Isolation, characterization and sequence analysis of the *scrK* gene encoding fructokinase of *Streptococcus mutans*

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A gene encoding an ATP-dependent fructokinase from *Streptococcus mutans* GS-5 was identified within a 2 kb DNA fragment immediately downstream from the *scrA* gene. The gene cloned in *Escherichia coli* also expressed mannokinase activity. Insertional inactivation of this gene in *S. mutans* markedly decreased both fructokinase and mannokinase activities. Nucleotide sequence analysis of the 2 kb fragment revealed an ORF starting 199 bp downstream from the *scrA* gene, preceded by potential ribosome-binding (Shine–Dalgarno) and promoter-like sequences. This ORF specified a putative protein of 293 amino acids with a calculated M_r of 31 681. The deduced amino acid sequence of the fructokinase gene, *scrK*, from *S. mutans* exhibited no significant similarity to fructokinase genes from *Klebsiella pneumoniae*, *E. coli* plasmid pUR400 or *Vibrio alginolyticus*, but was similar to a comparable gene from *Zymomonas mobilis*.

Introduction

Streptococcus mutans has been implicated as the principal etiological agent in human dental caries (Loesche, 1986). The mechanisms by which sucrose is transported and metabolized in this organism have been of particular interest because the resulting acid produced from dietary sugars, including sucrose, can demineralize the enamel tooth surface resulting in dental caries.

Sucrose is transported in this organism primarily by means of a phosphoenolpyruvate-dependent phosphotransferase system (PTS) (Slee & Tanzer, 1982; St Martin & Wittenberger, 1979). We have sequenced and characterized two genes, *scrA* and *scrB*, encoding sucrose Enzyme II of the PTS and sucrose-6-phosphate hydrolase (S6PH), respectively, from *S. mutans* (Sato *et al.*, 1989; Sato & Kuramitsu, 1988). Sucrose transported by the sucrose PTS with concomitant phosphorylation by sucrose Enzyme II at the 6' position of the glucose

moiety results in the formation of sucrose 6-phosphate in the streptococcal cells which is subsequently hydrolysed by S6PH to glucose 6-phosphate and fructose. Intracellular free fructose should then be phosphorylated for further glycolysis probably by an ATP-dependent fructokinase (ATP:D-fructose 6-phosphotransferase, EC 2.7.1.4). Genes encoding fructokinase activity from several organisms (Blatch *et al.*, 1990; Aulkemeyer *et al.*, 1991) have been isolated, sequenced and designated *scrK*. This gene appears to be part of the same regulon containing *scrA* and *scrB*. In addition, another gene, *frk*, from *Zymomonas mobilis* encoding a fructokinase which is not linked to any PTS has been cloned and sequenced (Zembrzuski *et al.*, 1992). In contrast, the *scrA* and *scrB* genes of *S. mutans* are tandemly arranged but are divergently transcribed from opposite DNA strands (Sato *et al.*, 1989). However, because intracellular free fructose would be generated by the sucrose PTS and S6PH as mentioned above, it was assumed that the fructokinase gene in this organism might be located near the *scrAB* region.

In this paper, we describe the isolation, characterization and nucleotide sequence of the *scrK* gene from *S. mutans*, encoding the fructokinase enzyme. The amino acid sequence deduced from the *scrK* gene was also compared with those for other bacterial fructokinases.

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Abbreviations: PTS, phosphoenolpyruvate-dependent phosphotransferase system; S6PH, sucrose-6-phosphate hydrolase.

The nucleotide sequence data reported in this paper have been submitted to DDBJ, EMBL and GenBank and assigned the accession number D13175.

Methods

Bacterial strains. *S. mutans* GS-5, its spontaneous colonization-defective strain, IS3, and *Escherichia coli* strains JM109 and MV1184 were maintained and routinely grown as described previously (Perry *et al.*, 1983; Yanisch-Perron *et al.*, 1985; Vieira & Messing, 1987).

Isolation of the *S. mutans* *scrK* gene. The marker rescue procedure using plasmid pVA891 (Macrina *et al.*, 1983) for isolating chromosomal DNA fragments was described previously (Sato *et al.*, 1992) and utilized to isolate the *scrK* gene. The recovered fragments flanking plasmid pVA891 were characterized following restriction endonuclease digestion with enzymes selected on the basis of the Southern blot hybridization patterns of this region of the chromosome.

Nucleotide sequencing and sequence analysis. All fragments to be sequenced were subcloned into pBluescript II KS(+)/SK(+) (Stratagene). Sequential deletion clones of the fragments in pBluescript were obtained by the exonuclease III and mungbean nuclease system (Takara) (Henikoff, 1984). Sequencing was accomplished by the dideoxy chain-termination method (Sanger *et al.*, 1977), using *Taq* DNA polymerase supplied with the *Taq* Dye Primer Cycle Sequencing Kit (Applied Biosystems), and a thermal cycler (Perkin Elmer Cetus, Model PJ2000). An automated DNA sequencer (Applied Biosystems Model 373A) was utilized to determine the nucleotide sequence of both strands of the entire region with the overlapping clones. Sequence analysis was carried out with the DNASYS-Mac using CD-ROM databases (Hitachi Software Engineering).

Expression of the *scrK* gene with a T7 RNA polymerase/promoter system. The *scrK* gene was subcloned into plasmid pBluescript II SK(+) in the opposite direction relative to the *lacZ* promoter (in the same direction as the T7 promoter). The resultant plasmid, pSRK2, was then introduced into *E. coli* strain K38 harbouring the plasmid pGP1-2 encoding the T7 RNA polymerase and expressed according to the procedure described by Tabor & Richardson (1985). The cells from 10 ml culture of the T7 RNA polymerase expression system were harvested, washed twice with 10 mM-potassium phosphate buffer, pH 6.5, and resuspended in 2 ml of the same buffer. A portion of the suspension was analysed by SDS-PAGE and an aliquot was used for enzyme and protein assays.

SDS-PAGE. A portion of the cell suspension was mixed with the same volume of the SDS sample buffer [125 mM-Tris/HCl, pH 6.8, 2% (w/v) SDS, 10% (v/v) 2-mercaptoethanol, 20% (v/v) glycerol, 0.02% bromophenol blue], boiled for 3 min and frozen until electrophoretic analysis using the gel system of Laemmli (1970) with 12% (w/v) acrylamide gels. The molecular mass of the protein encoded by the *scrK* gene was determined following electrophoresis and staining of the gels with 0.25% Coomassie brilliant blue R250 in methanol/acetate/water (9:2:9 by vol.).

Preparation of cell-free extracts. *E. coli* cells were grown and suspended in 10 mM-potassium phosphate buffer, pH 6.5 as described above. *S. mutans* cells were grown overnight at 37 °C in BTR-sucrose medium as described previously (Sato *et al.*, 1991). The overnight cultures were then inoculated (0.1%) into 250 ml of the same medium and incubated at 37 °C for 15 h. The cells were collected, washed and resuspended in potassium phosphate buffer at a concentration of 0.2 g wet weight ml⁻¹. Both *E. coli* and *S. mutans* suspensions were subjected to mechanical disruption with glass beads in a B. Braun MSK cell homogenizer for 5 and 15 min, respectively, at 4 °C. After the removal of the glass beads and cell debris by low speed centrifugation, the extract was centrifuged at 20000 *g* for 15 min, and the supernatant was subjected to an additional centrifugation at 100000 *g* for 30 min in a Hitachi Himac centrifuge CS120. The resultant supernatant fluid was used as a crude extract and was dispensed into small aliquots,

immediately frozen and stored at -80 °C until required for enzyme and protein assays.

Enzyme assays. Fructokinase activity was determined by a spectrophotometric assay described by Porter *et al.* (1980), with minor modifications. The standard assay mixture contained 2 mM-ATP, 10 mM-MgCl₂, 2 mM-NADP, 0.05–50 mM-fructose (usually 12.5 mM), 200 mM-Tris/HCl buffer, pH 7.4, 2.8 IU glucose-6-phosphate isomerase (EC 5.3.1.9), 1.5 IU glucose-6-phosphate dehydrogenase (EC 1.1.1.49), and 0.5 to 20 µl of crude enzyme preparation in a final volume of 0.5 ml incubated at 25 °C. For determination of manno-kinase activity, mannose was substituted for fructose, and 2.8 IU mannose-6-phosphate isomerase (EC 5.3.1.8) was added to the reaction mixture described above. Glucokinase activity was also determined by replacing fructose with glucose and omission of glucose-6-phosphate isomerase from the assay mixture. Kinase activities were completely dependent on the presence of ATP, NADP, Mg²⁺, the appropriate sugar and enzyme extract. 1- and 6-phosphofructokinase activities with *E. coli* crude extracts were determined with the NADH-coupled spectrophotometric method of Ferenci & Kornberg (1971) with minor modifications. Under these conditions, the amount of phosphorylated products formed in the assay is proportional to the amount of NADP reduced or NADH oxidized as measured by the change in absorbance at 340 nm. Protein concentrations of the crude extract were determined by the method of Lowry.

Results

*Isolation of the fructokinase gene immediately downstream from the *scrA* gene*

Our previous study (Sato *et al.*, 1989) showed that the *scrA* and *scrB* genes from *S. mutans* GS-5 were divergently transcribed on opposite strands of a 6.6 kb *EcoRI* fragment cloned in the plasmid pMH613 (Fig. 1). Two other potential ORFs, *ds1* and *ds2*, whose functions remain to be determined were located downstream from the *scrB* gene on the *EcoRI* fragment and a truncated ORF, ORF3, was located downstream from the *scrA* gene (Fig. 1). To clone the intact ORF3, a DNA fragment flanking the *EcoRI* site (map position 6.6, Fig. 1) was initially isolated from the *S. mutans* GS-5 chromosome by a marker rescue method. Following integration into and excision from the chromosome with the marker rescue plasmid, the region was isolated in pSW73B (Fig. 1). Since plasmid pSW73B did not contain the promoter and 5' region of ORF3, both the *PvuII*-*EcoRI* fragment (map position 5.8–6.6) from pMH613 and the *EcoRI*-*SacI* fragment (map position 6.6–7.9) from pSW73B were subsequently inserted into pBluescript II SK(+) in the opposite orientation to the *lacZ* promoter. The resultant plasmid containing the intact ORF3 fragment was designated pSRK2 (Fig. 1). *E. coli* JM109 harbouring pSRK2 expressed fructokinase activity probably by transcription from the promoter of ORF3. The activity was about ten times higher than that of the control strain, JM109 harbouring pBluescript II SK(+). Therefore, the ORF3 encoding this activity was designated the *scrK* gene.

Fig. 1. Restriction map of plasmids pMH613, pSW73B, pSRK2 and pSWM6. Plasmid pMH613 contains a 6.6 kb *EcoRI* fragment from GS-5. The location and direction of transcription of the *scrA*, *scrB*, *ds1* and *ds2* genes are indicated by arrows. Circles at the end of arrows represent putative promoter sites. ORF3 was truncated at the end of the fragment. Plasmid pSW73B was constructed from a GS-5 chromosomal fragment downstream from the *scrA* gene as described in Methods. Plasmid pSRK2 contains the intact *scrK* gene and was utilized for ScrK expression with a T7 RNA polymerase system. Plasmid pSWM6 was utilized for inactivation of the *scrK* gene on the GS-5 chromosome as described in Results. A, *AluI*; B, *BamHI*; E, *EcoRI*; EV, *EcoRV*; H, *HindIII*; Pv, *PvuII*; S, *SacI*; tet, tetracycline resistance gene.

Nucleotide sequence of the *scrK* gene

The 1404 bp nucleotide sequence shown in Fig. 2 begins at a *PvuII* site (map position 5.8, Fig. 1) and ends 240 bp downstream from the termination codon of the *scrK* gene. The sequence includes the 83 bp 3' region of the *scrA* gene, a 199 bp interval region, the 882 bp *scrK* ORF (position 283–1164, Fig. 2), a 168 bp putative inter-cistronic region and the 72 bp 5' region of a downstream ORF. The *scrK* gene encodes a 293 amino acid protein with a calculated M_r of 31681. A potential ribosome-binding site (AAGAGG) could be identified 6 bp upstream from the ATG initiation codon of the *scrK* gene. In addition, a promoter-like sequence (TTGAAA-N₁₅-TATAAT), which resembles the *E. coli* promoter consensus sequence, exists between positions 172 and 198, and is situated 53 bp downstream from the putative transcriptional terminator sequence of the *scrA* gene and 84 bp upstream from the initiator codon of the *scrK* gene. There appeared to be no inverted repeat sequences capable of forming stable hairpin loop structures between the termination codon of *scrK* and the putative initiation codon of the downstream ORF, although several promoter-like sequences were detected in this region (e.g. P1/P1', P2/P2'; Fig. 2).

Comparison of the *S. mutans* fructokinase sequence with other fructokinase sequences

The calculated M_r of the protein deduced from the *S. mutans scrK* gene was similar to proteins from a family of sugar kinases and sugar-phosphate kinases (references in Aulkemeyer *et al.*, 1991). It was therefore of interest to compare the deduced amino acid sequence of the *S. mutans* fructokinase with these related proteins. The

ScrK amino acid sequence was compared with protein databases containing the NBRF (R. 28.0) and SWISS-PROT (R. 18.0) entries, and only the xylose operon repressor protein of *Bacillus subtilis* exhibited significant similarity despite the presence of several fructokinase and phosphofructokinase genes. Recently, the sequence of the *frk* gene encoding fructokinase from *Zymomonas mobilis* has been reported (Zembrzusi *et al.*, 1992). Homology matrices with an 8 amino acid window indicated that the ScrK protein of *S. mutans* exhibited significant similarity to this protein throughout the entire sequence but no similarity to the ScrK protein of *Klebsiella pneumoniae* (data not shown). The computer-generated maximum-matched alignment of the ScrK protein from *S. mutans* with the Frk protein allowing for gaps (Fig. 3) indicated 38% identical and 58% similar amino acid residues with several highly similar regions. In addition, two ATP-dependent fructokinases have been purified recently from *Lactococcus lactis* (Thompson *et al.*, 1991) and *Fusobacterium mortiferum* (Thompson *et al.*, 1992). N-terminal amino acid sequences of the two enzymes were determined following Edman degradation. Eighteen of the first 25 amino acid positions in the former and 15 of 27 positions in the latter organism were identical to the N-terminal sequence deduced from the *S. mutans* fructokinase gene (data not shown).

Expression and characterization of the ScrK enzyme

To maximize *scrK* expression by pSRK2 which contains the intact *scrK* gene from *S. mutans*, a T7 RNA polymerase expression system was utilized. Cell extracts from each strain were subjected to SDS-PAGE, and a Coomassie blue-stained gel is presented in Fig. 4. Under these conditions, only K38(pGP1-2)(pSRK2) (lane 6)

<i>PvuII</i>	9	18	27	36	45	54
CAG CTG	ATT ATG CGT	TTG AAA	CCC TCG CTT	CTT CAG GAA	CTG TTG CTG	TAG
A D	Y A S V	E T L A	S S	G T V A	V V	G
GTG ATA	GCT TAC TTG	TTA AAA	AAT AAG AAA	TAT TAT CAG	AAA GAC CGT	AAG
D S	L L E V	K K	*..... <i>scrA</i>			
117	126	135	144	153	162	
GTC TTT TTG	ACT GCT TAA	AAG ATT CAG	TAA CAA TAG	TAT TAA AGC	CTT TTG GCT	
171	-35	180	189	-10	198	207
AAC TAA	TAC TTG AAA	TTT AGC AAA	TTA TGA TAT	AAT GTT AAG	TAG TCC TTA	AGG
225	234	243	252	261	270	
GTA GAT	TAA GGG TAT	TCA AAT CCA	AAA ATT GAT	TTG GTA AGT	TAA GTA AAA	TAT
279	288	297	306	315	324	
AAG AGG	TTT ATT ATG	TCT AAA TTA	TAT GGC AGC	ATC GAA GCT	GGC GGA ACA	AAA
S.D.	<i>scrK</i>	M S K L Y G S I	E A G G T K			
333	342	351	360	369	378	
TTT GTC	TGT GCT GTA	GAT GAA AAT	TTT CAA ATT	TTA GAA AAT	GTT CAG TTC	
F V C	A V G D E N	F Q I L E K	V V Q F			
387	396	405	414	423	432	
CCA ACA	ACA CCT TAT	GAA ACA ATA	GAA AAA ACA	GTT GCT TTC	TTT AAA AAA	
P T T	T T P E	T I E K	T V A F			
441	450	459	468	477	486	
TTT GAA	GCT GAT TTA	GCC AGT GTT	GCC ATT GGT	TCT GGC CCT	ATT GAT ATT	
F E A	A D L L	A S V A	I G T S	F P I D	I I	
495	504	513	522	531	540	
GAT CAA	AAT TCA GAC	ACT TAT GGT	TAC ATT ACT	TCA ACA CCA	AAG CCA AAC	TGG
D Q N	S D T Y	G Y I T	S T P K	P P N W		
549	558	567	576	585	594	
GCT AAC	GTT GAT TTT	GTC GGC TTA	ATT TCT AAA	GAT TTT AAA	ATT CCA TTT	TAC
A N V	D F V G L I	S K D F	K I P F	I P F Y		
603	612	621	630	639	648	
TTT ACG	ACA GAT GTT	AAT TCT TCT	GCT GAT GGC	ACA ATT GCT	CGT TCA AAT	
F T T	D V N S S A	Y T G E	T I A R	S N		
657	666	675	684	693	702	
GTT AAA	AGT CTG GTT	TAT TAT ACT	ATT GGA ACA	GGC ATT GGA	GCA GGC GCT	ATT
V K S	L V Y T	I G T G	I G A G	A G A I		
711	<i>EcoRI</i>	720	729	738	747	
CAA AAT	GGC GAA TTC	ATT GGC GGT	ATG GGA CAT	ACG GAA GCT	GGA CAC GTT	TAC
Q N G	E F I G	G M G H	T E A G	G H V Y		
765	774	783	792	801	810	
ATG GCT	CCG CAT CCC	AAT GAT GTT	CAT GGT TTT	GTA GGC ACC	TGT CCT TTC	
M A P	H P N D	V H E H	G T F			
819	828	837	846	855	864	
CAT AAA	GGC TGT TTA	GAA GGA CTT	GCA GCG GGT	CCT AGC TTA	GAG GCT CGT	ACT
H K G	C L E L	A A G F	S L E A	G A R T		
873	882	891	900	909	918	
GGT ATT	CGT GGT GAG	TTA ATT GAG	CAA AAC TCA	GAA GTT TGG	GAT ATT CAG	GCA
G I R	G E L I	E Q N S	E V W D	I Q A		
927	936	945	954	963	972	
TAC TAC	ATT GCT CAG	GCG GCT ATT	CAA GCG ACT	GTC CTT TAT	CGT CCG CAA	GTC
Y Y I	A Q A A	I Q A T	V L Y R	P P Q V		
981	990	999	1008	1017	1026	
ATT GTA	TTT GGC GGA	GGC GTT ATG	GCA CAA GAA	CAT ATG CTC	AAT CGG GTT	CGT
I V F	G G G V	M A Q E	H M L N	R V R		
1035	1044	1053	1062	1071	1080	
GAA AAA	TTT ACT TCA	CTT TTG AAT	GAC TAT CTT	CCA GTT CCA	GAT GTT AAA	GAT
E K F	T S L L	N D Y L	P V P D	V P D K	D	
1089	1098	1107	1116	1125	1134	
TAT ATT	GTG ACA CCA	GCT GTT GCA	GAA AAT GGT	TCA GCA ACA	TTG GGA AAT	CTC
Y I V	T F A E	N G S A	T L G N	L		
1143	1152	1161	1170	1179	P1'	1188
GCT TTA	GCT AAA	AAG ATA	GCA GCG CGT	TAA TTA	AAA ATG	AAT TGG AAG ATT AAA
A L A	K K I A	A R *				
1197	P1*	1206	1215	1224	1233	1242
GCA CCT	TCT AAT ATT	CAA TAT TAA	ACT GTT AGA ATT	TAC GTG AAC	GAA ATT TTC	
P2'	1251	1260	1269	P2'	1278	1287
ATT TTA	TGA GGA TAA	TGA AGT GAA	TAT AAT TAC	TCT TGA TTT	CCT CTG AAA	CTA
1305	1314	1323	1332	1341	1350	
GAT AGT	GGT ATA TTG	AAA AAC AGA	AAG GAG AAC	ACT ATG	GAA GGA	CCT TTG TTT
		S.D.		M E G P	L F	
1359	1368	1377	1386	1395	1404	
TTA CAA	TCA CAA	ATG CAT AAA	AAA ATC	TGG GGC	GGC AAT	CGG CTC
L Q S	Q M H K	K I W G	G G N R	L R K E		

Fig. 2. Nucleotide sequence and deduced amino acid sequence of the *scrK* region. Numbering begins at the *PvuII* site (map position 5·8, Fig. 1) and the sequence ends 240 bp downstream from the termination codon of the *scrK* gene. The sequence contains the 3' region of the *scrA* gene, the *scrK* gene and the 5' region of a putative ORF. The deduced amino acid sequences specified by the ORFs are presented below the nucleotide sequence. The opposing arrows downstream from the *scrA* gene denote an inverted repeat. -35 -10, P1/P1' and P2/P2' denote putative promoters of the *scrK* gene and the downstream ORF, and S.D. denotes ribosome-binding (Shine-Dalgarno) sequences.

ScrK	1	10	20	30	40	50	60	70
Frk	M---	SKLYSGTEAGG	TKFVCAVGDEN	FOILEKVOPFTT	TPYETIEKTVAF	FKK----	FEADLASVAIG	SF
	*	*****	*	*	*****	*	*	*****
	MKNDKKIY	GCIEGGG	TKFMLAL	IDSRLMLA	VERVPTT	PEETLGKSV	FFKKALPQY	ADSFASFGIA
	1	10	20	30	40	50	60	70
ScrK	71	80	90	100	110	120	130	140
Frk	GPIDIDQNSD	TYGYITST	PKFNMWNV	DVFLGSKDF	KIPFYFTT	DVNSSAYGETI	--ARSNVKSL	VYITI
	*	*	*****	*	*	*****	*	*****
	GPICLDRKSP	KWGYITN	PKFPMWNT	DVVTPFKEA	FGCPVEID	TDVNGAAL	ENFWGASK	GTHTSVYVTV
	71	80	90	100	110	120	130	140
ScrK	141	150	160	170	180	190	200	210
Frk	GTGIGAGAI	QNGEFIGM	GHTEAGHV	MAPNDVH	HGFVGTCP	PHKGCLE	GLAAGPS	LEAR--TGIRGE
	*	*	*****	*	*	*****	*	*****
	GTGFGGVL	IDGKPIB	GLAPFEM	GHGIP	IRHPDD--	RDFRGCC	PHYGGCYE	GLASGTAIR
	141	150	160	170	180	190	200	210
ScrK	211	220	230	240	250	260	270	280
Frk	LIEQNSEV	WDIQAY	YIAQA	IATVLYR	PQVIVFG	GGVMAQEH	MLNRVRE	KFTSLNDYLP
	*	*	*****	*	*	*****	*	*****
	EPAEERK	AREIIA	FLAHFV	ILQAFIS	PERIVFG	GGVMHVG	MLASVRQ	TAEIANSYF
	211	220	230	240	250	260	270	280
ScrK	281	290	300					
Frk	TPAVAENG	SATLGNL	LALAKKIA					
	*	*	*****	*	*	*****	*	*****
	LPGLGDC	--AGMGA	FALALAAE					
	281	290	300					

Fig. 3. Comparison of the amino acid sequence of *S. mutans* fructokinase (ScrK) with that of *Z. mobilis* fructokinase (Frk). Computer-generated maximum-matched alignment of the ScrK and Frk proteins allowing gaps (hyphens). Identical and similar amino acid residues are marked as asterisks and dots, respectively, between the two sequences. The numbers indicate the relative (not actual) position of each amino acid.

contains a protein band with an M_r of around 32000. The apparent molecular mass of this protein was almost the same as that calculated from the protein sequence deduced from the *scrK* gene. The crude extract of strain K38(pGP1-2)(pSRK2) phosphorylated 18·7 μmol of fructose and 4·27 μmol of mannose min^{-1} (mg protein^{-1}) (Table 1) when the sugar concentration of the assay mixtures was 12·5 mM, and apparently phosphorylated no glucose even when the glucose concentration was increased to 50 mM (data not shown). Fructokinase activity of the extract was inhibited by mannose but not by glucose (data not shown), whereas the *Z. mobilis* fructokinase is strongly inhibited by glucose (Zembrzuski *et al.*, 1992). The *scrK* gene expressed neither 1- nor 6-phosphofructokinase activities. Using crude extracts with relatively high specific activities prepared from the T7 RNA polymerase expression system, several kinetic parameters of the ScrK were determined. The apparent K_m and $V_{\max}$ values were 0·77 mM, 20·0 $\mu\text{mol min}^{-1}$ (mg protein^{-1}) for fructose and 1·00 mM, and 5·0 $\mu\text{mol min}^{-1}$ (mg protein^{-1}) for mannose, respectively. In addition, mannose acted as a competitive inhibitor of fructose phosphorylation. The apparent K_i value for mannose with respect to the fructokinase reaction was 0·44 mM. These K_m and K_i values were similar to those reported previously for the mannofructokinase partially purified from *S. mutans* SL-1 (*S. sobrinus* SL-1) (Porter *et al.*, 1980).

Inactivation of the *scrK* gene in *S. mutans*

To confirm whether or not the *scrK* gene codes for the sole fructokinase in *S. mutans* strain GS-5IS3, the *scrK*

Fig. 4. Coomassie blue-stained 12% (w/v) polyacrylamide gel of the T7 RNA polymerase expression products. Sample extracts were prepared as described in Methods. Lanes: 1 and 2, 5 μ l extracts from *E. coli* JM109(pBluescript II) (1) and JM109(pSRK2) (2) without the T7 RNA polymerase-expressing plasmid pGP1-2; 3, M_r markers; 4 to 6, 10 μ l extracts from *E. coli* K38(pGP1-2) (4), K38(pGP1-2)(pBluescript II) (5) and K38(pGP1-2)(pSRK2) (6) induced as described in Methods. The arrow indicates the ScrK protein.

Table 1. *Fructokinase (FK) and mannokinase (MK) activities of the E. coli clone and S. mutans*

Cells were grown and extracts were prepared and assayed as described in Methods. The experiments were repeated three times without significant differences: the results shown are representative.

Sugar* concn (mM)	Enzyme activity [μ mol min ⁻¹ (mg protein) ⁻¹]					
	<i>E. coli</i>		<i>S. mutans</i>			
	K38(pGP1-2)(pSRK2)		GS-5IS3		LSWMS2 (<i>scrK</i>)	
	FK	MK	FK	MK	FK	MK
50	ND	ND	0.426	0.157	0.021	0.052
12.5	18.7	4.27	0.348	0.117	0.006	0.018
1.25	13.2	2.65	0.239	0.101	< 0.002	< 0.002

ND, Not determined.

* Fructose or mannose concentration in the standard assay mixtures.

gene in pSWM6 was insertionally inactivated with the streptococcal tetracycline resistance gene (*tet*) (Perry & Kuramitsu, 1989) at the unique *Eco*RI site (Fig. 1). The plasmid pSWM6 was linearized and introduced back into *S. mutans* GS-5IS3 following transformation. After confirmation of the predicted double cross-over-integration event of the fragment into the chromosome by Southern analysis (data not shown), crude extracts

were prepared from the cells of the mutant, designated LSWMS2, and the parental strain GS-5IS3 grown with sucrose. The mutant was completely devoid of fructokinase as well as mannokinase activity when the sugar concentration of the assay mixtures was less than 1.25 mM and exhibited low activities compared to GS-5IS3 when tested with higher sugar concentrations (Table 1). Nevertheless, both strains LSWMS2 and GS-5IS3

reached the same growth yield with sucrose [$Y_{\text{scr}} = 0.2 \text{ mg dry weight (mg sucrose)}^{-1}$], although LSWMS2 grew slightly slower in BTR-sucrose broth.

Discussion

The nucleotide sequence of the region downstream from the *scrA* gene revealed that there was an 882 bp ORF in the same orientation as the *scrA* gene. This ORF is the *scrK* gene encoding the mannofructokinase activity since the cloned gene in *E. coli* expressed high fructokinase as well as mannokinase activities, but no 1- or 6-phosphofructokinase, or glucokinase activities. To our knowledge, this sequence represents the first complete sequence of a gene encoding mannofructokinase activity. The *scrK* gene specifies a protein with a M_r of 31 681 which agrees well with the value estimated from SDS-PAGE (Fig. 4). However, this value is different from the M_r (49 000) estimated previously by gel filtration for the purified mannofructokinase of *S. mutans* strain SL-1 (Porter *et al.*, 1980). Although this difference cannot be simply explained by dimerization of the protein, Thompson *et al.* (1991) observed a similar discrepancy for the *L. lactis* fructokinase I. This difference in molecular size estimates was apparently due to a concentration-dependent monomer-dimer equilibrium which confers the anomalous M_r deduced by gel filtration.

A kinetic study with the *scrK* gene product revealed that mannose was a competitive inhibitor of fructose phosphorylation and glucose was neither an inhibitor nor a substrate for the enzyme. On the other hand, the *K. pneumoniae* fructokinase (ScrK) did not phosphorylate mannose nor was the phosphorylation of fructose inhibited by mannose (Sprenger & Lengeler, 1988), whereas the *Z. mobilis* fructokinase is strongly inhibited by glucose (Zembrzusi *et al.*, 1992). In this respect, the *S. mutans* fructokinase encoded by the *scrK* gene appears to be distinct from both the *Z. mobilis* fructokinase (Frk) and the *scrK* gene products from *K. pneumoniae*, *E. coli* pUR400 and *V. alginolyticus*. Comparisons of the amino acid sequence of these fructokinase proteins revealed that the ScrK protein from *S. mutans* was most similar (58%) to the Frk protein from *Z. mobilis*. Moreover, the N-terminal amino acid sequence of this ScrK protein exhibited strong similarity to the comparable regions of the purified fructokinases from *L. lactis* and *F. mortiferum*. These results tentatively suggest that the *S. mutans* fructokinase together with the fructokinases from *L. lactis*, *F. mortiferum* and *Z. mobilis* might form a fourth subfamily in addition to the three sugar kinase subfamilies proposed by Aulkemeyer *et al.* (1991). However, such a classification will depend on the determination of the complete sequences of the fructokinases from *L. lactis* and *F. mortiferum*. Very recently,

nucleotide sequences of the *sacAB* genes coding for S6PH and sucrose PTS Enzyme II from *L. lactis* Tn5276 have been reported (Rauch & de Vos, 1992). The gene arrangement of this region was quite similar to that of the comparable chromosomal region of *S. mutans*. It will be of interest to determine whether or not the *scrK* gene of this organism is arranged similarly to that of *S. mutans*. It is also of interest that the gene arrangement of *scrK* relative to *scrA* and *scrB* in *S. mutans* is quite distinct from that of comparable genes from *K. pneumoniae* (*scrK/YAB*), *E. coli* pUR400 (*scrK/YAB*) or *V. alginolyticus* (*scrAKB*) (Wehmeier *et al.*, 1989; Schmid *et al.*, 1988; Blatch *et al.*, 1990).

The *S. mutans scrK* gene is preceded by a typical ribosome-binding (Shine-Dalgarno) sequence and promoter-like sequences were located 164 bp downstream from the putative *scrA* transcription terminator sequence. This result, together with expression of the gene in pSRK2, suggests that the *scrK* gene might be transcribed independently of *scrA* transcription. A 168 bp non-coding region was detected downstream from the *scrK* gene. However, no inverted repeat sequences characteristic of transcription terminators could be detected in this region, although several potential candidates for promoter regions were detected. This suggests that the ORF might be co-transcribed with the upstream *scrK* gene. Further analysis is necessary to elucidate the transcription start site and size of the *scrK* transcript together with determination of the complete sequence of the downstream ORF. These approaches are currently in progress in our laboratories.

Introduction of the inactivated *scrK* gene back into *S. mutans* severely decreased fructokinase activities. However, the *scrK* mutant of *S. mutans*, LSWMS2, gave the same Y_{scr} as parental strain GS-5IS3. This suggested that the fructose moiety of sucrose could be phosphorylated in LSWMS2 by another enzyme(s) exhibiting fructokinase activity or by the fructose PTS following hydrolysis of sucrose with a variety of extracellular enzymes of this organism (e.g. glucosyltransferases, fructosyltransferase and fructanase). The latter possibility is unlikely since almost 90% of the sucrose metabolized by *S. mutans* strains is transported into the cells (Tanzer *et al.*, 1972). Alternatively, intracellular fructose generated by the sucrose PTS and S6PH could be released back to medium by an unknown mechanism, in order to be phosphorylated by the fructose PTS. A more likely explanation is based on the observation of Thompson *et al.* (1991) that *L. lactis* expresses a second fructokinase II synthesized independently of the fructokinase I linked to the sucrose PTS. Therefore, the presence of a second fructokinase with a weak affinity for fructose in *S. mutans* is compatible with the results indicating that extracts of LSWMS2 exhibited low but

significant fructokinase activity when the fructose concentration of assay mixture was raised to 50 mM (Table 1). However, further investigation will be necessary to clarify the pathway of fructose phosphorylation in *S. mutans*.

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