Cloning and DNA Sequencing of the Dextranase Inhibitor Gene (*dei*) from *Streptococcus sobrinus*

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Received 5 May 1994/Accepted 13 September 1994

Some dextranase-deficient (Dex⁻) mutants of *Streptococcus sobrinus* UAB66 (serotype g) synthesize a substance which inhibits dextranase activity (S.-Y. Wanda, A. Camilli, H. M. Murchison, and R. Curtiss III, J. Bacteriol. 176:7206-7212, 1994). This substance produced by the Dex⁻ mutant UAB108 was designated dextranase inhibitor (Dei) and identified as a protein. The Dei gene (*dei*) from UAB108 has been cloned into pACYC184 to yield pYA2651, which was then used to generate several subclones (pYA2653 to pYA2657). The DNA sequence of *dei* was determined by using Tn5seq1 transposon mutagenesis of pYA2653. The open reading frame of *dei* is 990 bp long. It encodes a signal peptide of 38 amino acids and a mature Dei protein of 292 amino acids with a molecular weight of 31,372. The deduced amino acid sequence of Dei shows various degrees of similarity with glucosyltransferases and glucan-binding protein and contains A and C repeating units probably involved in glucan binding. Southern hybridization results showed that the *dei* probe from UAB108 hybridized to the same-size fragment in *S. sobrinus* (serotype d and g) DNA, to a different-size fragment in *S. downei* (serotype h) and *S. cricetus* (serotype a), and not at all to DNAs from other mutans group of streptococci.

The ability to synthesize extracellular glucans is known to be one of the virulence properties of *Streptococcus sobrinus* and related oral streptococci which contributes to plaque formation and to the subsequent development of dental caries (8, 17, 18, 55). This ability depends on two different kinds of enzymes: glucosyltransferases (GTFs) (13, 24, 27, 43, 63) and dextranase (5, 13, 42, 48, 59). The GTFs fall into two groups: those that synthesize a water-soluble glucan (WSG), primarily α -1,6-linked glucan (GTF-S) (14, 34, 36), and those that synthesize a water-insoluble glucan (WIG), primarily α -1,3-linked glucan (GTF-I) (2, 10, 24, 28). Clinical and biochemical studies have shown that WIG produced by oral streptococci contributes significantly to the induction of dental caries and plaque formation (17, 55, 63). The precise role of dextranase is as yet poorly understood. Several hypotheses as to its function have been offered (5, 42, 48, 55, 59). It seems that the GTF-to-dextranase ratio regulates WIG production (55, 59), but there remain many unanswered questions. For this reason, much attention has been given to the importance of dextranase (5, 30, 44, 61). The dextranase of *S. sobrinus* has been purified, its gene has been cloned and sequenced (6, 61). In 1982, Hamelik and McCabe found another protein in oral streptococci which can inhibit the activity of dextranase (19). We previously isolated many *S. mutans* and *S. sobrinus* mutants defective in synthesis of WIG which were devoid of dextranase activity (i.e., were Dex⁻) (37). We more recently discovered that many of these mutants possess dextranase protein but have a high level of an endogenous dextranase inhibitor (Dei) which inhibits dextranase activity (60).

This report describes the cloning, sequencing, and characterization of the *dei* gene from *S. sobrinus* UAB108, a mutant that is phenotypically Dex⁻, produces exclusively WSG, is nonadherent and noncariogenic, and inhibits adherence by and cariogenicity of wild-type strains (38, 54).

MATERIALS AND METHODS

Bacterial strains and growth media. The bacterial strains used in this study are listed in Table 1. *Escherichia coli* strains were grown in Luria (L) broth (31) with additives as indicated. Complex media were supplemented with thymidine at 40 μ g/ml for all strains with a *thyA* mutation. The antibiotic concentrations used were 100 μ g/ml for ampicillin, 30 μ g/ml for chloramphenicol, 12.5 μ g/ml for tetracycline, and 50 μ g/ml for kanamycin. The mutans group of streptococci were grown in brain heart infusion (BHI; Difco) medium or modified FMC medium (37). Small cultures were incubated anaerobically as previously described (37), whereas large cultures for batch production of proteins were incubated as standing cultures at 37°C.

Assays. Dextranase was assayed by Nelson's modification (40) of Somogyi's procedure (50) for the determination of glucose. The assay consisted of incubation of protein fractions in 50 mM sodium acetate (pH 5.5) with dextran T-2000 (Sigma, St. Louis, Mo.) at 37°C under conditions of substrate excess (61). The assay for inhibitor activity was the same as for dextranase except for adding Dei preparation and preincubating for 5 min at 37°C before adding substrate dextran to start the reaction. Dei activity was determined by the reduction of dextranase activity (60). Protein was determined with the bicinchoninic acid protein assay reagent (Pierce, Rockford, Ill.).

Preparation of DNA and protein. Overnight cultures of oral streptococci in BHI broth were diluted 100-fold into 500 ml of the same medium in 500-ml flasks and incubated standing for 2 days at 37°C. Cells were harvested and washed twice with TE (10 mM Tris-HCl, 1 mM EDTA [pH 7.5]) buffer. About 1 g (wet weight) of bacteria was suspended in 5 ml of TE buffer containing 20 mg of lysozyme (Sigma) and 100 U of mutanolysin (Sigma) per ml. The cell suspensions were incubated for 1 h at 37°C. Approximate 2.5 mg of proteinase K (Sigma) was added, and the suspensions were further incubated for 1 h at 37°C. Cells were lysed by adding sodium dodecyl sulfate (SDS) to a 2.5% (wt/vol) final concentration. Total DNAs were then purified by one extraction with phenol, three with phenol-chloroform (1:1), and two with chloroform and then precipi-

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TABLE 1. Bacterial strains used in this study

Strain	Phenotype or genotype ^a	Source
<i>E. coli</i> K-12		
χ925	<i>thr-1 ara-13 leu-6 azi-8 tonA2 lacY1 minA1 glnV44 gal-6 minB2 rpsL135 xyl-7 mtl-2 thi-1</i>	7
χ1849	<i>fhuA53 dapD8 minA1 purE41 glnV42 Δ40(gal-uvrB) λ⁻ minB2 his-53 nalA25 metC65 oms-1</i> <i>Δ29(bioH-asd) ilv-277 cycB2 cycA1 hsdR2</i>	21
χ2819	<i>lacY1 glnV44 galK2 galT22 λ(c1857 b2 red3 S7) recA56 ΔthyA57 metB1 hsdR2</i>	26
χ2831	<i>lacY1 glnV44 galK2 galT22 λ(c1857 b2 redb3) recA56 ΔthyA57 metB1 hsdR2</i>	26
CC118	<i>araD139 Δ(ara-leu)7697 ΔlacX74 ΔphoA20 galE galK thi rpsE rpoB argE_{am} recA1</i>	32
JM83	<i>ara Δ(lac-proAB) rpsL φ80 lacZΔM15</i>	65
JM109	<i>recA1 endA1 gyrA96 thi hsdR17 glnV44 relA1 λ⁻ Δ(lac-proAB) [F' traD36 proAB lacI^q lacZΔM15]</i>	65
LE392	<i>glnV44 tyrT58 hsdR514 galK2 galT22 metB1 trpR55 lacY1</i>	46
Mutans group streptococci		
<i>S. cricetus</i> HS6	Serotype <i>a</i>	23
<i>S. rattus</i> BHT	Serotype <i>b</i>	23
<i>S. mutans</i> Ingbritt	Serotype <i>c</i>	23
<i>S. sobrinus</i> OMZ176	Serotype <i>d</i>	23
<i>S. mutans</i> LM7	Serotype <i>e</i>	23
OMZ175	Serotype <i>f</i>	23
<i>S. sobrinus</i> 6715	Serotype <i>g</i> , Str ^r	23
<i>S. downei</i> MFe28	Serotype <i>h</i>	10
<i>S. sobrinus</i> UAB66	Serotype <i>g</i> , mutant of 6715, Str ^r Spc ^r Adh ⁺ Dex ⁺	38
UAB108	Serotype <i>g</i> , mutant of UAB66, Str ^r Spc ^r Adh ⁻ Dex ⁻	38

^a Str^r, streptomycin resistant; Spc^r, spectinomycin resistant; Adh⁻, nonadherent.

tated with ethanol. DNA pellets were dissolved in TE buffer containing DNase-free pancreatic RNase (20 μg/ml; Sigma) and kept at -20°C. Plasmid DNAs from *E. coli* strains were isolated by the alkaline lysis procedure or by centrifugation in CsCl-ethidium bromide gradients (46). The DNA fragment used as a probe was purified from agarose gel by using a GeneClean kit (Bio 101, La Jolla, Calif.). Periplasmic protein fractions of *E. coli* cells were prepared by the cold osmotic shock procedure described by Hazelbauer and Harayama (22). Extracellular proteins from oral streptococci were obtained from culture supernatant fluid as previously described (37).

Gel electrophoresis and isoelectric focusing. SDS-polyacrylamide gel electrophoresis (PAGE) was carried out essentially by the method of Weber and Osborn (62) with a 7.5 or 10% gel. Coomassie brilliant blue was used to detect protein bands. The demonstration of the Dei bands on blue dextran (BD)-SDS-PAGE was carried out as previously described (60); after the gel was washed completely, about 2,000 U of a dextranase preparation from the periplasmic fraction of recombinant *E. coli* clone χ2819(pYA3009), which possesses the *dex* gene from *S. sobrinus* UAB108 (see below), was added in 150 ml of washing solution and incubated at 37°C with the gel for 1 to 2 days. Blue bands on the clear background gel corresponded to the Dei proteins. Isoelectric focusing was performed as described in reference 61.

Construction of a genomic library and detection of clones. An *S. sobrinus* UAB108 genomic library was constructed by ligating 4- to 10-kb *Sau*3AI partially cleaved DNA fragments to the *Bam*HI site of bacterial alkaline phosphatase (Bethesda Research Laboratories, Gaithersburg, Md.)-dephosphorylated vector pACYC184. The ligated DNA fragments were used to transform host strain *E. coli* χ2819 containing the *dex* plasmid pYA3011, which was derived from pUC8 (61). Transformants obtained on L agar with thymidine, ampicillin, and chloramphenicol were screened for tetracycline sensitivity on L-agar plates with thymidine, ampicillin, and tetracycline. At the same time, the plates with transformants were overlaid with 12 ml of 1% BD top agar (1% agar, 50 mM sodium acetate [pH 5.5])

and incubated at 42°C for 4 h and then at 37°C for 8 to 20 h until halos (clearing zones) around the colonies were clearly visible. Screening was done for the transformants which gave halos of either reduced or increased size. Further assays were carried out with these transformants, using BD-SDS-PAGE (4) and renaturation soaking of BD-SDS-polyacrylamide gels in dextranase solution (60). The transformant colonies which gave both Dei and dextranase bands were selected and stored.

Tn5seq1 insertion mutagenesis. CC118 containing the recombinant plasmid pYA2653 was grown in 40 ml of TYM (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.4% maltose) medium with chloramphenicol until the optical density at 600 nm reached 0.3 to 0.4. Bacteria were pelleted by centrifugation, suspended in 4 ml of TMGS (10 mM Tris-HCl [pH 7.0], 10 mM MgSO₄, 0.01% gelatin, 100 mM NaCl) and infected with λ::Tn5seq1 (39) at a multiplicity of 0.1 to 0.5. Phages were allowed to adsorb for 30 min at room temperature, then 4 ml of L broth (containing chloramphenicol) was added, and phages were incubated at 37°C for 2 to 3 h with gentle shaking to allow expression of kanamycin resistance (Km^r). The cultures were added to 35 ml of L broth containing chloramphenicol and kanamycin and incubated overnight at 37°C to enrich for cells with Tn5seq1 insertions in plasmids. The next day, plasmid DNA was extracted and transformed into *E. coli* CC118 with selection for Km^r and chloramphenicol resistance (Cm^r). Restriction maps of plasmids in Km^r Cm^r transformants were analyzed to verify insertion of Tn5seq1 into DNA sequences in pYA2653. Results from BD-SDS-PAGE and Somogyi assays identified clones with Tn5seq1 in the *dei* gene or its promoter which did not express Dei activity. These clones and others with Tn5seq1 nearby *dei* gene were selected for DNA sequencing.

Nucleotide sequencing. DNAs used for sequencing were CsCl purified. Transposon Tn5seq1 was used to define primer sites. The bidirectional chain termination nucleotide sequencing procedure (39) was carried out with a modified T7 DNA polymerase supplied with the Sequenase kit (U.S. Biochemical Corp., Cleveland, Ohio) and [α-³⁵S]dATP (1,000 Ci/mmol;

NEN, Du Pont Co., Boston, Mass.). Double-stranded templates were used directly for sequencing. The primers for the SP6 and T7 promoters (39) were synthesized with a DNA synthesizer. The sequence was confirmed with overlapping clones, and the entire gene and its control region were sequenced in both orientations. The nucleotide and amino acid sequences were analyzed with the University of Wisconsin Genetics Computer Group sequence analysis program.

Minicell protein analysis. The *E. coli* minicell-producing strain χ 925 was used to identify plasmid-encoded proteins as previously described (7, 16). Minicells were isolated from late-log-phase cultures by sucrose gradient centrifugation. Proteins synthesized by purified minicells were labeled with [³⁵S]methionine (1,000 Ci/mmol; NEN, Du Pont), analyzed by SDS-PAGE, and identified by radioautography.

Hybridization analysis. DNAs in 0.8% agarose gels were denatured, transferred to nitrocellulose paper, and hybridized with labeled probe DNA by the method of Southern (51). The probe was labeled with [α -³²P]dCTP (800 Ci/mmol; NEN, Du Pont), using a nick translation kit (Amersham Corp., Arlington Heights, Ill.) as recommended by the manufacturer. Hybrids were visualized by radioautography.

Nucleotide sequence accession number. The GeneBank accession number for the *dei* sequence is L34406.

RESULTS

Construction of an *S. sobrinus* genomic library in pACYC184 and identification and subcloning of a *dei* gene clone. The experimental plan and results are summarized in Fig. 1. From 3,169 Cm^r tetracycline-sensitive transformants, 60 with reduced halos and 9 with increased halos, using the BD agar overlay screening method, were selected, and protein extracts from each were analyzed by BD-SDS-PAGE. Only three clones showed Dei bands (weak band at 50 kDa and strong band at 43 kDa), and one expressed a greatly enhanced dextranase activity band (100 kDa). Partial physical maps were obtained by restriction endonuclease analysis. The clones which expressed Dei activity carried plasmids with identical physical maps, and one was designated pYA2651. The clone which contained the dextranase gene was designated pYA3009. pYA2651 DNA was partially digested by *Hind*III and religated to yield a new clone (pYA2653) which has a smaller insertion carrying the *dei* gene. Using *Tn5seq1* mutagenesis, we localized the *dei* gene to a *Dra*I-*Eco*RV fragment (see below), and this fragment was cloned into the *Hinc*II site of pUC19. The ligation mixture was introduced into *E. coli* JM83, and two clones (pYA2656 and pYA2657) that carry the insert in opposite orientations were obtained. JM83(pYA2656) cells gave much higher Dei activity than did cells of JM83(pYA2657); thus, we deduced that the direction of *dei* transcription in pYA2656 is the same as that of *lacP* and *lacZ* of pUC19 (Fig. 1).

Activity bands of Dei proteins in different *dei* clones and different serotypes of the mutans group of streptococci on BD-SDS-PAGE. In recombinant *E. coli* clones, two bands of Dei activity (50 and 43 kDa) were visible in the early incubation stage of the gel with dextranase (Fig. 2). After 2 days of incubation, only the 43-kDa band remained readily visible except for the extract from the clone carrying pYA2656, which displayed the larger form as well as some breakdown products. For the mutans group of streptococci, Dei bands were visible in HS6 (serotype *a*), OMZ176 (serotype *d*), UAB66 (serotype *g*), UAB108 (serotype *g*), and MFe28 (serotype *h*). Strains OMZ176, UAB66, UAB108, and MFe28 produced a 43-kDa

band and some low-molecular-weight bands which are probably produced by degradation, while strain HS6 (serotype *a*) has a band at 40 kDa and another at 85 kDa. This finding suggests that HS6 may have two *dei* genes or that the gene products are different from those in the other strains. The intensity of the Dei bands from the extract of clone pYA2656 is greater than that from other clones. Since only half the amount of protein from the pYA2656-containing clone (11.5 μ g) was loaded compared with the other strains (23 μ g), it appears that the Dei protein in the extract from the pYA2656-containing clone has a higher specific activity. These results are consistent with those from Somogyi assays (data not shown).

Identification of recombinant plasmid-encoded proteins. A comparison of protein synthesis in *E. coli* minicells that contained only the vector plasmid pUC19 or pACYC184 with those that contained pYA2651, pYA2653, pYA2656, and pYA2657 allowed us to determine the specific polypeptides associated with cloned *S. sobrinus* DNA. A radioautograph of radioactively labeled minicell proteins after SDS-PAGE is shown in Fig. 3. With the exception of the bands specified by the vectors, the 25.6-kDa Cm^r gene product in pACYC184 and 31- and 28-kDa ampicillin-resistant gene products in pUC19 (47), all plasmids which contain the *dei* gene gave two extra bands at 50 and 43 kDa. These findings are consistent with the results from renatured BD-SDS-PAGE.

Effects of IPTG on *dei* expression in different clones. Table 2 shows the effects of isopropyl- β -D-thiogalactopyranoside (IPTG) on expression of *dei* in different clones. IPTG enhanced the ability of *E. coli* JM109(pYA2656) extract to inhibit dextranase from 29.2 to 87.3% but reduced the ability of the *E. coli* JM109(pYA2657) extract to inhibit dextranase from 25.9 to 3.0%. Thus, release of *lacI*^q repression in JM109 increases *dei* expression when the *dei* gene orientation is the same as that of *lacP* and decreases its expression when it is in the opposite orientation. In JM83 (Table 2), which does not have *lacI*^q, the presence of pYA2656 leads to constitutive high-level expression of *dei* and maximal inhibition of dextranase independent of growth in the presence of IPTG. In contrast, constitutive transcription initiation from *lacP* in JM83(pYA2657) leads to minimal expression of *dei* following growth with or without IPTG. Collectively, these results provide evidence for the direction of transcription of the *dei* gene and indicate that *dei* probably possesses its own promoter. The DNA sequence of the control region of *dei* gene proves these inferences (see below).

Transposon *Tn5seq1* insertion mutagenesis of pYA2653 and *dei* gene location. pYA2653 in CC118 was subjected to transposon *Tn5seq1* insertion mutagenesis. In accordance with the restriction map of *Tn5seq1* (39), *Nco*I, *Sac*II, and *Hind*III were chosen to indicate the insertion position and orientation of each *Tn5seq1* insertion. Figure 4 shows the results from analysis of transformants showing the *Tn5seq1* insertion positions in pYA2653. By determining the Dei activity by both the Somogyi assay and renatured BD-SDS-PAGE, only insertions in the area identified by the open box inactivated *dei*. This finding indicates that the *dei* gene is in this region.

DNA sequence. Clones 9, 4, 19, and 23 (Fig. 4) were chosen for DNA sequencing. The complete nucleotide sequence of a 1,180-bp fragment carrying the *dei* gene and its control region was determined on both strands and is shown in Fig. 5. The *dei* gene has an open reading frame of 990 bp preceded by a ribosome-binding site (TAAGGAGGTA) 6 bases upstream from the ATG start codon. Several promoter-like sequences can be found upstream of the gene. We speculate that a possible -35 region (TTACGA) at positions 70 to 75 separated by 19 bp from a proposed -10 region (TACAAT) at

FIG. 1. Preparation of a genomic library of *S. sobrinus* UAB108 DNA and generation of clones and subclones containing *dei*. Relevant restriction sites, antibiotic resistance coding regions, and genes are indicated. Details are described in the text. Abbreviations: BAP, bacterial alkaline phosphatase; CIAP, calf intestine alkaline phosphatase; B, *Bam*HI; C, *Cla*I; D, *Dra*I; E, *Eco*RI; EV, *Eco*RV; H, *Hind*III; Hn, *Hinc*II; P, *Pvu*II; S, *Sau*3AI. The sites designated B/S* and Hn/D* are no longer cleaved by *Bam*HI and *Sau*3AI and by *Hinc*II and *Dra*I, respectively.

positions 95 to 100, 10 bp upstream from the ribosome-binding site, may be a promoter. As mentioned above, only this part of the control region was cloned into *E. coli* recombinants JM83(pYA2656) and JM83(pYA2657); the *dei* gene was expressed in both but displayed a great difference in expression level according to the orientation of *dei*. Downstream from the termination codon TAA, there exists a potential stem-loop structure with a ΔG of -9.2 kcal (ca. -38.5 kJ) (56) plus a string of T's suggestive of a transcription terminator.

Amino acid composition. The deduced amino acid sequence,

commencing with the ATG start codon and extending to the termination codon TAA, contains 330 amino acids and has a molecular weight of 36,148. The amino acid composition indicates that Dei is a highly hydrophilic protein. The N-terminal region resembles a typical signal sequence for secretory proteins. The first 15 residues are strongly basic (8 of 15), and these are followed by a highly hydrophobic region (12 of 17 residues). On the basis of the $-3, -1$ rule proposed by von Heijne (58), the putative cleavage site following Ala-38 was assigned. To verify this, the 43-kDa Dei protein expressed in *E.*

FIG. 2. BD-SDS-PAGE analysis showing bands of Dei active proteins in different serotypes of mutans group of streptococci (A) and in different *dei* clones in *E. coli* (B). The same amounts of protein (23 μ g) were loaded on all lanes except that for JM83(pYA2656) (B, lane 6), which contained only 11.5 μ g of protein. (A) Lanes: 1, molecular weight markers; 2, HS6 (serotype *a*); 3, BHT (serotype *b*); 4, Ingbritt (serotype *c*); 5, OMZ176 (serotype *d*); 6, LM7 (serotype *e*); 7, OMZ175 (serotype *f*); 8, 6715 (serotype *g*); 9, UAB66 (serotype *g*); 10, UAB108 (serotype *g*); 11, MFe28 (serotype *h*). (B) Lanes: 1, molecular weight markers; 2, χ 925(pACYC184); 3, χ 925(pYA2651); 4, χ 925(pYA2653); 5, JM83(pUC19); 6, JM83(pYA2656); 7, JM83(pYA2657).

coli JM83(pYA2656) was purified by electroelution from an SDS-polyacrylamide gel, transferred onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore Corp., Bedford, Mass.), and subjected to N-terminal amino acid analysis (33, 35); the first 13 amino acids were identified as DEQTESTV.

FIG. 3. Identification of proteins encoded by Dei⁺ recombinant plasmids, which were synthesized in purified minicells, radiolabeled with [³⁵S]methionine, resolved in an SDS-10% polyacrylamide gel, and identified by radioautography. Lanes: 1, χ 925; 2, χ 925(pUC19); 3, χ 925(pYA2657); 4, χ 925(pYA2656); 5, χ 925(pYA2653); 6, χ 925(pYA2651); 7, χ 925(pACYC184). Arrows indicate positions of Dei proteins. Size standards are indicated at the left. The amounts of total protein extracts loaded on the gel were different for displaying the protein bands encoded by different recombinant plasmids.

TABLE 2. Effects of IPTG on expression of *dei* in different clones^a

Strain	IPTG (2 mM)	Amt of Dei extract added (mg)	OD ₅₂₀	Inhibition (%)
JM109(pYA2656)	—	6	0.584	29.2
	+	6	0.105	87.3
JM109(pYA2657)	—	6	0.611	25.9
	+	6	0.800	3.0
JM83(pYA2656)	—	6	0.037	95.5
	+	6	0.00	100
	—	3	0.071	92.8
	+	3	0.093	90.6
JM83(pYA2657)	—	6	0.759	8.0
	+	6	0.738	10.5

^a Bacterial cultures were grown in L broth with ampicillin for 4 h, and then IPTG (Sigma) was or was not added to a final concentration of 2 mM. Incubation was continued for another 5 h, the cells were harvested, and periplasmic protein fractions were made from the pellets. Finally the Dei activity was measured for each sample as the percent reduction in dextranase activity in 3 μ g of the periplasmic protein fraction from χ 2819(pYA902) (61) as measured by Somogyi assays. The optical density at 520 nm (OD₅₂₀) for dextranase when no Dei was present was 0.825 (equal to 0% inhibition).

VRADS. This sequence is identical to that of the deduced amino acid sequence directly following the postulated cleavage site. Thus, the 38-amino-acid signal peptide of Dei is in the general size range of other streptococcal signal peptides (10) and that reported for other gram-positive organisms (41). The mature Dei protein contains 292 amino acids and has a molecular weight of 31,372. The acidic residues (32.6%) are much more prevalent than the basic residues (9.9%), which implies that Dei is an acidic protein. The pI was determined to be 4.15 by isoelectric focusing.

Comparison of A and C repeat units and their locations in GBP, GTF, and Dei proteins. From the comparison of the amino acid sequence of Dei with those for GTFs and glucan-binding protein (GBP) (3), we found that with the exception of some homology in their signal peptide regions (26% identity and 45% similarity with GBP; 24 to 34% identity and 37 to 54% similarity with GTFs), there is another domain of homology located in the C-terminal region (44% identity and 59% similarity with GBP; 34 to 43% identity and 46 to 61% similarity with different GTFs). Furthermore, we found that this homology corresponds to the A and C repeat sequences in GBP and GTFs (3, 10, 45). However, Dei has only two A and C repeat consensus sequences. Figure 6 presents a comparison of amino acid sequences of the A and C repeats and the spacing intervals separating the A repeats from the C repeats in Dei, GTF, and GBP molecules.

Hybridization of the *dei* gene probe with DNAs from different serotypes of the mutans group of streptococci. The *dei* probe was a 0.7-kb fragment obtained after double digestion of pYA2653 DNA with *Hind*III and *Cla*I and is located within the *dei* gene (Fig. 4) on the basis of the DNA sequence. The DNAs from streptococci of different serotypes were digested by *Hind*III and separated by agarose gel electrophoresis. The Southern hybridization results (Fig. 7) indicated that after 20 h of exposure, hybridization could be detected to a 0.95-kb band in OMZ176 (serotype *d*), UAB66 (serotype *g*), and UAB108 (serotype *g*) and to a 3-kb band in MFe28 (serotype *h*; data not shown). Strain HS6 (serotype *a*) showed only weak homology with a 4-kb fragment after 3 days of exposure (data not shown). The DNAs from other strains gave negative results even after long exposure times.

FIG. 4. Map of the restriction enzyme cleavage sites in the recombinant plasmid pYA2653 and transposon Tn5seq1 insertions. Bold lines represent the vector pACYC184. Numbers and arrows indicate the different locations of Tn5seq1 insertions. Only insertions in the open box region inactivated Dei activity.

DISCUSSION

Extracellular polysaccharides produced by the mutants group of streptococci are known to play an important role in stable colonization of streptococci on the tooth surface and are thus important for dental caries formation (8, 17, 55). Studies have shown that two classes of GTFs, one which produces WSG (GTF-S) and another which produces WIG (GTF-I), can be identified (28, 34). Many studies have shown that WIG significantly contributes to the induction of dental caries and plaque formation (17, 55, 63). Separation of these two enzymes has led

to the observation that both are necessary for the production of adherent WIG (14, 18).

Several lines of evidence suggest that another enzyme (dextranase) is involved in the virulence of oral streptococci (5, 13, 15, 59). Some Dex⁻ mutants produce copious amounts of WSG which inhibit plaque formation by the wild-type mutants group of streptococci (29, 37, 38, 54). It seems that both GTFs and dextranase are important in the formation of WIG (13, 55). The precise role of dextranase is as yet not understood. Several hypotheses as to its function have been offered (5, 15, 48, 55, 59), but there still remain many unanswered questions.

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1      ATTAACCTTC TTGCAAAAGA GTAAGATTTC TCTCATCGAG ATTACAATCT GTAATCGTTT TAAAGACTAT TACGATAGGC TTTCATTAGT ATGATACAAT
      SD
101     AGGCCCTGCA TAAGGAGGTA TGTTTTATGC AAGGAAAGAA AACTTATAAG ATGCACAAGG TTAATAAGCA TTGGGTAAAG ATTGCTGGTA CAGCAACAGT
      M Q G K K T Y K M H K V K K H W V S I A G T A T V
201     CTTATCTGTC GCTTTATTAG CGAATAATCA GGTCAAGGCT GATGAACAAA CAGAATCAAC TGTGTTCGG GCCGATAGCG CTGCTGTTGT AACCAAGCCT
      L S V A L L A N N Q V K A D E Q T E S T V V R A D S A A V V T K P
301     GCTGATGAGA CCAAGTCAGC TGACCAAGCA CAGCCAGCGA CTGCTGAACA AACGGCTACA GCTAATCAAA ATCAGCAAGC TTCTGCTAAT ACTGCTGATC
      A D E T S Q T D Q A Q P A T A E Q T A T A N Q N Q Q A S A N T A D Q
401     AAGCCCAAGA GCAAGACAG GATACAGCCA ATCAAGATAA ATGGCAAGCT GTTGATCAAG CTAGCCAACC TGAACAAATC GCTACTGCTG TCGATCAAGT
      A Q E Q R Q D T A N Q D K W Q A V D Q A S Q P E Q V A T A V D Q V
501     TCAAAACGCA GCTAAGACGC ATGCTAACCA AGTAGTAAGT ACGGATGTAA AAGATAGCCA TGCTGTTGTC AGCAAGGATG ATGCCAAGTC ATCTTCAGAC
      Q N A A K S D A N Q V V S T D V K D S H A V V S K D D A K S S S D
601     CAAGCAGCTG AGCAAGCTGG CTTCTACACA ACTGGTAATA ATGACTGGTA TTATAACAAA GAAGATGGTA ATTTAGCTAA AGGATTACAA ACCATCAACG
      Q A A E Q A G F Y T T G N N D W Y Y K Q E D G N L A K G L O T I N G
      "A" repeat
701     GTCAAACCTT TTAATTCGAT ACCAATACGG GTAAACAAGT TAAAGGTTCT GCTGTTACAA TTGATGGTAA AGAATACTAT TTCGACCAAG ATACTGGTGA
      Q T L Y F D T N T G K Q V K Q S A V T I D G K E Y Y F D Q D T G D
      "C" repeat
801     CATGTGGAAG GATCGTTTCC GTCAAATTGA TAAGCAAGAC TATCGCGGTG TTGCTCCAGG TTCGAAGGTT GGGATTGCTT GGCTCTATTA CCAAGCAGAT
      M W K D R F R Q I D K Q D Y R G V A P G S K V G I A W L Y Y Q A D
      "A" repeat
901     GGTTCGGTTG CTTCAAGGTT GACCAATACA CCTGATGGCC GCACTCTCAT GTTTAATACT TATAATCAG AGCAAGTCAA GGGTAAACTG GTCAATACCG
      G S V A S G L T N T P D G R T L M F N T Y N H E Q V K G K L V N T D
1001    ACGGTAGCAA CTATCGTTAC TTGACCTGAC ATACCGGTGA CATGTTGCGT AATACAAGCA TCTATGATGG TAGCCAAAAA TACAACATCG ATGAGAACCG
      G S N Y R Y F D L H T G D M L R N T S I Y D G S Q K Y N I D E N G
      "C" repeat
1101    TATTGCAACC AAGGCCTAAG CAACAATTAT TTTCTACTC TAACGTGAGA ACCTTCTTTT GAGGAGGTTT TTTTGTGATG
      I A T K A *
  
```

FIG. 5. Nucleotide sequence of coding and control regions of the *dei* gene from pYA2653, which is derived from *S. sobrinus* UAB108 (serotype g). Only the sequence of the anticoding strand is shown. Numbering is from the 5' end. The potential Shine-Dalgarno ribosome-binding site is underlined and labeled SD. The translation initiates from ATG (Met) and is underlined. Two opposite arrows downstream of the termination codon (identified by *) represent a potential stem-loop structure. The signal sequence cleavage site is shown by the vertical arrow. The two A and C repeat regions are underlined and labeled.

FIG. 6. Amino acid compositions of the A and C repeats and the spacing intervals separating them in Dei, GTFs, and GBP. GTF-I is the *gtf* gene product from *S. downei* MFe28 (10), GTF-B and GTF-C are from *S. mutans* GS-5 (49, 57), GTF-S is from *S. downei* MFe28 (14), GTF-T is from *S. sobrinus* OMZ176 (20), and GBP is from *S. mutans* Ingbritt (3). The numbers at the left indicate locations of the repeats in the proteins. The shaded residues correspond to amino acids which are conserved through the majority of the repeats and intervals. a.a., amino acid residues.

It had been found that another protein which can inhibit the activity of dextranase exists in oral streptococci (19). Our previous work also indicated that some Dex⁻ mutants possessed dextranase but produced a Dei (60). We previously obtained the Dei protein from a Dex⁻ Dei⁺ mutant UAB108 of *S. sobrinus* and showed that it is heat stable: after 15 min of

boiling, it still maintains 100% of inhibiting activity when assayed at 37°C. It can be degraded by trypsin, however, and thus possesses characteristics of a protein (60). In this study, the *dei* gene has been cloned and sequenced. The recombinant plasmid can specify synthesis of the Dei protein either in minicells or in *E. coli* cells. The Dei protein possesses dextran-

FIG. 7. Southern hybridization of a 0.7-kb fragment, located inside the *dei* gene recovered after *Hind*III-*Cla*I double digestion of pYA2653 DNA, to DNAs from some *E. coli* recombinant clones and to various chromosomal DNAs from strains of the mutans group of streptococci. All DNA preparations were digested to completion with *Hind*III, and the amount of DNA loaded on the gel was adjusted according to the fluorescence intensity of ethidium bromide-stained gel to make the concentration of the DNA from different serotypes as consistent as possible. Lanes: 1, χ 2819(pYA3009; *dex* clone); 2, χ 2819(pYA2653; *dei* clone); 3, HS6 (serotype *a*); 4, BHT (serotype *b*); 5, Ingbritt (serotype *c*); 6, OMZ176 (serotype *d*); 7, LM7 (serotype *e*); 8, OMZ175 (serotype *f*); 9, UAB66 (mutant of 6715, serotype *g*); 10, UAB108 (mutant of UAB66, serotype *g*). (A) Ethidium bromide-stained gel; (B) autoradiogram of the blotted DNA after 20 h of exposure.

ase-inhibiting activity, as revealed by both renatured BD-SDS-PAGE or Somogyi assay, and is heat stable. The *dei* gene encodes a single mature protein chain of 292 amino acids with molecular weight of 31,372 and a signal peptide of 38 amino acids. Further experiments are being carried out to identify the mechanism of Dei inhibition of Dex and the contribution of Dei to WSG synthesis and to determine why these Dex⁻ Dei⁺ mutants, such as UAB108, can produce huge amounts of WSG which inhibit plaque formation and adherence by the mutans group of streptococci.

The molecular weight of intact Dei synthesized by the recombinant *E. coli* clone as determined from the deduced amino acid sequence is 36,148. This value is smaller than that determined by SDS-PAGE with extracts from the clones or from minicells, both yielding two bands of 50 and 43 kDa. As with *nusA* and many other gene products (3, 25, 66), this discrepancy in molecular size is probably due to a large number of acidic residues which give a low pI value of 4.15. The Dei band of 43 kDa on BD-SDS-PAGE appears to be the mature Dei protein after secretion into the periplasm, whereas

the 50-kDa form probably contains the signal sequence. This is likely to be the reason why we see a much higher ratio of 50-kDa band to 43-kDa band for a total protein sample than that from a periplasmic fraction sample on BD-SDS-PAGE (data not shown). The low-molecular-mass bands shown in Fig. 2 are thought to be breakdown products. It appears that a single *dei* gene, and furthermore a single *dei* gene product, is present in *S. sobrinus*, since the subclones of *dei* derived from *S. sobrinus* expressed in *E. coli* display proteins of the same molecular masses on BD-SDS-PAGE compared with those of *S. sobrinus* (Fig. 2). Almost no degraded products can be observed if we grow the bacteria in THB or BHI medium instead of in FMC medium (60).

Southern hybridization (Fig. 7) and BD-SDS-PAGE (Fig. 2) results revealed no differences in the *dei* genes or gene products from strains OMZ176 (serotype *d*) and 6715, UAB66, and UAB108 (serotype *g*), all of which belong to the species *S. sobrinus*. However, differences were observed in strain MFe28 (*S. downei*, serotype *h*) in Southern hybridization and in strain HS6 (*S. cricetus*, serotype *a*) in both Southern hybridization and BD-SDS-PAGE patterns. Recently, we found that Dei possesses high specificity (53): Dei derived from *S. sobrinus* UAB108 (serotype *g*) can inhibit Dex only from *S. sobrinus* (serotypes *d* and *g*), *S. downei* (serotype *h*), and *S. macacae* (serotype *h*). These results probably reflect the relationship among different species of the mutans group of streptococci in taxonomy: in this category, serotype *g* is most closely related to serotype *d*, then to serotype *h*, then to serotype *a*, and then to other serotypes.

There are several similarities in amino acid composition and properties among Dei, GTFs, and GBP (Table 3). Ferretti and colleagues discovered that the A and C repeat units are widespread in GTFs and GBP and suggested that they are probably involved in glucan binding (3, 10, 45). These A and C repeats are also present in the Dei protein. However, the spacing intervals which separate the A repeat from the C repeat, and the length of the C repeat, are somewhat different in these different proteins (Fig. 6). The repeat structures in Dei are similar to those in GTFs, with spacing intervals of five amino acids, but with a longer C repeat region as in GBP and some GTFs. Some repeats have been reported to exist in the C termini of some other proteins from *S. pneumoniae* (12), pneumococcal phage (11), and *Clostridium difficile* (9), but the homologies of those repeats with Dei are limited. Wren suggested that there is some evolutionary relationship among them (64). Yother and Briles observed the homologies among the repeats of PspA and other proteins and suggested a direction of evolution, i.e., PspA $\leftrightarrow$ the lysins of *S. pneumoniae* and phages $\leftrightarrow$ ToxA of *C. difficile*, GBP, and GTF-S (66). We think that Dei could probably be placed between GBP and GTF in their evolution map.

We have also found that Dei can bind to a Sephadex or BD-Sepharose 4B column and be eluted by 6 M guanidine as

TABLE 3. Comparison of Dei specified by pYA2656 with other cloned *gtf* and *gbp* gene products^a

Protein	Source	Serotype	Mol wt	No. of residues	Signal peptide length (residues)	pI	No. of A repeats	No. of C repeats	Reference
Dei	<i>S. sobrinus</i> UAB108	<i>g</i>	31,372	292	38	4.15	2	2	This report
GTF-I	<i>S. downei</i> MFe28	<i>h</i>	172,983	1,559	38		6	5	9
GTF-B	<i>S. mutans</i> GS-5	<i>c</i>	162,300	1,441	34	6.87	6	4	44
GTF-C	<i>S. mutans</i> GS-5	<i>c</i>	149,000	1,341	34	9.4	3	2	52
GTF-S	<i>S. downei</i> MFe28	<i>h</i>	147,408	1,328	36/37	5.28	4.5	4	11
GBP	<i>S. mutans</i> Ingbritt	<i>c</i>	59,039	528	35		5.5	4	3

^a None of the proteins contained cysteine, and all had glucan-binding ability.

described for GTFs and GBP. The glucan-binding ability of Dei is even stronger than of GBP or GTFs (unpublished data). It is an attractive hypothesis that these enzymes or proteins concerned with glucan metabolism in oral streptococcal strains contain the same structure for glucan binding. They differ, however, in molecular mass, with GTF-S being 160 kDa, GBP being 60.5 kDa (36), and Dei being only 31.4 kDa. It is unlikely that Dei is a deletion derivative of GTF or GBP since there is no significant homology between Dei and these proteins apart from the signal peptide and the A and C repeat regions. In addition, no GTF activity or GTF-inhibiting activity could be detected in Dei samples, and we also did not find any Dei activity in GTFs and GBP prepared from recombinant *E. coli* clones synthesizing GTFs or GBP (data not shown). These data indicate that Dei is a special kind of GBP which can uniquely inhibit dextranase activity. Recently we have obtained data concerning the characterization of the *dei* gene product, its inhibition mechanism of dextranase, and the analysis of its contribution to glucan metabolism (52, 53). These results will be published elsewhere. However, elucidation of the regulation mechanism for the synthesis of GTFs, GBP, Dex, and Dei and of the functional interactions of these proteins leading to the formation of dental plaque will require further research.

ACKNOWLEDGMENTS

This work was supported by grant DE06673 from the National Institutes of Health.

We thank A. L. Honeyman for help with computer DNA sequence analysis and discussions during this work and P. Brown for critical review of the manuscript.

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