucans (Table 1). However, in contrast to the crude enzyme preparations, the presence of the primer somewhat inhibited insoluble glucan synthesis by the purified enzyme. Finally, SDS-PAGE analysis of the purified GTF revealed the same active species (150 and 145 kDa) as observed in ADS-76 lysates (Fig. 2). However, the 150-kDa active band was very weak in the purified enzyme preparation.

Restriction mapping of the ADS-76 insert. The DNA from ADS-76 was purified from C600 lysates and subjected to restriction mapping (Fig. 4). After the construction of a restriction map, it could be determined that ADS-76 contained a 15.4-kb insert from strain GS-5. Since the GTF must be coded by a gene of at least 4.1 kb (corresponding to a 150-kDa protein), it was of interest to subclone the gene to further characterize the coding region.

Subcloning of the GTF gene. Based on the restriction map of the ADS-76 insert, several strategies for subcloning the GTF gene were evaluated. It was observed that an active subclone carrying plasmid pMH76 could be isolated by cleaving pACYC184 and ADS-76 DNA with CclI followed by ligation and transformation into C600. The resulting Cm′ Te′ transformants then were directly assayed for sucrase activity, and five active subclones were isolated. One of these, MH76, was chosen for further analysis; restriction mapping of its chimeric plasmid revealed that pMH76 carried a 7.7-kb insert from ADS-76 (Fig. 4). Furthermore, subsequent analysis revealed that the entire insert originated...
from the strain GS-5 fragment. This subclone also synthesized large amounts of visibly detectable insoluble glucan when crude extracts were incubated with sucrose. It was also of interest that MH76 grew very slowly in media containing sucrose, suggesting that the synthesis of glucan by the subclone is deleterious to the cells.

In order to isolate a GTF active subclone carrying the smallest GS-5 insert, λDS-76 DNA was subjected to partial Sau3A1 digestion and ligated to BamHI-cleaved pACYC184. The resultant Cm^R Tc^R transformants were screened for their ability to grow on LB-sucrose agar, and 12 colonies which grew poorly in this medium were identified. Each displayed GTF activity when assayed enzymatically and displayed the same GTF activity bands after SDS-PAGE (data not shown). Restriction mapping revealed that each carried a PstI-BamHI fragment in common (Fig. 4). The inserts in the GTF positive subclones ranged in size from 4.5 to 9.2 kb. In addition, the GS-5 insert was carried in both orientations relative to the vector (exemplified by pTss and pTS18; Fig. 4), suggesting that the GTF promoter was present on the inserts.

Subsequent deletion analysis suggested that the GTF promoter was located near the common PstI site of the inserts, since relatively small deletions in this area resulted in complete loss of enzymatic activity and the disappearance of GTF polypeptide bands (assayed after Western blotting of SDS-PAGE gels with anti-GTF-S). In contrast, Bal31 nuclelease digestion at the opposite end of the insert led to truncated GTF polypeptides with varying degrees of GTF activity (T. Shiroza and H. K. Kuramitsu, manuscript in preparation). These results strongly suggested that the cloned gene was transcribed from the PstI region toward the internal EcoRI site of the common inserts (Fig. 4).

**Southern blot analysis.** In order to demonstrate that the cloned gtfB gene represented a contiguous fragment of DNA from the GS-5 chromosome as well as the number of copies of the gene in strain GS-5, Southern blot analysis was carried out. Chromosomal DNA was digested to completion with several restriction endonucleases, and the fragments were transferred to nitrocellulose paper. Complete digestion with each enzyme was confirmed by the demonstration that a probe made from the gtfA plasmid pYA601 (26) hybridized to only one fragment of chromosomal DNA cleaved with BamHI, EcoRI, PstI, ClaI, and to two fragments after cleavage with HindIII (data not shown). In addition, no hybridization between gtfA and fragments corresponding to portions of the gtfB gene could be detected.

When a 32P-labeled probe made from the 4.5-kb fragment containing the entire gtfB gene, (Fig. 5A), probe a) was hybridized to fragments produced from cleavage with a variety of enzymes, positive bands compatible with the restriction map of gtfB were detected (i.e., 1.6- and 1.9-kb HindIII fragments hybridized with the probe; Fig. 5B). The 6.6-kb HindIII fragment represents the carboxyl terminal portion of the gtfB gene and downstream sequences on the GS-5 chromosome, since probe b but not the amino terminal probe c also reacted with the same size fragment. In addition, in each case an additional positive fragment was detected (4.5-kb HindIII, 3.2-kb BamHI, 4.6-kb EcoI, and 7.0-kb PstI fragments). This indicated that either a portion of the gtfB gene contained a "scrambled" fragment (a noncontiguous piece of chromosomal DNA inserted during ligation of the GS-5 fragments into the λA47.1 vector) or that another homologous gene existed on the GS-5 chromosome. That the latter was responsible for the additional hybridizable fragment was indicated by the utilization of additional probes from different portions of the gtfB gene. When probes b and c (Fig. 5A), composed of the 1.6-kb BamHI-BamHI and the 2.0-kb PstI-EcoRI fragments, respectively, were hybridized to the restriction fragments, one additional band in addition to those compatible with the restriction maps was again detected (Fig. 5C and D). Therefore, a scrambled fragment did not exist within the 3.8-kb cloned region encompassing the b plus c regions. If such a fragment existed, probe a and either probe b or c, but not both probes b and c, would detect this extra band. A noncontiguous fragment of chromosomal DNA was also not present in the remaining portion of the gtfB gene (not encompassed by probes b and c), since all three probes hybridized with only

Table 2. Localization of GTF activity in E. coli

<table>
<thead>
<tr>
<th>Cell compartment</th>
<th>Relative distribution (%)</th>
</tr>
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<tbody>
<tr>
<td>Periplasm</td>
<td>1.0 - 43</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>91 - 57</td>
</tr>
<tr>
<td>Cytoplasmic membrane</td>
<td>8 - 0</td>
</tr>
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</table>

*e Cell extracts of MH76 were prepared, fractionated, and assayed as described in Materials and Methods.

FIG. 5. Southern blot analysis of GS-5 chromosomal DNA. (A) Restriction map of λDS-76 and location of probe fragments. Restriction enzymes are as described in the legend to Fig. 3. Approximate location of the gtfB gene. Probe a, encompassing the entire gtfB gene, was constructed after Bal31 deletion of the PstI-Sall fragment containing the gtfB gene incorporated into vector pUC9; probe b was obtained by cloning the PstI-EcoRI fragment into pUC9; probe c was isolated after ligation of the 1.6-kb BamHI fragment into pUC9. (B) Hybridization with probe a. (C) Hybridization with probe b. (D) Hybridization with probe c. Chromosomal DNA was cleaved with HindIII (lane 1), BamHI (lane 2), EcoRI (lane 3), and PstI (lane 4). Arrows, Fragments generated from the gene partially homologous to gtfB (for the PstI fragments, it was not possible to determine which of the two fragments originated from the homologous gene). Molecular size markers (kilobases) are indicated at the left.
numbers fructosyltransferase. However, the size difference in the GS-5 culture fluids in the GS-5 culture fluids was caused by fructosyltransferase. Numbers on right and left are molecular size markers, in kilodaltons.

two PstI fragments. If such scrambling had occurred at the COOH-terminal region of the gtfB gene (deduced from the localization of the promoter as described above), probe a would have hybridized with three fragments: the gtfB fragment, the homologous gene fragment, and the noncontiguous fragment from the GS-5 chromosome.

Location of GTF expressed in E. coli. Since the GTFs are synthesized as extracellular enzymes in GS-5, it was of interest to determine the cellular location of the enzyme in subclone MH76. Fractionation of the cells revealed that essentially none of the GTF activity traversed the E. coli membrane and localized in the periplasmic space (Table 2). Most of the activity was associated with the cytoplasmic compartment (60%), whereas a large amount was also found associated with the cell membrane (38%). The synthesis of insoluble glucan by the membrane-associated GTF might explain why all of the subclones containing intact gtfB genes grew poorly on sucrose-containing media.

Activity staining of subclone extracts. When extracts of MH76 were subjected to SDS-PAGE followed by activity staining, it was observed that two bands of activity corresponding to the same molecular weights (150 and 145 kDa) observed in ADS-76 lysates could be detected (Fig. 6). In addition, a third band of activity corresponding to a molecular size of approximately 135 kDa was observed in the MH76 extracts and was barely detectable in the ADS-76 lysates. However, this latter lower-molecular-weight enzyme could be readily detected after periodate Schiff base staining. Likewise, all of the twelve GTF active subclones (pTS3, pTS18, etc.) displayed the same activity bands as did MH76. No difference in the number and intensity of the GTF bands was noted in the presence or absence of dextran T10 in the reaction mixtures. Concentrated culture fluids of strain GS-5 displayed a single major band of GTF activity of approximately 155 kDa and a barely detectable band at 135 kDa (Fig. 6). Inclusion of the protease inhibitor phenylmethylsulfonyl fluoride did not affect this pattern. These results suggested that the product of the gtfB gene may have undergone different degrees of proteolysis in E. coli extracts (MH76 and ADS-76), compared with the GS-5 extracellular compartment.

Insertional inactivation of S. mutans GS-5 GTF activity. Since the cloned enzyme synthesized both soluble and insoluble glucans and reacted with both anti-GTF-S and anti-GTF-I, it was not clear which enzyme was coded by the gtfB gene. To unequivocally identify the gene product, the cloned gene was inactivated after the insertion of an Em' gene fragment to specifically inactivate the cloned gene product in strain GS-5 after subsequent transformation.

The linearized gtfB gene containing the Em' gene substituting for an internal HindIII fragment derived from pTS61 (Fig. 1) was transformed into strain GS-5. Approximately 98% of the resultant Em' transformants displayed the smooth colonial morphology on mitis salivarius agar plates that is characteristic of mutants defective in insoluble glucan synthesis (13). When these transformants were assayed directly for GTF activity and compared with the wild-type activities, it was observed that the transformants displayed markedly reduced insoluble glucan synthesizing activity. One typical transformant, LN61, displayed only 28% of the insoluble glucan synthetic activity of strain GS-5 (Table 3). This activity was not increased by the addition of primer dextran T10 to the assay mixtures. In contrast, the smooth transformant synthesized higher levels of soluble glucan, compared with the parental organism. The smooth transformants also displayed reduced sucrose-dependent colonization of glass surfaces when assayed in an in vitro system. Finally, when chromosomal DNA was extracted from LN61 and transformed into parental GS-5, more than 99% of the resultant Em' transformants displayed the smooth colonial morphology on mitis salivarius agar plates. This indicated that this latter property was dependent upon the insertion of the Em' gene into the chromosomal GTF-I gene.

DISCUSSION

The present results indicate that a gene coding for a GTF-I activity synthesizing insoluble glucan in strain GS-5 has been cloned. This conclusion is contrary to that of our previous report regarding this gene derived from earlier data (H. Aoki and H. K. Kuramitsu, J. Dent. Res. 64:258, 1985), which suggested that the gtfB gene coded for GTF-S activity. This latter conclusion was based primarily on the observations that the cloned gene product reacted with anti-GTF-S sera and that the purified cloned enzyme synthesized water-soluble glucans. However, the results of the present com-

<table>
<thead>
<tr>
<th>Strain</th>
<th>Soluble glucan</th>
<th>Insoluble glucan</th>
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<tbody>
<tr>
<td></td>
<td>Without dextran T10</td>
<td>With dextran T10</td>
</tr>
<tr>
<td>GS-5</td>
<td>370</td>
<td>509</td>
</tr>
<tr>
<td>LN61</td>
<td>1,511</td>
<td>1,189</td>
</tr>
</tbody>
</table>

* Parental GS-5 and Em' smooth transformant LN61 were grown to mid-log phase in 5.0 ml of Todd Hewitt broth. After centrifugation, 0.10-ml samples were assayed for GTF activity as described in Materials and Methods.
munication demonstrate that the cloned enzyme also reacted with antisera directed against a purified serotype c GTF-I enzyme (20). Either the GTF-S and GTF-I enzymes of strain GS-5 share sufficient cross-reactivity to be detected after Western blotting but not by immunodiffusion (20), or one of the two enzymes used to prepare the antibodies might be contaminated with small amounts of the other enzyme. Furthermore, the pl of the purified cloned enzyme (pH 5.0) resembles that estimated for the GTF-I activity of strain GS-5 and is quite distinct from the alkaline pl estimated for the corresponding GTF-S activity (14).

The observation that the gtfB gene product synthesized both water-soluble and insoluble glucans did not allow differentiation between a GTF-S and GTF-I gene product. Recent results have indicated that serotype c GTF-S enzymes can also synthesize insoluble glucan under conditions of enzyme aggregation (14, 21). In addition, the purified GTF-I from another serotype c strain synthesizes both soluble and insoluble glucans (20). Therefore, the nature of the glucan product synthesized by the cloned gene enzyme alone could not be used unequivocally to identify the nature of the enzyme.

In addition, crude extracts of E. coli MH76 containing the subcloned gene produced large amounts of insoluble glucan relative to soluble glucan. It is not clear why the purified enzyme and crude phage lysates synthesized lower ratios of insoluble glucan/soluble glucan relative to the crude subclone extracts (Table 1). It may be possible that the crude and purified ADS-76 GTF-I may be altered in a different way than is the enzyme present in crude MH76 extracts. Currently, further attempts to purify the enzyme and its iso-
zymes from subclone MH76 are in progress.

Previous results from this laboratory have also indicated that the partially purified GTF-I activity of strain GS-5 was dependent on the addition of exogenous dextran (14). However, both the homogeneous GTF-I from serotype c strain Ingbritt (20) and the cloned enzyme are apparently primer independent. In fact, the addition of primer inhibited insoluble glucan synthesis by the purified enzyme (Table 1). It is likely that the addition of α-1,3-glucose units to the soluble primer shifted the product glucans to a more water-soluble form. These differences may also result from different types of processing which might occur in the culture fluids of S. mutans strains relative to E. coli extracts. The molecular weight of the GS-5 GTF-I was estimated as 155 kDa (14), that of the active GTF-I fragment from strain Ingbritt was estimated as 99 kDa (20), and the cloned enzyme exhibited molecular weights of 135 to 150 kDa.

The most convincing evidence to indicate that the gtfB gene coded for a GTF-I activity came from the in vivo inactivation experiments. Inactivation of the gtfB gene of strain GS-5 after transformation with the altered gene clearly results in a major decrease in soluble glucan-synthesizing activity and a corresponding loss of sucrose-dependent colonizing activity. Since a low level of insoluble glucan-synthesizing activity still remains in the transformants, it is possible that more than one GTF-I gene is produced in serotype c strains or conversely, that this residual activity results from aggregated GTF-S activity (14, 21). Attempts to isolate other putative GTF genes in strain GS-5 are continuing in this laboratory to evaluate these possibilities.

The possibility that some of the properties of the cloned gtfB gene which differed from the GTF-I of GS-5 resulted from scrambling of the GTF-I gene during the construction of the clone bank was eliminated by the results of the Southern blotting experiments. These results indicated that the nucleotide sequences encompassing the GTF gene were contiguous on the GS-5 chromosome. Furthermore, these results suggested the presence of another gene sharing homology with the gtfB gene. This additional gene did not appear to be merely a duplication of the gtfB gene, since its apparent restriction map differed significantly from that of gtfB (Fig. 5). It is possible that this latter gene could be another closely related GTF-I gene and may be responsible for the residual insoluble glucan synthesizing activity detected after insertional inactivation of the gtfB gene. Alternatively, this gene might code for GTF-S activity and could explain the cross-reactivity of the gtfB gene product with both anti-GTF-I and anti-GTF-S. Furthermore, the detection of a few (<1%) Em' rough colonies displaying wild-type insoluble glucan synthetic activity after transformation with pTS61 could also be explained by assuming that the Em' gene inserted into the homologous gene and not into gtfB. It will be of interest to isolate the homologous gene from the L47.1 clone bank by using probes constructed from the putative fragments of this gene (Fig. 5). Furthermore, a recent report (M. Pucci and F. Macrina, personal communication) indicated that a GTF gene probe from S. mutans LM-7 also detects two distinct genes from this organism.

Localization experiments (Table 2) suggested that the cloned GTF enzyme was not able to traverse the E. coli cytoplasmic membrane. However, a significant portion of the GTF activity was isolated that was associated with the membrane fraction. In fact, a higher proportion of GTF activity (>38%; Table 2) may be associated with the membrane, since a substantial amount of the periplasmic enzyme alkaline phosphatase is found associated with the cytoplasmic fraction with the cell fractionation protocol utilized in the present investigation. Since transcription of the GTF gene in MH76 appears to originate from its own promoter, these results suggest that the intact signal sequence (2) is present on the chimeric plasmid. It may be possible that the GTF signal sequence may allow association of the cloned enzyme with the E. coli cytoplasmic membrane but does not allow the heterologous protein to traverse this barrier. It is of interest that recent results from this laboratory (29) suggest that the majority of a cloned GS-5 fructosyltransferase exoenzyme can be secreted into the periplasmic space of E. coli C600.

The availability of the cloned gtfB gene from GS-5 now makes it possible to examine its role in cariogenicity in greater detail. Currently, the relative position of the gene on the chromosome is being determined following transfer to the mapping vector pVA891 (15) followed by transformation as was done recently to map the gtfA gene (23). In addition, the nucleotide sequence of the gene and its regulatory regions will be determined to explore the fine structure of the enzyme and investigate the regulation of its expression in S. mutans. Finally, the gtfB gene will be compared with the other GTF genes cloned from GS-5 in order to determine the relationship between these enzymes.

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