Identification of the Streptococcus mutans frp gene as a potential regulator of fructosyltransferase expression

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Abstract

Four putative open reading frames (ORFs) were previously identified in the regions flanking the Streptococcus mutans GS-5 fructosyltransferase (FTF) gene. One of these, ORF 3, appeared to code for a low-molecular-mass protein containing amino acid sequences sharing homology with several Gram-positive bacterial DNA-binding proteins and it was suggested that the ORF 3 gene product might be an FTF regulatory protein (FRP). In order to characterize this protein, we have purified the biotinylated tag-FRP fusion protein using the PinPoint protein purification system and this fusion protein was used in gel shift assays with DNA fragments containing the ftf promoter region. FRP bound specifically to the upstream region of the ftf promoter containing the inverted repeat structure that is present upstream of the -35 sequence. In contrast, FRP did not bind to DNA fragments lacking the inverted repeat structure. The results of these experiments suggest that FRP interacts with the inverted repeat region upstream of the ftf promoter and such interactions may regulate FTF expression.

Keywords: Streptococcus mutans; DNA binding protein; Fructosyltransferase; Regulation

1. Introduction

Streptococcus mutans has been implicated as the primary etiological agent in the development of human dental caries and is frequently isolated from human dental plaque [9,12]. This organism expresses several extracellular sucrose-metabolizing enzymes including three glucosyltransferases (GTFs) and a fructosyltransferase (FTF). FTF synthesizes a high-molecular-mass fructan polymer of the inulin type from sucrose consisting mostly of β-(2-1)-linked fructose residues with some branching in the 6 position [2,3,5,9,15,20] and the fructans may serve as a reserve extracellular carbohydrate under conditions of nutrient starvation [8]. Moreover, inactivation of the ftf gene results in reduced virulence of S. mutans in the rat dental caries model [13,17].

Previously, a gene coding for FTF activity has been isolated from S. mutans GS-5 [16] and its nucleotide sequence has been determined [19]. Furthermore, the ftf gene was preceded by an inverted repeat structure which might function in the regulation of FTF expression. In addition, sequencing of the regions flanking the ftf gene revealed the presence of four other putative open reading frames (ORFs) [19]. ORF 3, which was located in the region...
downstream of the *ftf* gene, appeared to code for a low-molecular-mass protein containing amino acid sequences sharing homology with several DNA-binding proteins such as the sigma 37 subunit [4], *spoOF* [23], *phoP* [18], and *penI* [10] from Gram-positive bacteria [19]. These results suggested that ORF 3 might be involved in regulating the expression of the *ftf* gene as an FTF regulatory protein (FRP). In order to confirm this possibility, the protein was purified as a fusion protein and gel shift analyses were carried out.

### 2. Materials and methods

#### 2.1. Bacterial strain and cultivation

*Escherichia coli* JM 109 was maintained and grown routinely as previously described [1].

#### 2.2. DNA manipulations

DNA isolation, endonuclease restriction, ligation, and transformation of competent *E. coli* cells were carried out as previously described [1].

#### 2.3. Purification of FRP

The PinPoint Xa System (Promega) was used for purification of the recombinant protein. For utilization of this system, plasmid pYS6 (Fig. 1) was constructed by site-directed mutagenesis. Briefly, at the *Bgl*II site in pTS102 [19] containing the 4.3 kb *HindIII* fragment, a *HindIII* site was constructed by addition of a *HindIII* linker. The *SpeI*-*HindIII* fragment containing ORF 3 was then ligated to pBluescript II SK+, yielding pYS2 (Fig. 1). For construction of the *NcoI* site, the ATG site of ORF 3 in pYS2 was altered by site-directed mutagenesis from AT*ATG*T to CC*ATG*G. A mutagenic 28-mer oligonucleotide (5′-TTG ACT TGA AAC CAT GGA CTC CTC ATA A-3′) was synthesized by the oligonucleotide synthesis facility at the Department of Biochemical Pharmacology, State University of New York at Buffalo. Site-directed mutagenesis of the DNA fragment was carried out by the method of Eckstein et al. [14,22]. Subsequently, at the *SacI* site in the MCS of pYS2 (upstream of the *NcoI* site), a *HindIII* site was constructed by addition of a *HindIII* linker. The *HindIII* fragment containing ORF 3 was then ligated to vector PinPoint pXa-2, yielding plasmid pYS5. Plasmid pYS5 was then digested with *NcoI*, and the truncated fragment was filled in with the Klenow enzyme. After digestion with *NruI*, the fragment was self-ligated and plasmid pYS6 was obtained.

*E. coli* JM109 (pYS6) was harvested by centrifugation after growth in Luria-Bertani broth (10 liters) at 37°C. The cell pellet was washed with 50 mM Tris·HCl (pH 7.5)–50 mM NaCl–5% glycerol containing 0.1 mM phenylmethylsulfonyl fluoride and resuspended in 10 volumes of the same buffer (ml

![Fig. 1. Structures of plasmids pYS2 and pYS6. To construct plasmid pYS2, the DNA fragment containing the *frp* gene isolated from pTS102 [19] was ligated to plasmid vector pBluescript II SK+. For pYS6, the site-directed mutagenized *HindIII*-*HindIII* fragment was ligated to the PinPoint pXa-2 vector and self-ligated following *NruI* and *NcoI* cleavage and filling in of the *NcoI* site.](https://academic.oup.com/femsle/article-abstract/140/1/49/542266/Identification-of-the-Streptococcus-mutans-frp)

of cell paste) at 4°C. The bacterial cells were disrupted by sonication and centrifuged to remove the cellular debris, the resultant crude extract was applied to an equilibrated SoftLink Resin (Promega) column, and detection of the recombinant fusion protein was carried out in accordance with the manufacturer's instructions. Briefly, the recombinant extract was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to a polyvinylidene-difluoride membrane (Immobilon-P; Millipore). The biotinylated proteins were detected with streptavidin-alkaline phosphatase and nitroblue tetrazolium-5-bromo-4-chloro-3-indolylphosphate toluidinium (salt) reagent for alkaline phosphatase activity.

2.4. Preparation of the DNA fragments carrying the ftf promoter region

Two potential inverted repeat structures are present upstream of the ftf promoter region [21]. The first is located between nucleotides 471 and 518, and the second is present from nucleotides 565 to 593. Three DNA fragments were produced by PCR for the gel shift assays. Probe A is a 278-bp fragment containing the two inverted repeat structures from nucleotides 422 to 697 (Fig. 2). Probe B is a 260-bp fragment lacking both inverted repeat structures from nucleotides 608 to 867 (Fig. 2). Probe C is a 261-bp fragment containing only the first inverted repeat structure from nucleotides 302 to 562 (Fig. 2). These fragments were labeled with [γ-32P]ATP.

2.5. Gel shift assay

We used gel shift assays to demonstrate FRP binding to the ftf promoter region. The gel shift assay was based on the methods of Fried and Crothers [6] as well as Garner and Revzin [7]. Binding reactions for the gel shift assays contained 10 mM Tris·HCl (pH 7.5), 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 5% glycerol, and 1.6 µg

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\text{Fig. 2. The structure of the ftf promoter region. The transcription start site (+1) and the } -10 \text{ and } -35 \text{ promoter sequence elements were determined previously [21]. The opposing arrows denote two inverted repeat structures. The base positions are numbered corresponding to [19]. Boundaries of probes: } \triangle, \text{A, } \triangle, \text{B, } \bigcirc, \text{C.}
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HaeIII-digested pBluescript SK+. 1 ng of a labeled DNA fragment and various amounts of protein were added to the reaction mixtures. Reaction mixtures were allowed to equilibrate for 30 min at room temperature and immediately loaded onto pre-electrophoresed 4% polyacrylamide gels and electrophoresed in Tris-acetate–EDTA buffer. DNA and DNA–protein complexes were detected by autoradiography.

3. Results and discussion

3.1. Purification of FRP

The biotinylated proteins were detected with streptavidin–alkaline phosphatase and nitroblue tetrazolium–5-bromo-4-chloro-3-indolylphosphate toluidinium (salt) reagent for alkaline phosphatase activity (Fig. 3). E. coli strains normally synthesize a 22.5-kDa biotinylated protein which could be readily detected on the blots (lanes 1, 2, and 3). In addition, the 12.5-kDa subunit of the transcarboxylase complex (Tag protein) was also detected in transformants containing plasmid vector pXa-2 (lane 2). However, in the extract containing plasmid pYS6 expressing the recombinant fusion protein, a biotin-containing protein band of approximately 43 kDa was detected (lane 1). This size is slightly larger than that predicted for a fusion of the Tag protein (13 kDa) with FRP (26.5 kDa) [19]. The lower-molecular-mass bands detected likely represent degradation products of the fusion protein.

3.2. Assay of FRP binding to the ftf regulatory region

Various concentrations of FRP were incubated with the 32P-labeled probe A containing two invert repeat regions (Fig. 4). Only a single shifted band was detected. To further confirm that FRP binds to the ftf promoter specifically, excess amounts of unlabeled probe A (P_{ftf}) and the rpsJ promoter (P_{rpsJ}), the rpsJ gene is present directly downstream of the frp gene (Shibata and Kuramitsu, submitted), were added to a series of binding reactions in which 1 ng of 32P-labeled P_{ftf} was used as a probe. Fig. 5 shows that as little as a 5-fold excess of unlabeled P_{ftf} DNA begins to compete with 32P-labeled P_{ftf} DNA for FRP binding (lane 3), whereas a 50-fold excess of unlabeled P_{rpsJ} DNA does not (lane 8). Two inverted repeat sequences are present upstream of the ftf promoter region. Therefore, to determine whether or not FRP binds to the inverted repeat

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Fig. 4. Gel shift assay of P_{ftf} (probe A) with increasing amounts of purified FRP fusion protein. Protein content per lane was as follows: 1. no protein; 2. 0.24 μg; 3. 0.48 μg; 4. 1.2 μg. The arrow indicates the retarded DNA fragment.

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Fig. 3. Detection of the expression of fusion constructs following SDS-PAGE analysis. Lanes: 1, E. coli subclone pYS6; 2, E. coli with vector pXa-2; 3, E. coli. The numbers at the right indicate molecular sizes in kDa.
binding was detected between FRP and probe C (C). It appeared that FRP primarily bound to the region that was within 118 bp of the ATG initiation codon of the fif gene and the inverted repeat structure present in this region might play an important role in the regulation of fif expression.

A recent study has demonstrated the complexity of fif gene regulation in S. mutans [11]. FTF expression was induced by sucrose and the region involved in this regulation was located to within 135 bp of the ATG initiation codon and encompassed an inverted repeat. It was suggested that the inverted repeat structure (nucleotides 565–593; Fig. 2) was involved in the control of fif expression in response to carbohydrates. The present results suggest that FRP may play a role in such regulation. Furthermore, regulation of fif gene expression was suggested to involve the sucrose phosphotransferase system (PTS).

It is not clear whether or not FRP acts as a positive or negative regulator of fif expression since attempts to insertionally inactivate the gene were unsuccessful. Additional approaches will be required to directly demonstrate the role of FRP in FTF expression.

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References


