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Characterization of the Major Promoter for the Plasmid-Encoded Sucrose Genes *scrY*, *scrA*, and *scrB*

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Sucrose genes from a *Salmonella thompson* plasmid were cloned in *Escherichia coli* K-12. A physical map and a genetic map of the genes were constructed, revealing strong homology with the *scr* regulon from the *Salmonella typhimurium* plasmid pUR400. Two promoters were examined after being subcloned into transcriptional fusion vectors. Primer extension analysis and site-directed mutagenesis were used to identify the precise location of the promoter of *scrY*, *scrA*, and *scrB*. Transcription from this promoter was regulated over a 1,000-fold range by the combined effects of ScrR-mediated repression and catabolite repression. A putative cyclic AMP receptor protein binding site centered 72.5 bp upstream of the start point of transcription of *scrY* appeared to be essential for full activity of the *scrY* promoter. Transcription from the putative *scrK* promoter was far less sensitive to repression by ScrR. In ScrR$^+$ cells, readthrough transcription from the putative *scrK* promoter into *scrY* accounted for less than 10% of *scrY* expression.

The utilization of sucrose has been extensively studied in gram-positive (*Bacillus subtilis* [33] and *Streptococcus mutans* [24]) and gram-negative (*Klebsiella pneumoniae* [30, 35] and *Vibrio alginolyticus* [4, 5, 28]) bacteria. In these organisms, sucrose is taken up via the phosphoenolpyruvate-dependent carbohydrate phosphotransferase system and enters the cell as sucrose 6-phosphate, which is cleaved by an intracellular hydrolase to yield glucose 6-phosphate and fructose.

Klebsiellae are the only members of the family *Enterobacteriaceae* to demonstrate a stable sucrose-positive (Scr$^+$) phenotype. *Escherichia coli* and salmonellae are normally unable to ferment sucrose, but Scr$^+$ isolates of both species have been described, the Scr$^+$ phenotype in most cases being specified by conjugative or mobilizable plasmids (3, 22, 29, 36). One well-characterized example is the *Salmonella typhimurium* plasmid pUR400 (10, 14, 16, 18, 25–27), which carries an *scr* system closely related to that of *K. pneumoniae* (14, 26). The pUR400 system (Fig. 1A) contains five genes, three of which (*scrY*, *scrA*, and *scrR*) have been sequenced (10, 14, 16, 26). Expression of *scrKYAB* is regulated by the ScrR repressor and is inducible by growth on sucrose, fructose, and fructose-containing oligosaccharides (27). Catabolite repression is also involved in the control of the expression of at least two of the genes (*scrA* and *scrB*). Three physiological promoters (*scrKp*, *scrYp*, and *scrRp*) are known (26), but although the location of one (*scrYp*) has been inferred by primer extension (14), none has been subjected to mutational analysis. It is also not known whether transcriptional readthrough from *scrK* into the downstream genes is significant.

We examine here the organization and regulation of sucrose genes carried on a large conjugative plasmid (Sac) from *Salmonella thompson* and establish that these genes form a regulon very similar to that of pUR400. This article also describes the analysis of (i) the regulatory region upstream of *scrY*, including identification of the promoter, and (ii) the degree of transcription termination between *scrK* and *scrY*.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and phage. *E. coli* K-12 strain JP3561 (*thr-1 leuB6 ΔlacZM15 supE44 tonA379 aroL478*::Tn*10*), used as an Scr$^-$ and Lac$^-$ host in this work, has been described previously (37). Plasmids are listed in Table 1, except for derivatives of pMU3001 and pMU3006, which are described in Table 4. The phages M13tg130 and M13tg131 have been described elsewhere (17).

Media and chemicals. The minimal medium (MM) used was half-strength buffer 56 (21), supplemented with the appropriate sugar at 0.2%, thiamine (10 μg/ml), and necessary growth factors. Chemicals and enzymes were obtained commercially and were not purified further. Ampicillin and chloramphenicol were used at a final concentration of 50 μg/ml; kanamycin, trimethoprim, and tetracycline were used at final concentrations of 20, 10, and 5 μg/ml, respectively. [α-^{35}S]dATP (1,200 Ci/mmol) and [U-^{14}C]sucrose were from Amersham.

Recombinant DNA techniques. The isolation and manipulation of DNA were performed essentially as described by Maniatis et al. (19). DNA sequence was determined by the chain termination method of Sanger et al. (23). The MELBDBSYS suite of computer programs, adapted by A. Kyne from the DB system of Staden (31, 32), was used for sequence analysis.

Enzyme assays. Sucrose 6-phosphate hydrolase and sucrose uptake activities were determined essentially as described by Schmid et al. (27). β-Galactosidase assays were performed as described by Miller (20). The results given are the averages of at least two separate assays.

Primer extension analysis. The primer extension method of Hudson and Davidson (15) was used. Total cellular RNA was prepared by the method of Aiba et al. (1). The [α-^{35}S]dATP-labelled 154-base primer was isolated after annealing the universal 17-base M13 sequencing primer to

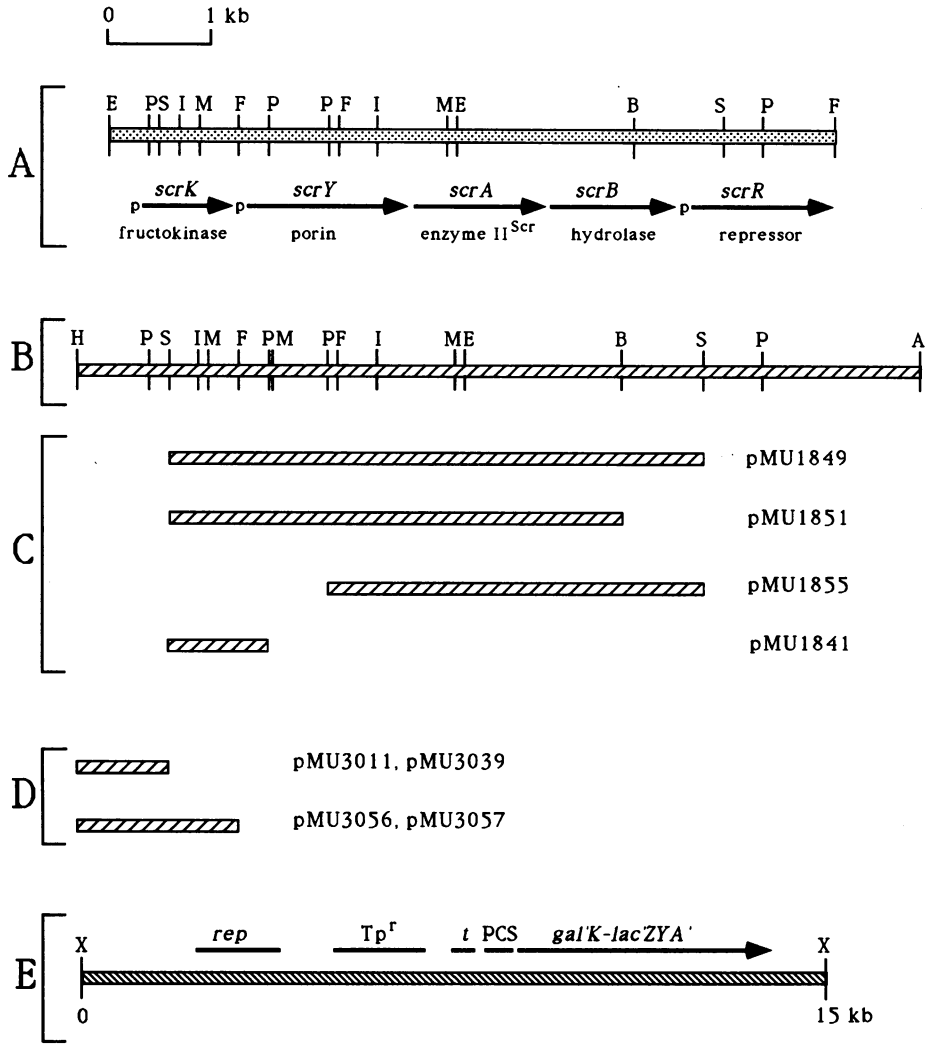

FIG. 1. (A) Restriction and genetic maps of the *scr* regulon of pUR400, adapted from Schmid et al. (25, 26). (B) Restriction map of part of the Scr$^+$ insert carried by pMU1852. (C) Regions of pMU1852 cloned in pMU575 to create the designated plasmids. (D) Regions of pMU1852 cloned in pMU578 or pMU3012 to create the designated plasmids. (E) Restriction and genetic map of pMU575 (note different scale). Key to features (not drawn to scale): *rep*, replication region; Tp^r, trimethoprim resistance gene; *t*, transcription terminator; PCS, polycloning site; *gal'K-lac'ZYA'*, translational fusion necessary for promoter-cloning function of pMU575. Abbreviations for restriction endonuclease sites: E, *Eco*RI; P, *Pst*I; S, *Sal*I; I, *Pvu*I; M, *Sma*I; F, *Sph*I; B, *Bam*HI; H, *Hin*dIII; A, *Hpa*I; X, *Xho*I.

single-stranded mpMU580 (which contains the 1.0-kb fragment shown in Fig. 1C cloned into M13tg130), extending briefly under labelling conditions, cleaving with *Rsa*I at base 835, and denaturing at 100°C.

Site-directed mutagenesis. Site-directed mutagenesis with synthetic oligonucleotides was performed by using a commercially available kit (Amersham). The template used was mpMU579, which was constructed by cloning the *scrY* regulatory region on a 190-bp *Sau*3AI-*Rsa*I fragment (Fig. 2) into M13tg131. Fragments carrying the desired mutations were completely sequenced before being recloned into the pMU575 derivatives pMU578 (*scrR*) and pMU3012 (*scrR*$^+$).

Nucleotide sequence accession number. The nucleotide sequence shown in Fig. 2 has been submitted to GenBank (accession number M63038).

RESULTS

Subcloning and mapping of the Sac plasmid sucrose genes. Smith and Parsell (29) described the transfer from *S. thompson* into *E. coli* K-12 of genes for sucrose utilization via an element referred to as the Sac plasmid. Analysis of the *E. coli* transconjugant strain, kindly provided by H. W. Smith, indicated that the Sac plasmid was very large (approximately 150 kb). The Scr$^+$-determining region was transferred from Sac onto RP4 by in vivo recombination (data not shown), creating RP4-*scr*; subcloning of Scr$^+$ on a 21.5-kb *Hin*dIII fragment from this plasmid into the low-copy-number vector pMU575 (Fig. 1E) created pMU1852.

JP3561/pMU1852 expressed sucrose uptake and hydrolase activities (Table 2), indicating that genes corresponding to *scrA* and *scrB* had been cloned. The inducibility of expres-

TABLE 1. Plasmids used in this study

Plasmid	Relevant phenotype[a]	Source, derivation[b], or reference
Sac	Scr^+	29
RP4	Ap^r Km^r Tc^r	7
RP4-*scr*	Ap^r Km^r Tc^r Scr^+	Scr^+ genes from Sac recombined onto RP4
pACYC184	Cm^r Tc^r	6
pMU575	Tp^r Lac^-	Low-copy-number promoter cloning vector (37)
pMU578	Tp^r Lac^-	$LacY^-$ derivative of pMU575 (J. Praszkier)
pMU1841	Tp^r Lac^+	*scrY* promoter on a 1.0-kb *SalI-PstI* insert in pMU575
pMU1849	Tp^r Scr^+	*scrYAB* on a 5.4-kb *SalI* insert in pMU575
pMU1851	Tp^r Scr^-	0.8-kb *Bam*HI-*Sal*I deletion in pMU1849
pMU1852	Tp^r Scr^+	*scrKYABR* on a 21.5-kb *Hin*dIII insert in pMU575
pMU1855	Tp^r Scr^-	1.6-kb *SalI-Pst*I deletion in pMU1849
pMU1874	Cm^r $ScrR^+$	*scrR* on a 3.0-kb *Bam*HI-*Hpa*I insert in pACYC184
pMU3001	Tp^r Lac^+	*scrY* promoter on a 0.19-kb *Sau*3AI-*Rsa*I insert in pMU578
pMU3006	Tp^r Lac^+ $ScrR^+$	*scrY* promoter on a 0.19-kb *Sau*3AI-*Rsa*I insert in pMU3012
pMU3011	Tp^r Lac^+	*scrK* promoter on a 0.9-kb *Hin*dIII-*Sal*I insert in pMU578
pMU3012	Tp^r $ScrR^+$	*scrR* on a 3.0-kb *Bam*HI-*Hpa*I insert in pMU578
pMU3039	Tp^r Lac^+ $ScrR^+$	*scrK* promoter on a 0.9-kb *Hin*dIII-*Sal*I insert in pMU3012
pMU3056	Tp^r Lac^+	*scrK* on a 1.6-kb *Hin*dIII-*Sph*I insert in pMU578
pMU3057	Tp^r $Lac^{+/-}$ $ScrR^+$	*scrK* on a 1.6-kb *Hin*dIII-*Sph*I insert in pMU3012

[a] The nomenclature for genetic symbols follows that of Bachmann (2).
[b] Derived in this study unless otherwise noted.

sion suggested that *scrR* was also present. When restriction endonuclease sites at one end of the Scr^+ insert were mapped (Fig. 1B), it was apparent that the *scrA*, *scrB*, and *scrR* genes from the Sac plasmid were closely related to those of pUR400 (Fig. 1A). From the conservation of sites upstream of *scrA*, it appeared likely that *scrK* and *scrY* were also contained on pMU1852.

By cloning a 5.4-kb *Sal*I fragment into pMU575 to create pMU1849 (Fig. 1C), it was established that this fragment contained the genes *scrA* and *scrB* but not *scrR* (Table 2). Although reference to the pUR400 *scr* map suggested that pMU1849 did not contain all of *scrK*, strains containing pMU1849 were able to grow on 0.2% sucrose, presumably because the *scrK*-encoded fructokinase is not essential for sucrose metabolism in *E. coli* K-12 (25). Introduction of specific deletions (pMU1851 and pMU1855) (Fig. 1C) provided evidence for the putative gene order *scrA-scrB* (Table 2). Similarly, the filling in by Klenow polymerase of the *Eco*RI site predicted to be within *scrA* resulted in loss of sucrose uptake activity (data not shown).

TABLE 2. Sucrose enzyme activities of JP3561 containing Scr plasmids

Plasmid in JP3561	Added sugar[a]	Uptake of sucrose[b]	Sucrose 6-phosphate hydrolase activity[c]
pMU1852	None	100	3
	Glucose	100	2
	Sucrose	1,400	112
	Fructose	2,700	91
pMU1849	None	3,300	279
	Sucrose	3,300	151
pMU1851	None	7,500	<1
	Fructose	6,500	<1
pMU1855	None	0	6
	Fructose	ND[d]	5

[a] Strains were grown in MM containing 0.2% glycerol, and other sugars were added at 0.2% where indicated.
[b] Expressed as picomoles per minute per milligram (dry weight) of cells.
[c] Expressed as nanomoles per minute per milligram of protein.
[d] ND, not determined.

Subcloning and sequencing of the *scrY* regulatory region. If the gene order in pMU1849 is indeed the same as in pUR400, the putative promoter for *scrY*, *scrA*, and *scrB* should be located on a 1.0-kb *SalI-PstI* fragment (Fig. 1B). Accordingly, pMU1841 (Fig. 1C) was constructed by cloning this fragment into the polycloning site of the promoter-cloning vector pMU575.

Expression of β-galactosidase in glycerol-grown JP3561/pMU1841 was about 2,500 U, fivefold higher than when glucose was the carbon source (Table 3). Addition of cyclic AMP (cAMP) in the latter case abolished this difference. When ScrR was provided in *trans* by introducing RP4-*scr* into JP3561/pMU1841, expression was repressed during growth on glucose or glycerol and induced by growth on sucrose. Interestingly, the addition of cAMP also stimulated β-galactosidase synthesis in the case of sucrose-grown cells.

The nucleotide sequence of the 1.0-kb insert in pMU1841 is presented in Fig. 2. Primer extension using RNA from glycerol-grown JP3561/pMU1841 (Fig. 3, lane 1) mapped the transcription start point of *scrY* to the A at base 776 (Fig. 2), suggesting that the hexamers TTAAGA (bases 740 to 745) and TAACAT (bases 763 to 768) were the −35 and −10 boxes, respectively, of the *scrY* promoter. The same start point was observed when RNA from glucose-grown cells was used (data not shown). When RNA from JP3561/pMU1841 transformed with a multicopy $scrR^+$ plasmid and grown on glycerol was used, the same extension product could be observed, but the intensity of the band was reduced to the extent that it was barely visible (Fig. 3, lane 2).

From the map of the pUR400 *scr* regulon, one would expect the sequenced region to contain part of *scrK*. Confirming this, the deduced amino acid sequence of an incomplete open reading frame (ORF) detected upstream of the *scrY* promoter (Fig. 2) showed 50.7% identity in a 219-residue overlap with the ScrK fructokinase of *V. alginolyticus* (4). The ORF was closely followed by a potential stem-and-loop structure (ΔG°, −10.2 kcal/mol [ca. −42.7 kJ/mol] [11]), with a T-rich stretch a further 3 bases downstream, suggestive of a rho-independent transcription terminator (8).

Notable features in the intergenic region between *scrK*

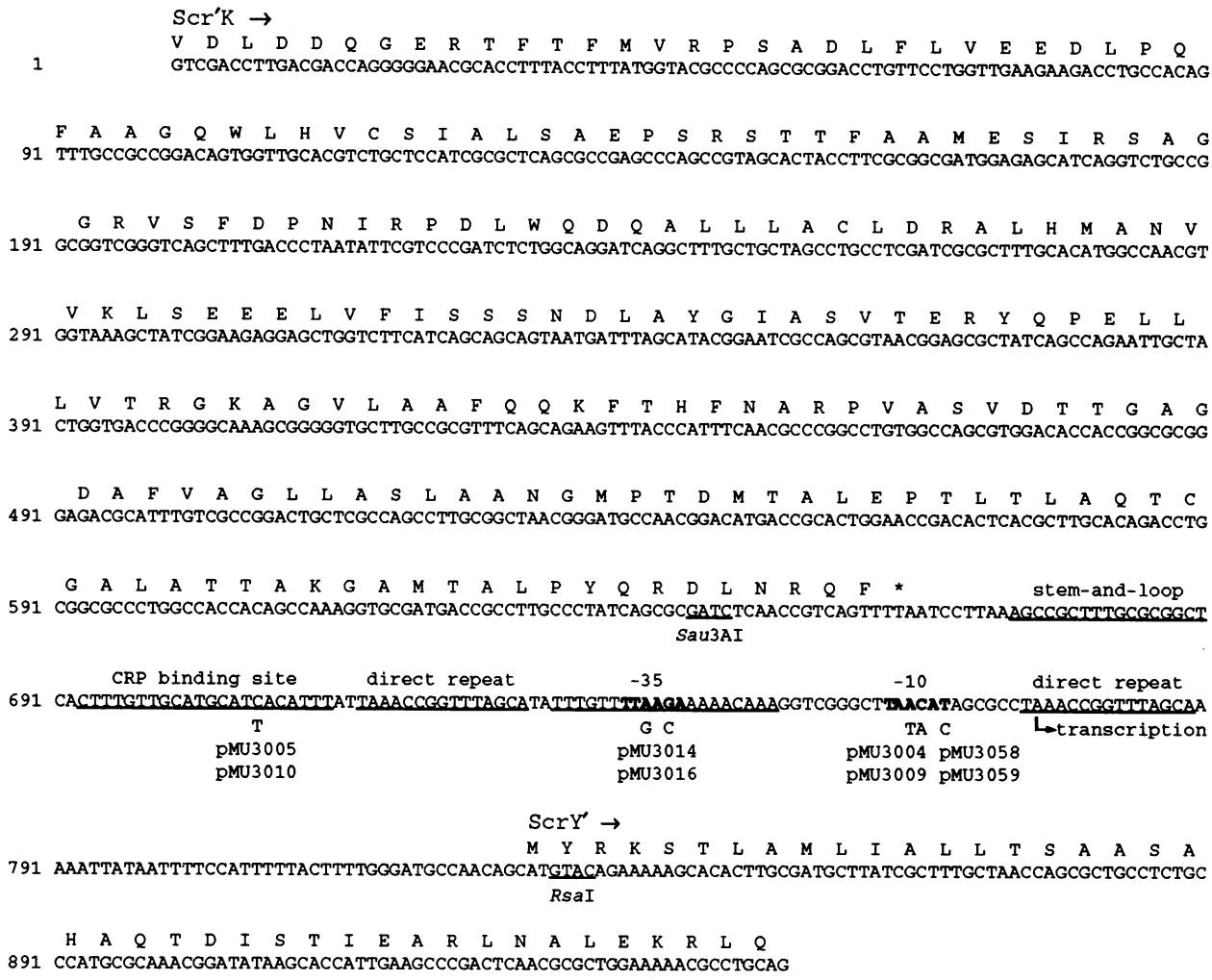

FIG. 2. Nucleotide sequence of the antisense strand of the insert in pMU1841. The *scrY* promoter is shown in bold type. Several features are underlined: (i) *Sau*3AI and *Rsa*I sites used for subcloning the *scrY* regulatory region; (ii) stem-and-loop structure of the putative transcription terminator of *scrK*; (iii) putative CRP-binding site; (iv) direct repeat on either side of the *scrY* promoter; and (v) 20-bp palindromic sequence encompassing the −35 box of the *scrY* promoter. Mutational changes described in Table 4 are shown below the sequence, as is the transcription start point of *scrY*. The right-hand terminus of the *scrK-lacZ* fusion in pMU3056 and pMU3057 is the G at base 704.

and *scrY* included a potential cyclic AMP receptor protein (CRP)-binding site upstream of the *scrY* promoter, a largely palindromic 15-bp sequence repeated on either side of the promoter, and a 20-bp palindrome encompassing the −35 box.

Mutational analysis of the *scrY* regulatory region. To facilitate further analysis, the *scrY* promoter was subcloned on a 190-bp *Sau*3AI-*Rsa*I fragment (Fig. 2) into the pMU575 derivatives pMU578 (*scrR*) and pMU3012 (*scrR*$^+$) to create pMU3001 and pMU3006, respectively. The β-galactosidase activities of JP3561/pMU3001 and JP3561/pMU3006 (Table 4) showed that the complete *scrY* regulatory region had been cloned. Site-directed mutagenesis was used to generate mutations in several of the features within this region.

Mutation of the "invariant" T in the putative −10 box of the promoter almost completely abolished promoter activity in both *scrR* and *scrR*$^+$ backgrounds (pMU3058 and pMU3059; Table 4). Conversely, changing the −10 box to the consensus for *E. coli* promoters (TATAAT) increased promoter strength, with little effect on catabolite repression or regulation by ScrR (pMU3004 and pMU3009). Changing the −35 box to the consensus (TTGACA) caused an even greater increase in promoter strength (pMU3014 and pMU3016); in this case, ScrR repression was intact, but the effect of catabolite repression appeared to have been reduced or abolished.

Mutation of the C at base 708, in the downstream arm of the putative CRP-binding site, caused a drastic reduction in promoter activity (pMU3005 and pMU3010). Repression by ScrR, however, was still evident.

Base 782, the second G in the palindromic repeat sequence downstream of the promoter (Fig. 2), was mutated to A or C. Neither of these changes significantly affected the expression level or regulation of the promoter (data not shown). Mutations were also introduced into either the left arm (G to A or C at base 737) or the right arm (C to T at base 750) of the larger palindrome encompassing the promoter −35 box. Although one mutation (G to A at nucleotide 737) reduced expression four- to eightfold, neither this nor the other

TABLE 3. β-Galactosidase activities of strains containing the *scrY'-lacZ* fusion plasmid pMU1841

Plasmid(s) in JP3561	*scrR* genotype	Carbon source[a]	Added cAMP[b]	β-Galactosidase activity (U)
pMU1841	*scrR*	Glycerol	−	2,546
		Glucose	−	542
		Glucose	+	3,077
pMU1841 and RP4-*scr*	*scrR*$^+$	Glycerol	−	126
		Glucose	−	67
		Glucose	+	172
		Sucrose	−	608
		Sucrose	+	1,809

[a] Strains were grown in MM containing the designated sugar at 0.2%.
[b] +, Addition of 6 mM cAMP to the growth medium; −, no cAMP added.

changes had a significant effect on operator function (data not shown).

Contribution of the *scrK* promoter to the transcription of *scrY*. A 0.9-kb fragment expected, by comparison with pUR400, to contain the *scrK* promoter was cloned into pMU578 and pMU3012, creating pMU3011 and pMU3039, respectively (Fig. 1D). Expression of β-galactosidase by JP3561/pMU3011 and JP3561/pMU3039 grown under various conditions (Table 5) indicated that the *scrK* promoter was regulated by ScrR, but over a smaller range than the *scrY* promoter and with a higher basal level.

To determine the relative level of readthrough transcription beyond the putative *scrK* terminator, the entire *scrK* gene (including the terminator) was cloned on a 1.6-kb fragment into pMU578 and pMU3012, creating pMU3056 and pMU3057, respectively (Fig. 1D). Comparison of β-galactosidase activities (Table 5) showed that, in *scrR*$^+$ cells, extending the point of fusion with *lacZ* from within *scrK* (pMU3039) to just downstream of the putative terminator (pMU3057) caused a 100-fold reduction in the transcription of *lacZ* under repressed conditions and a 5-fold reduction under derepressed conditions. In *scrR* cells, however, there was little evidence of termination (pMU3011 and pMU3056).

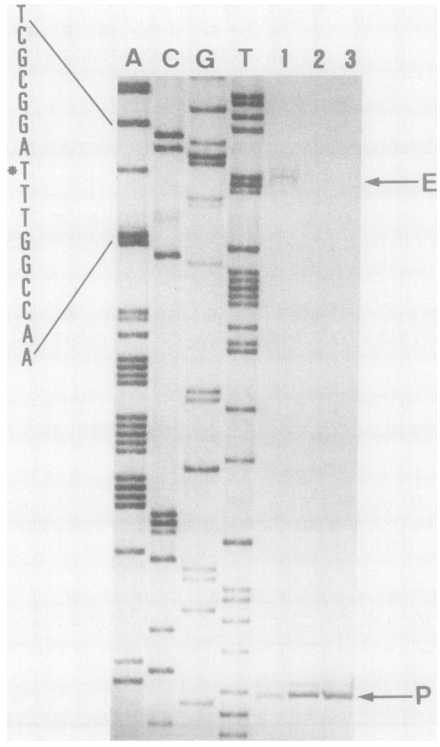

FIG. 3. Determination of the transcription start point of *scrY* by primer extension. The primer is indicated by the arrow labelled P. The product of extension, corresponding to the asterisk-marked T in the sequencing ladder of the sense strand of the *scrY* regulatory region, is indicated by the arrow labelled E. Lane 1, primer extended in the presence of 25 μg of RNA from glycerol-grown JP3561/pMU1841; lane 2, primer extended in the presence of 25 μg of RNA from glycerol-grown JP3561/pMU1841/pMU1874; lane 3, primer extended in the absence of RNA.

DISCUSSION

In the first part of this study, we described the subcloning and mapping of sucrose catabolic genes from the 150-kb Sac plasmid of *S. thompson*. On the basis of the similarities of both restriction maps and gene order, we concluded that these genes were very closely related to the *scr* regulon of the 78-kb *S. typhimurium* plasmid pUR400. The recently published sequence of *scrY* from pUR400 (14, 26) differs at only one position in a 273-base overlap with the corresponding sequence from Sac (bases 682 to 954; Fig. 2), providing further evidence for the relationship.

Transcriptional fusions with *lacZ* were used to show that the four structural genes were divided into two quite distinct transcriptional units. *scrK* was transcribed from a promoter regulated over a three- to fivefold range by ScrR (Table 5). The promoter of the *scrY scrA scrB* transcriptional unit, on

TABLE 4. β-Galactosidase activities of strains containing plasmids with wild-type and mutant *scrY'-lacZ* fusions

Plasmid in JP3561	Mutation in *scrY* regulatory region	*scrR* genotype	β-Galactosidase activity (U) with[a]:		
			Glucose	Glycerol	Fructose
pMU3001	None	*scrR*	885	4,370	6,108
pMU3006	None	*scrR*$^+$	3.3	12	2,986
pMU3058	T-768→C	*scrR*	4.4	10	10
pMU3059	T-768→C	*scrR*$^+$	0.3	0.5	12
pMU3004	−10 box→TATAAT	*scrR*	1,111	6,057	10,816
pMU3009	−10 box→TATAAT	*scrR*$^+$	12	19	5,160
pMU3014	−35 box→TTGACA	*scrR*	14,948	13,327	16,976
pMU3016	−35 box→TTGACA	*scrR*$^+$	81	210	5,897
pMU3005	C-708→T	*scrR*	45	106	173
pMU3010	C-708→T	*scrR*$^+$	2	3	119

[a] Strains were grown in MM containing the designated sugar at 0.2%.

TABLE 5. β-Galactosidase activities of strains containing *scrK'-lacZ* and *scrK-lacZ* fusion plasmids

Plasmid in JP3561	Description of fusion	*scrR* genotype	β-Galactosidase activity (U) with[a]:		
			Glucose	Glycerol	Fructose
pMU3011	*scrK'-lacZ*	*scrR*	977	2,110	2,305
pMU3039	*scrK'-lacZ*	*scrR*$^+$	361	615	1,098
pMU3056	*scrK-lacZ*	*scrR*	899	1,910	1,360
pMU3057	*scrK-lacZ*	*scrR*$^+$	3.0	6.9	206

[a] Strains were grown in MM containing the designated sugar at 0.2%.

the other hand, was regulated over a 1,000-fold range by the combination of ScrR-mediated repression and catabolite repression (Table 4). Between the *scrK* coding sequence and the *scrY* promoter was a G+C-rich self-complementary sequence followed after three bases by a T-rich stretch. Although this region satisfied several conditions proposed to be important in defining *E. coli* rho-independent transcription terminators (8), including the "quality" of the stretch of T residues (n_T), the quality of the potential RNA hairpin (Y), the number of bases in the loop (n_L), and the number of GC base pairs in the stem (n_{GC}), it would not be predicted as a rho-independent terminator by the algorithm of d'Aubenton Carafa et al. (8). Despite this, transcription termination was evident in *scrR*$^+$ cells (pMU3057; Table 5). There was little or no termination in *scrR* cells (pMU3056; Table 5), a puzzling finding that we are currently investigating.

Readthrough transcription from the *scrK* promoter into the downstream genes appeared to be relatively insignificant in *scrR*$^+$ cells, where under derepressed conditions the *scrK-lacZ* fusion in pMU3057 specified 206 U of β-galactosidase, less than 7% of that specified by the *scrY'-lacZ* fusion in pMU3006.

Primer extension was used to infer the location of the *scrY* promoter. Transcription initiated at a point very close to that proposed for the pUR400 *scrY* transcript (14). We unequivocally identified the *scrY* promoter by isolating both down and up mutations (Table 4). Interestingly, changing the −35 box to consensus caused a more pronounced increase in promoter strength than did changing the −10 box to consensus, and it had the additional effect of reducing or abolishing catabolite repression. This is consistent with the observation that the −35 boxes of cAMP-CRP-activated promoters are generally further from the consensus than the −10 boxes (9). In fact, it has been shown that a silent −10 hexamer-like sequence can be converted into a strong cAMP-CRP-activated promoter simply by positioning a CRP-binding site at an appropriate distance upstream (34).

Even in the absence of binding data, we believe that the following data strongly supports the contention that cAMP-CRP activates the *scrY* promoter by binding to the site shown in Fig. 2. (i) Certain bases in CRP-binding sites are believed to be directly involved in contacting the CRP dimer and are thus critical to binding (13). Mutation of one such base in the site upstream of the *scrY* promoter caused a 20- to 40-fold decrease in promoter activity. (ii) In most cAMP-CRP-activated promoters, the distance between the center of the binding site and the transcription start point is close to 41.5, 61.5, or 72.5 bp (9), and this spacing has been shown to be critical for activation (12). The spacing for the *scrY* promoter is 72.5 bp.

The location of the *scrY* operator is unclear. It has been reported recently that ScrR binds to the palindromic sequences contained within the 15-bp repeated sequence in the regulatory region of *scrY* and is released upon induction (unpublished results of K. Jahreis, reported in reference 26). This conclusion was based on the results of gel retardation assays, but the data was not given. We found that the mutation of a base in the palindrome downstream of the *scrY* promoter had very little effect on operator function. Similarly, mutations in either arm of the 20-bp palindrome encompassing the −35 box of the promoter failed to affect operator function.

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