MOLECULAR AND CELLULAR BIOLOGY, Dec. 1984, p. 2750–2757
0270-7306/84/122750-08$02.00/0

Vol. 4, No. 12

Upstream Region Required for Regulated Expression of the Glucose-Repressible *SUC2* Gene of *Saccharomyces cerevisiae*

LAURA SAROKIN AND MARIAN CARLSON*

Department of Human Genetics and Development, College of Physicians and Surgeons, Columbia University, New York, New York 10032

Received 30 July 1984/Accepted 18 September 1984

The *SUC2* gene produces two mRNAs with different 5′ ends that encode two forms of invertase. The 1.9-kilobase mRNA encoding secreted invertase is regulated by glucose repression (carbon catabolite repression), and the 1.8-kilobase mRNA encoding intracellular invertase is produced constitutively at low levels. To identify 5′ noncoding sequences essential for regulated expression of *SUC2*, we constructed in vitro a series of deletions and inserted them into the yeast genome at the chromosomal *SUC2* locus. Analysis of the effects of each deletion on *SUC2* gene expression identified an upstream region required for derepression of secreted invertase synthesis. The 3′ boundary of this region is near −418. The 5′ boundary does not appear to be sharply defined, but lies ca. 100 base pairs upstream. A deletion extending from −418 to −140 allowed high-level derepression, indicating that no essential sequences lie between the upstream region and the TATA box at −133 and that the upstream region can be moved 279 base pairs closer to the transcriptional start site. Interactions between the deletions and several unlinked mutations affecting the regulation of *SUC2* gene expression were examined. Sequences between −1,900 and −86 are dispensable for expression of the 1.8-kilobase mRNA.

Glucose repression (carbon catabolite repression) regulates the expression of a multitude of genes in *Saccharomyces cerevisiae*, including the *SUC2* structural gene for invertase. The *SUC2* gene provides a convenient model system for studying glucose repression because glucose repression appears to be the only regulatory mechanism affecting expression of *SUC2* and because expression is modulated over a greater than 100-fold range. In addition, the gene product can be readily assayed.

The *SUC2* gene encodes two differently regulated mRNAs and two forms of the sucrose-hydrolyzing enzyme invertase. The secreted, glycosylated form of the enzyme is responsible for the extracellular hydrolysis of sucrose, and its synthesis is regulated by glucose repression. The intracellular, nonglycosylated form has no known role in sucrose utilization, and it is produced constitutively at a low level. The two forms are encoded by two differently regulated *SUC2* mRNAs, which differ only at their 5′ ends (3). A 1.9-kilobase RNA, which is regulated by glucose repression, encodes a signal peptide-containing precursor to the secreted invertase (6, 15). A 1.8-kb mRNA, which is synthesized constitutively, does not include the complete signal sequence and is translated to yield a cytoplasmic invertase. The nucleotide sequence of the entire *SUC2* coding region has been determined (20).

Unlinked mutations that affect expression of the *SUC2* gene have been isolated. Mutations in the *SNF1* through *SNF6* (sucrose nonfermenting) genes cause defects in the derepression of secreted invertase in response to glucose limitation (4; L. Neigeborn and M. Carlson, Genetics, in press). The *snf1*, *snf2*, *snf4*, and *snf5* mutants show pleiotropic defects, suggesting that these *SNF* gene products are required for derepression of a variety of glucose-repressible genes. The defect in *snf1* and *snf2* mutants lies in their failure to derepress synthesis of the 1.9-kb mRNA (3; L. Neigeborn and M. Carlson, unpublished data). Other unlinked mutations that cause constitutive (glucose-insensitive) expression of *SUC2* have been isolated. Mutations at the *SSN6* locus cause constitutive high-level synthesis of secreted invertase and suppress *snf1*, *snf3*, *snf4*, and *snf6* mutations (5; Neigeborn and Carlson, in press). The *SSN6* gene product appears to act, directly or indirectly, as a repressor of *SUC2* gene expression. No unlinked mutations that alter the levels of cytoplasmic invertase have been found.

To identify regions flanking the *SUC2* gene that are required for its expression, we constructed a series of deletions in the 5′ noncoding region. The deletions were constructed in vitro by BAL 31 treatment of cloned *SUC2* DNA and were then introduced into the yeast genome at the chromosomal *SUC2* locus so as to replace the wild-type sequence. The effects of these deletion mutations on the expression of the *SUC2* gene were analyzed both in a wild-type genetic background and in the presence of unlinked regulatory mutations.

MATERIALS AND METHODS

Strains and genetic methods. All *S. cerevisiae* strains used in this study were isogenic or congenic to strain S288C. Strains MCY624 (*MATa SUC2 ura3-52 his4-539*) or MCY638 (*MATa SUC2 ura3-52 his4-539 lys2-801*) were transformed (12) with the plasmids containing deletions. Ura$^-$ derivatives of the transformants were selected by a modification of the procedure of Winston et al. (22); 10^5 cells were plated onto selection medium and incubated at 30°C for 1 to 2 weeks. Some Ura$^-$ colonies acquired a clumpy phenotype and were discarded.

Double mutants were constructed by standard genetic procedures of crossing, sporulation, and tetrad analysis (18), and their genotypes were confirmed by complementation analysis. Media and methods for scoring ability to utilize carbon sources have been described previously (4). Sucrose and raffinose utilization were scored by spotting cell suspensions on YEP media containing 2% of the sugar and incubating them under anaerobic conditions.

* Corresponding author.

Preparation and analysis of DNA. Plasmid DNAs were prepared by cesium chloride-ethidium bromide cntrifugation, except for screening of *Escherichia coli* transformants to identify colonies containing particular plasmids. In that case, plasmid DNAs were prepared by a modification of the alkaline lysis method (13). Yeast DNA was prepared by the method of Davis et al. (8). Enzymes were purchased from New England BioLabs unless otherwise indicated. Gel electrophoresis was carried out in 89 mM Tris-hydrochloride (pH 8.3)–89 mM boric acid–2.5 mM EDTA, except where noted.

Construction of plasmids. Plasmid pLS1 was constructed by inactivating the *Eco*RI site in the plasmid vector YIp5 (2), which carries the yeast *URA3* gene inserted into pBR322. YIp5 DNA was digested with *Eco*RI, treated briefly with S1 nuclease (Miles Laboratories, Inc.), and ligated with T4 DNA ligase (gift of J. van Oostrum) in 50 mM Tris-hydrochloride (pH 7.8)–10 mM $MgCl_2$–20 mM dithiothreitol–0.5 mM ATP at 14°C overnight. Plasmids were recovered by transforming *E. coli* HB101 to ampicillin resistance by the $CaCl_2$ procedure (13), and plasmid pLS1 was identified.

Plasmid pLS2 was constructed by subcloning the 4.5-kb *Bam*HI-*Bgl*II fragment from pRB54 (3), which contains the 5′ half of the *SUC2* gene (*suc2-215* amber allele) and 3.7 kb of adjacent upstream sequence, into the *Bam*HI site of pLS1. The *suc2-215* amber mutation in pLS2 was then replaced with the wild-type sequence by replacing the *Bam*HI-*Xho*I fragment of pLS2 with the corresponding fragment from pRB58, which carries a *SUC2*$^+$ gene (3). Plasmid pLS27 (Fig. 1) was recovered. To prove that pLS27 contained the wild-type sequence, pLS27 DNA was used to transform a *suc2-215 ura3* strain to uracil independence; stable Suc$^+$ transformants were recovered.

Construction of deletions. Deletion mutations extending from the *Xho*I site in pLS27 progressively farther towards the *SUC2* structural gene were constructed as outlined in Fig. 1a. Plasmid pLS27 was linearized by digestion with *Eco*RI, which cleaves at a single site 0.9 kb upstream from the *SUC2* gene, and was treated with BAL 31 nuclease in 20 mM Tris-hydrochloride (pH 8.0)–600 mM NaCl–12 mM $CaCl_2$–12 mM $MgCl_2$–1 mM EDTA at 25°C. Samples were removed at 60-s intervals and pooled. *Xho*I linkers were phosphorylated with polynucleotide kinase and ligated to the plasmid DNA. Plasmid DNA was then separated from excess linkers by electrophoresis in an agarose gel in 40 mM Tris-acetate (pH 7.5)–2 mM EDTA and was recovered with glass powder (21). The plasmid DNA was digested with *Xho*I to activate the linkers and cleave the plasmid at a site located ca. 1 kb upstream from the *Eco*RI site, then was circularized by ligation and used to transform *E. coli*. Plasmid DNAs were prepared from transformants, and the approximate size of the deletion carried by each plasmid was determined by digestion with *Hin*dIII and *Xho*I and sizing of the fragment carrying the deletion by electrophoresis on a polyacrylamide gel. The exact endpoints of most of the deletions were determined by nucleotide sequence analysis (14).

Deletion mutations with a 3′ endpoint at −223 or −140 and extending away from the *SUC2* gene were constructed as

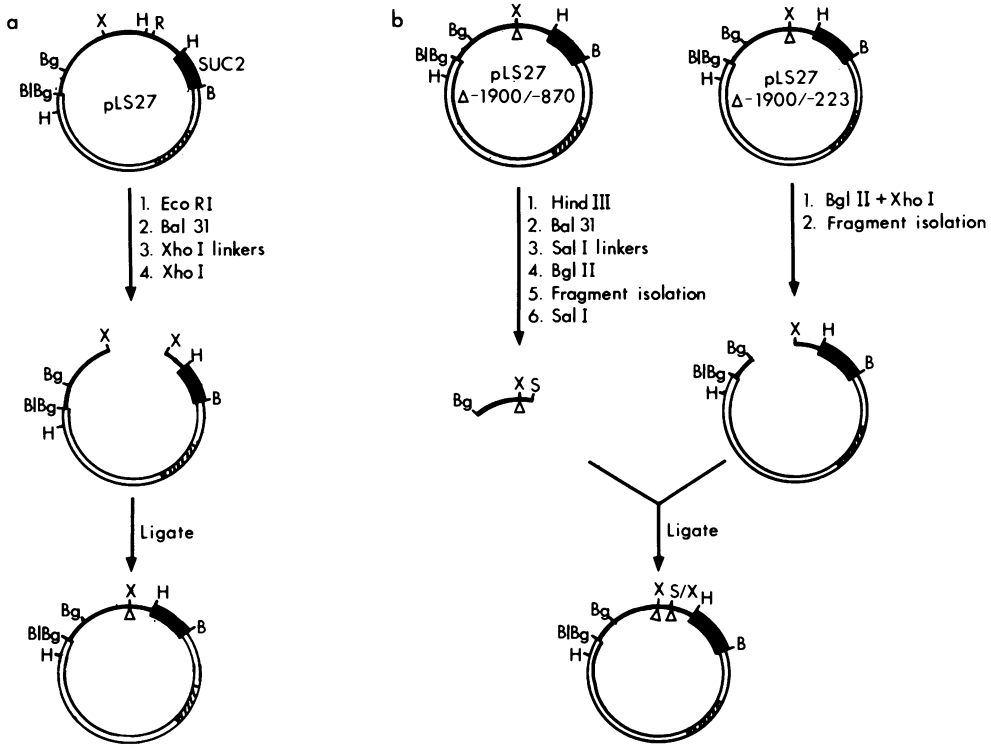


FIG. 1. Construction of deletions. (a) Construction of the series of deletions with a fixed 5′ endpoint at the *Xho*I site at −1,900. See the text for explanation. (b) Construction of the series of deletions with a fixed 3′ endpoint at −223. Plasmids pLS27Δ−1,900/−870 and pLS27Δ−1,900/−223 were recovered from the experiment outlined in a. It was convenient to use pLS27Δ−1,900/−870 (instead of pLS27) because the deletion removed a *Hin*dIII site. Deletion Δ−418/−140 was constructed by the same procedure, using pLS27Δ−1,900/−140 as the starting plasmid instead of pLS27Δ−1,900/−223. Solid bar: 5′ half of the *SUC2* structural gene. Thin line: flanking sequences 5′ to the *SUC2* gene. Open bar: the pBR322 DNA in the YIp5 vector. Hatched bar: the yeast *URA3* gene in YIp5. Restriction sites: B, *Bam*HI; Bg, *Bgl*II; H, *Hin*dIII; R, *Eco*RI; S, *Sal*I; X, *Xho*I.

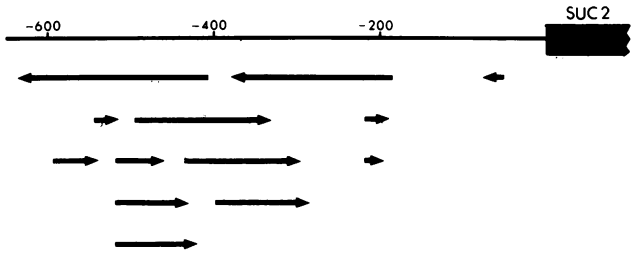


FIG. 2. Sequencing strategy. The nucleotide sequence shown in Fig. 3 was determined by sequence analysis of plasmids carrying deletions. Sequence analysis was carried out on 5′ end-labeled fragments by the procedure of Maxam and Gilbert (14) as previously described (20). Arrows indicate the direction and extent of the nucleotide sequence read from each 5′ end-labeled site. The sequence from −300 through the *SUC2* structural gene was reported previously (6, 20).

follows (Fig. 1b). Plasmid pLS27Δ−1,900/−870 was digested with *Hin*dIII, which cuts near the 5′ end of the *SUC2* gene (at positions +10 and −27) and once within the vector sequence. The DNA was treated with BAL 31 as described above and ligated to *Sal*I linkers. DNA was digested with *Bgl*II and electrophoresed in a 0.7% agarose gel in 40 mM Tris-acetate (pH 7.5)–2 mM EDTA. DNA was recovered from the diffuse band of fragments ca. 1.8 to 2.5 kb in size by the use of glass powder and digested with *Sal*I to activate the linkers. Plasmids pLS27Δ−1,900/−140 and pLS27Δ−1,900/−223 were digested with *Bgl*II and *Xho*I, and the 6.7-kb fragment from each was isolated after electrophoresis. These fragments were ligated to the 1.8- to 2.5-kb *Bgl*II-*Sal*I fragments from pLS27Δ−1,900/−870 and used to transform *E. coli*. Plasmids were recovered and analyzed as described above. A plasmid carrying a deletion of the sequence between the runs of thymidylate residues beginning at −183 and at −85 was also recovered from this experiment; its origin is obscure. This deletion has been arbitrarily designated Δ−180/−86.

Invertase assays. Preparation of glucose-repressed and derepressed cells was as described by Celenza and Carlson (7). Repressed cells were prepared by growing cells to exponential phase in rich medium (YEP) containing 2% glucose, and derepressed cells were prepared by shifting repressed cells to YEP medium containing 0.05% glucose for 2.5 h. In the case of clumpy yeast cultures, cell density was determined by measuring dry weight (5). Secreted invertase was assayed (10) in whole cells as previously described (7). Intracellular invertase activity was assayed by permeabilizing cells in 10 mM NaN_3 containing 0.3% Triton X-100 for 5 min at 25°C. The gel assay was carried out as described before (4).

RESULTS

Construction of delections in the 5′ noncoding region of *SUC2*. Two series of deletion mutations in the *SUC2* 5′ noncoding region were constructed by BAL 31 treatment of cloned *SUC2* DNA, as shown in Fig. 1 and described above. The first series of deletions was constructed in plasmid pLS27, which carries the 5′ half of the *SUC2* coding region and 3.7 kb of upstream sequences cloned into an integrative yeast vector (Fig. 1a). These deletions share a fixed 5′ endpoint at the *Xho*I site 1,900 bp upstream from the *SUC2* structural gene (position −1,900 relative to the translational initiation codon for the precursor to secreted invertase) and extend progressively farther towards the gene. The 3′ endpoints of these deletions range from −870 to −140. The

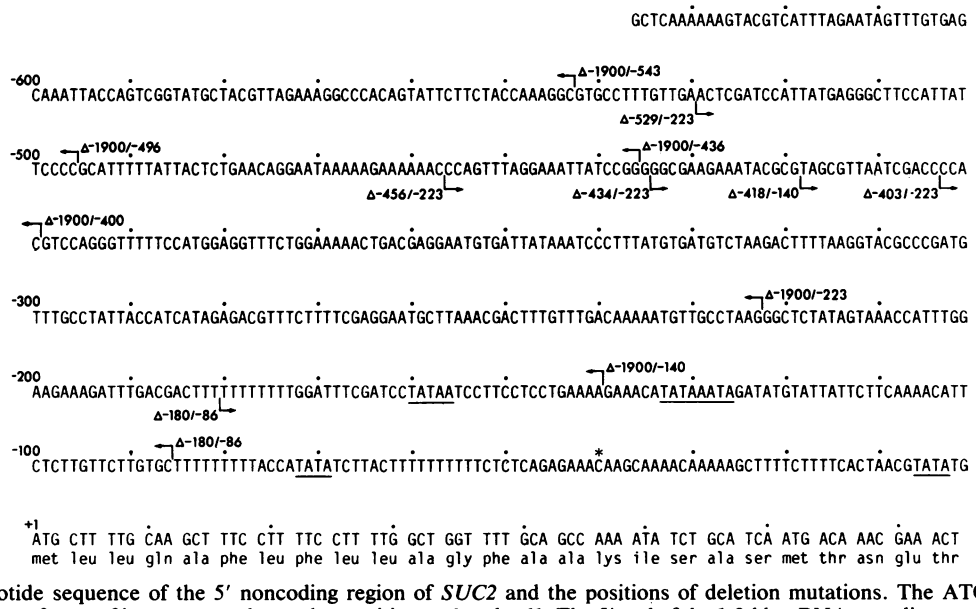


FIG. 3. Nucleotide sequence of the 5′ noncoding region of *SUC2* and the positions of deletion mutations. The ATG codons initiating translation of the two forms of invertase are located at positions +1 and +61. The 5′ end of the 1.9-kb mRNA encoding secreted invertase is at position −40 (asterisk); the heterogeneous 5′ ends of the 1.8-kb mRNA lie in the region between +50 and +58 (6). The TATA boxes mentioned in the text are underlined. Arrows above the sequence mark the 3′ endpoints of those deletions having a 5′ end at −1,900. Arrows below the sequence indicate the 5′ endpoints of deletions with a 3′ end at either −223 or −140. Both endpoints for Δ−180/−86 are shown; the exact placement of the endpoints within the runs of T residues is arbitrary. The expected linker sequence was present at the site of each deletion, except that Δ−434/−223 and Δ−529/−223 contained no linker sequence and Δ−418/−140 and Δ−403/−223 contained an extra *Sal*I linker. The nucleotide sequence is in accord with that determined by C. N. Chang (personal communication), except for a single base difference at position −312; the nucleotide we find at that position is part of a *Rsa*I site that is present in our DNA.

endpoints of most of these deletions were determined by sequence analysis (Fig. 2 and 3). The deletion mutations and the plasmids containing them carry designations corresponding to the deletion endpoints: Δx/y indicates that the deletion has a 5′ endpoint at position x and a 3′ endpoint at position y.

The second series of deletion mutations was constructed by using each of two plasmids from the first series that contain *Xho*I linkers at position −223 or −140; both sites are upstream from the TATAAATA box at −133 (6) (Fig. 1b). These deletions have a fixed 3′ endpoint at position −223 or −140 and extend away from the *SUC2* gene with 5′ endpoints ranging from −403 to −650. Deletion Δ−180/−86 was also recovered (see above). The plasmids carrying these deletions also carry another deletion, Δ−1,900/−870, which has no effect on expression of *SUC2* (see below); for technical reasons, its presence facilitated construction of the deletions of interest (see the legend to Fig. 1).

Construction of yeast strains carrying deletions at the chromosomal *SUC2* locus. To determine the effect of each deletion mutation on expression of the *SUC2* gene, we constructed strains in which the wild-type *SUC2* sequence was replaced by each of the deletion mutations. A two-step procedure (17) was used: first, the plasmid containing a deletion was inserted at the yeast chromosomal *SUC2* locus by integrative transformation (12), and then the integrated vector sequences and the wild-type copy of the duplicated yeast DNA were excised. The strains that were recovered carried a single copy of the mutated *SUC2* region at its normal chromosomal location and lacked any vector DNA sequences. This approach precludes the possibility that our analysis of *SUC2* gene expression could be affected by such factors as copy number, chromosomal location, the proximity of vector sequences, or the duplication of yeast DNA sequences at the chromosomal *SUC2* locus.

The first step of the procedure, the insertion of each plasmid containing a deletion at the chromosomal *SUC2* locus, was accomplished by using the plasmid DNA to transform a *SUC2 ura3* yeast strain to uracil prototrophy. Those crossover events at the *SUC2* locus occurring on the 3′ side of the deletion resulted in placement of the deletion mutation immediately upstream from the complete *SUC2* gene, and those occurring 5′ to the deletion left this upstream region intact (Fig. 4). From these transformants, we then derived strains in which the deletion mutation replaced the wild-type *SUC2* upstream sequence. Because homologous recombination between the duplicated *SUC2* sequences would result in excision of the vector DNA, including the *URA3* gene, and one copy of the duplicated upstream region, we selected for loss of the *URA3* marker (see above). Depending on the position of the crossover, either the deleted or the intact 5′ noncoding region was retained, and Ura⁻ strains retaining the deletion were identified by analysis of the physical structure of the *SUC2* locus, following the strategy outlined in Fig. 5. For most deletions, we identified at least two independent Ura⁻ strains in which the wild-type

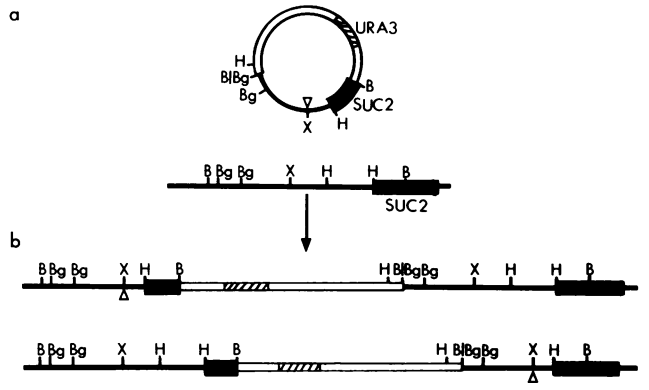


FIG. 4. Integration of plasmids containing deletions at the *SUC2* locus by homologous recombination. (a) Structures of plasmid containing deletion and wild-type chromosomal *SUC2* locus before integration of plasmid. (b) Two possible configurations of *SUC2* locus after integration of plasmid. Depending on the position of the crossover, recombination can result in placement of the deletion in front of either the intact *SUC2* gene or the truncated 5′ segment of the gene. Integration of a plasmid from the first series (Fig. 1a) is illustrated. Plasmids from the second series (Fig. 1b) contain an additional deletion, which may also be placed in front of either *SUC2* sequence, depending again on the position of the crossover. Solid bar: *SUC2* gene. Thin line: yeast DNA sequences flanking the *SUC2* gene. Open bar: the pBR322 DNA in the YIp5 vector. Hatched bar: the yeast *URA3* gene in YIp5. Restriction sites: B, *Bam*HI; Bg, *Bgl*II; H, *Hin*dIII; S, *Sal*I; X, *Xho*I.

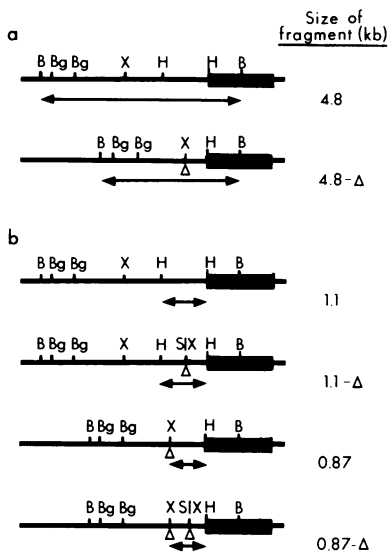
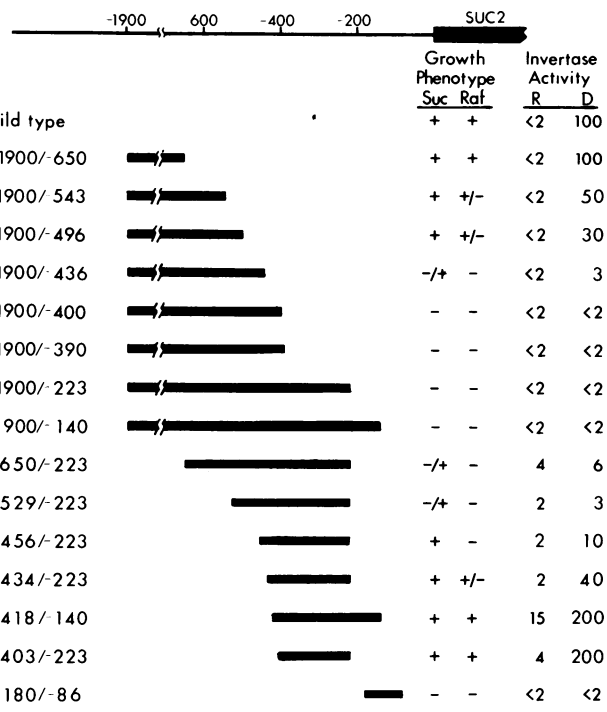


FIG. 5. Identification of deletion mutations at the *SUC2* locus. Ura⁻ derivatives of strains transformed with each plasmid were analyzed for identification of strains in which the deletion mutation replaced the wild-type sequence at *SUC2*. (a) For identification of strains carrying a deletion from the first series (Fig. 1a), the DNA was digested with *Bam*HI and the fragments were separated by agarose gel electrophoresis and transferred to nitrocellulose (19). The fragments indicated by arrows were detected by hybridization with radioactively labeled (16) pRB117 DNA (3); pRB117 contains the *Bam*HI to *Hin*dIII fragment encompassing the 5′ half of the *SUC2* gene. The wild-type *SUC2* locus generated a 4.8-kb fragment, and a mutant locus generated a fragment smaller than 4.8 kb by the size of the deletion. (b) Strains carrying a deletion from the second series (Fig. 1b) were identified by a similar procedure. For this analysis, the DNA was digested with *Hin*dIII plus *Xho*I, and fragments were detected by probing with pRB118 (3), which includes *SUC2* DNA extending from the *Bam*HI site to the *Hin*dIII site 1.9 kb upstream. The wild-type *SUC2* locus gave rise to a 1.1-kb *Hin*dIII fragment, and a locus carrying one of the deletions with a 3′ endpoint at −223 or −140 gave rise to a *Hin*dIII fragment smaller than 1.1 kb by the size of the deletion. A strain carrying Δ−1,900/−870 generated a 0.87-kb *Xho*I to *Hin*dIII fragment, and a strain carrying Δ−1,900/−870 and one of the deletions of interest generated an appropriately smaller fragment. Symbols are as indicated for Fig. 4.



	Growth Phenotype Suc	Raf	Invertase Activity R	D
Wild type	+	+	<2	100
Δ-1900/-650	+	+	<2	100
Δ-1900/-543	+	+/-	<2	50
Δ-1900/-496	+	+/-	<2	30
Δ-1900/-436	-/+	-	<2	3
Δ-1900/-400	-	-	<2	<2
Δ-1900/-390	-	-	<2	<2
Δ-1900/-223	-	-	<2	<2
Δ-1900/-140	-	-	<2	<2
Δ-650/-223	-/+	-	4	6
Δ-529/-223	-/+	-	2	3
Δ-456/-223	+	-	2	10
Δ-434/-223	+	+/-	2	40
Δ-418/-140	+	+	15	200
Δ-403/-223	+	+	4	200
Δ-180/-86	-	-	<2	<2

FIG. 6. Properties of deletion mutants. The extent of each deletion is indicated by its bar. The endpoints of all deletions except for Δ−1,900/−650, Δ−1,900/−390, and Δ−650/−223 were determined by sequence analysis. The ability of deletion mutants to utilize sucrose (Suc) and raffinose (Raf) for anaerobic growth is indicated. Secreted invertase activity was assayed in cells grown under glucose-repressing (R) and derepressing (D) conditions. Units are micromoles of glucose released per minute per 100 mg (dry weight) of cells. At least two independently derived mutants carrying each deletion were assayed, and essentially the same level of activity was detected. In those cases tested, the presence of Δ−1,900/−870 together with one of the deletions from the second series had, as expected, no effect.

SUC2 upstream sequence was replaced by the deletion of interest. These deletion mutants were then examined for alterations in the expression of the *SUC2* gene.

Growth properties of deletion mutants. The deletion mutants were first tested for their ability to utilize sucrose and raffinose as carbon sources (Fig. 6). Raffinose is a poorer substrate than sucrose for the enzyme invertase, so growth on raffinose is a more sensitive indicator of secreted invertase levels. Strains carrying deletions extending from −1,900 to −650 and from −418 to −140 were phenotypically wild type. Deletions extending from −1,900 to −543, or farther towards the *SUC2* gene, caused defects in raffinose utilization. Deletions with a 3′ endpoint at −223 and extending in the 5′ direction to position −434 or farther also resulted in defects in raffinose fermentation. These findings suggested that a large region upstream of −418 is required for normal derepression of secreted invertase synthesis.

Derepression of secreted invertase in deletion mutants. For a more quantitative measure of the effects of each deletion on synthesis of secreted invertase, the deletion mutants were assayed for secreted invertase activity after growth under derepressing conditions (Fig. 6). Mutants carrying deletion Δ−1,900/−650 produced wild-type levels of secreted invertase. Deletions Δ−1,900/−543 and Δ−1,900/−496 caused significant reductions in the derepressed level of secreted invertase relative to wild type. Deletions extending from −1,900 to −400 or farther prevented derepression of detectable secreted invertase activity. Deletions Δ−418/−140 and Δ−403/−223 had no detrimental effects on derepression of secreted invertase; in fact, strains carrying either mutation produced approximately twofold more invertase activity than did the wild type. Deletions with 3′ endpoints at −223 and 5′ endpoints at −434 or farther upstream caused defects in derepression. Δ−650/−223 and Δ−529/−223 allowed synthesis of low amounts of invertase, but the levels were not regulated by glucose repression (see below), suggesting that these deletions abolish glucose-regulated expression of *SUC2*. Taken together, these date indicate (i) that sequences upstream of position −650 and sequences between −418 and −140 are dispensable for the derepression of secreted invertase to wild-type levels, and (ii) that a region required for derepression lies between −650 and −418.

Glucose repression of invertase synthesis in mutants. For a determination of whether any of the deletions caused constitutive (glucose-insensitive) synthesis of secreted invertase, the deletion mutants were grown under glucose-repressing conditions and assayed for secreted invertase (Fig. 6). No significant activity was detected in any mutant carrying a deletion with a 5′ endpoint at −1,900, and only low levels were detected in other mutants. Thus, none of the deletions conferred high-level constitutivity to the synthesis of secreted invertase. The synthesis of low levels of invertase by mutants carrying Δ−650/−223 and Δ−529/−223 under both glucose-repressing and derepressing conditions suggests that sequences upstream of −650 promote low-level, unregulated expression of the *SUC2* gene when a deletion is present; no such expression is observed from a wild-type *SUC2* locus. The *SUC2* gene expression observed in the Δ−529/−223 mutant is independent of the *SNF1* gene (see below and Table 1). Deletions Δ−456/−223, Δ−434/−223, Δ−418/−140, and Δ−403/−223 also allowed low-level constitutive synthesis of secreted invertase. It is again possible that sequences upstream of −650 are responsible; however, in these cases it is also possible that the change in position of the upstream region necessary for regulated expression of *SUC2* results in aberrant constitutive expression. Deletion Δ−418/−140 caused significantly higher constitutive expression than the other deletions. We have shown that in the case of Δ−418/−140, a functional *SNF1* gene product is not required for the constitutive expression (see below and Table 1).

Identification of the TATA sequence for expression of secreted invertase. Previous studies identified TATA-like sequences located 120, 93, and 32 nucleotides upstream from the 5′ end of the 1.9-kb mRNA, at positions −160, −133, and −72, respectively (6). Comparison with other yeast genes suggested that the TATAAATA element at −133 was the relevant sequence for transcription of the 1.9-kb mRNA encoding secreted invertase. Analysis of the effects of deletion mutations provided additional evidence. Δ−180/−86, which removes the TATA sequences at −160 and −133, prevented expression of secreted invertase. Δ−418/−140, which eliminates the TATA sequence at −160 without substituting another TATA sequence at an equivalent position, allowed high-level synthesis of secreted invertase. These results indicate that the TATA box at −133 can be utilized for expression, but they do not rule out the possibility that the sequence at −160 is also a functional element.

Deletions do not affect constitutive synthesis of intracellular invertase. The intracellular, nonglycosylated form of invertase is encoded by a different mRNA than is the secreted

TABLE 1. Properties of strains carrying a deletion mutation and an unlinked regulatory mutation

Relevant Genotype[a]	Growth phenotype[b]		Invertase activity[c]	
	Suc	Raf	Repressed	Derepressed
ssn6 SUC2	+	+	300	380
ssn6 Δ−1,900/−496	+	+	40	100
ssn6 Δ−1,900/−436	+/−	−/+	4	40
ssn6 Δ−1,900/−400	−	−	<1	<1
ssn6 Δ−1,900/−390	−	−	<1	<1
ssn6 Δ−1,900/−140	−	−	<1	<1
ssn6 Δ−418/−140	+	+	280	360
snf1 SUC2	−	−	<1	<1
snf1 Δ−1,900/−496	−	−	ND	ND
snf1 Δ−1,900/−400	−	−	ND	ND
snf1 Δ−1,900/−390	−	−	ND	ND
snf1 Δ−1,900/−140	−	−	ND	ND
snf1 Δ−529/−223	ND	−	4	6
snf1 Δ−418/−140	ND	−	19	24
snf2 SUC2	+/−	−	<1	4
snf2 Δ−1,900/−496	+/−	−	ND	ND
snf2 Δ−1,900/−390	−	−	ND	ND

[a] Alleles are *ssn6-1* (5), *snf1-28* (4), *snf1-3* (J. Celenza and M. Carlson, unpublished data), and *snf2-141* (Neigeborn and Carlson, in press).
[b] Anaerobic growth with sucrose (Suc) or raffinose (Raf) as carbon source.
[c] Secreted invertase activity is expressed in units of micromoles of glucose released per minute per 100 mg (dry weight) of cells. ND, Not determined.

invertase, and both the mRNA and the intracellular enzyme are expressed constitutively at low levels (3). The mRNA encoding the intracellular enzyme does not have the same 5′ end as the mRNA for secreted invertase and is presumably transcribed from a different promoter that is not regulated by glucose repression (6). To determine whether the deletion mutations affected expression of the intracellular invertase, glucose-repressed cells carrying each of the deletions with a 5′ endpoint at −1,900 and Δ−180/−86 were examined for the presence of the nonglycosylated, intracellular form of invertase by means of a gel assay. The two forms of the enzyme were separated by electrophoresis in a polyacrylamide gel and then were detected by staining the gel for activity (9). In all cases, the nonglycosylated form was present in approximately normal amounts and, as expected, no glycosylated enzyme was detected (data not shown). Assays of permeabilized cells confirmed that the deletions caused no dramatic alterations in intracellular activity. Values for the mutants ranged from 0.2 to 2 units, and values for the wild type ranged from 0.2 to 0.6 units. These results indicate that deletion of sequences between positions −1,900 and −86 does not prevent expression of the intracellular invertase, and that therefore all sequences required for expression of intracellular invertase lie downstream of nucleotide −86.

Interaction of deletion mutations at *SUC2* and unlinked regulatory mutations. Mutations that affect the regulation of secreted invertase expression have been isolated in genes unlinked to *SUC2*. Mutations in the *SSN6* gene cause constitutive high-level production of secreted invertase; *ssn6* *SUC2*$^+$ strains produce as much invertase as derepressed wild type when grown under glucose-repressing conditions (5). For a determination of the relationships between *ssn6* and the deletion mutations in the 5′ noncoding region of *SUC2*, double mutants were constructed and assayed for secreted invertase activity (Table 1). No secreted invertase was detected in strains carrying *ssn6* and a deletion that prevented secreted invertase synthesis in a wild-type (*SSN6*$^+$) background (Δ−1,900/−140, Δ−1,900/−390, Δ−1,900/−140). Thus, the upstream region that is required for *SUC2* gene expression in a wild-type background is also required in the absence of a functional *SSN6* gene; an *ssn6* mutation did not restore expression. In contrast, *ssn6* did confer constitutivity when combined with a deletion that allowed some invertase synthesis in a wild-type background (Δ−1,900/−496, Δ−1,900/−436, or Δ−418/−140). However, *ssn6* did not restore invertase synthesis to wild-type levels in these cases. The *ssn6* mutation caused higher derepressed levels of invertase in the double mutants than were observed in the corresponding parent deletion mutants; a similar effect was observed when *ssn6* *SUC2*$^+$ and *SSN6*$^+$ *SUC2*$^+$ strains were compared (5).

The *snf1* mutations prevent derepression of the *SUC2* gene and other glucose-repressible genes in response to reduced availability of glucose (4). Several strains carrying a *snf1* allele and a deletion mutation were constructed and tested for their ability to utilize sucrose and raffinose (Table 1). In the presence of the *snf1* mutation, the Δ−1,900/−496 allele no longer conferred a sucrose- or raffinose-fermenting phenotype; thus, the *SNF1* gene product is still required for derepression of *SUC2* when sequences between −1,900 and −496 are deleted. Strains carrying *snf1* and a deletion that abolished invertase synthesis in a wild-type (*SNF1*$^+$) background (Δ−1,900/−400, Δ−1,900/−390, and Δ−1,900/−140) showed a nonfermenting phenotype; these mutations did not compensate for one another. Strains carrying *snf1* and Δ−529/−223 produced low invertase activity under both glucose-repressing and derepressing conditions, as did the *SNF1*$^+$ Δ−529/−223 strains (Fig. 6). This result suggests that this deletion abolishes glucose-regulated expression of *SUC2* and that the expression observed in these deletion mutants is due to another unregulated promoter, as discussed above. The *snf1* Δ−418/−140 double mutants were unable to derepress secreted invertase, indicating that this deletion does not obviate the requirement for a functional *SNF1* gene. The double mutants did, however, produce the same low amounts of invertase under both repressing and derepressing conditions as were detected in the *SNF1*$^+$ Δ−418/−140 parent strain under repressing conditions.

The *SNF2* gene is also required for derepression of the *SUC2* gene and other glucose-repressible genes (Neigeborn and Carlson, in press). The *snf2* mutants produce only low levels of invertase and are capable of slow growth on sucrose but not on raffinose. A strain carrying a *snf2* allele and Δ−1,900/−496 was constructed and tested for growth phenotype. The double mutant displayed the growth characteristics of the *snf2* parent rather than those of the deletion mutant parent, indicating that the *SNF2* gene is required for optimal expression of the Δ−1,900/−496 allele. A double mutant carrying *snf2* and Δ−1,900/−390 was also constructed; it displayed the sucrose-nonfermenting phenotype of the deletion mutant parent.

DISCUSSION

We constructed a series of deletions in the 5′ noncoding region of the *SUC2* gene and inserted these deletions into the yeast genome at the chromosomal *SUC2* locus. We then assessed the effect of each deletion on the expression of both the secreted invertase, which is encoded by a glucose-repressible 1.9-kb *SUC2* mRNA, and the intracellular invertase, which is encoded by a constitutive 1.8-kb mRNA.

This analysis identified an upstream region between nucleotides −650 and −418, relative to the translational initiation codon for secreted invertase, that is required for derepression of secreted invertase. The 3′ boundary of the region required for derepression lies near −418. Deletions △−418/−140 and △−403/−223 allowed derepression of invertase to high levels (200% of the wild-type level), and a deletion extending another 16 nucleotides farther upstream, △−434/−223, caused a fivefold decrease in derepression (40% of the wild-type level). The 5′ boundary does not appear to be sharply demarcated; removal of sequences between −650 and −543 caused a twofold decrease in derepression, further deletion to −496 reduced derepression to 30% of the wild-type level, and deletion to −436 nearly abolished derepression. These data suggest that the most crucial sequences lie downstream of position −496.

Deletion △−418/−140 shows that no sequences essential for expression lie between positions −418 and −140, and also that the upstream region can be moved as much as 279 base pairs closer to the transcriptional start site without deleterious effect. In fact, △−418/−140 and △−403/−223 allowed derepression of invertase to levels twice that of wild type, and we speculate that this elevation in expression may result from the increased proximity of the upstream region to the gene.

What is the role of this upstream region in derepression of *SUC2* gene expression? One possibility is that this region mediates the regulation of *SUC2* expression in response to the availability of glucose in the environment. Our data would then suggest that such regulation operates by positive control; deletion of the proposed regulatory region prevented derepression. A second possibility is that regulatory sequences lie downstream of position −140 (interspersed with elements of the promoter such as the TATAAATA sequence at −133) and that the upstream region is required for promoter function or for enhancement of gene expression. Because neither secreted invertase nor the 1.9-kb mRNA encoding it can be detected in glucose-repressed cells (3), we cannot distinguish between defects in expression and defects in regulation of expression. Further experiments are under way to address this issue.

We found no evidence for direct negative regulation of *SUC2* gene expression in response to external glucose concentration: none of our deletion mutations caused the high-level constitutive phenotype that would be expected from the deletion of a negative regulatory site. We cannot, of course, exclude the possibility that such a site is located downstream of −140. In fact, it is possible that the significantly higher levels of constitutive expression observed in the △−418/−140 mutant result from partial deletion of a negative regulatory element or even from the complete deletion of a negative element that plays a minor role in modulation of expression. However, an alternative explanation is that the elevated constitutivity is a consequence of particular features of this deletion, such as the novel junction sequence or the changes in relative positions of flanking sequences. Although the properties of the *ssn6* mutations suggest that the *SSN6* gene product acts as a repressor of *SUC2* gene expression (5), genetic studies of the interactions of *ssn6* with other mutations affecting *SUC2* expression are consistent with an indirect mode of action for *SSN6* (Neigeborn and Carlson, in press).

Because it is likely that regulated expression of the *SUC2* gene requires interaction of regulatory molecules with the 5′ noncoding region, we investigated the relationships between deletion mutations and the unlinked regulatory mutations *ssn6*, *snf1*, and *snf2*. These mutations did not compensate for defects caused by deletions of the 5′ noncoding region, nor did the deletions alleviate the requirement for functional *SNF1*, *SNF2*, and *SSN6* gene products. The only possible exception is that the *ssn6* △−1,900/−436 double mutant derepressed invertase to a much greater extent than might have been expected from the phenotype of the *SSN6*$^+$ deletion mutant; however, the significance of this finding is not clear because *ssn6* causes derepression of a wild-type *SUC2* gene to higher levels than those observed in an *SSN6*$^+$ background and the explanation for this effect is not yet evident.

None of our deletions affected expression of the intracellular invertase, suggesting that sequences between −1,900 and −86 are dispensable for expression of the 1.8-kb mRNA. This result is not surprising because the 5′ end of this mRNA, which is heterogeneous, lies well downstream; major species start at +50, +54, and +58 (6). A TATA sequence that may be used for transcription of this mRNA is located at position −6.

These studies also show that the intracellular invertase does not confer a sucrose-fermenting phenotype. Mutants lacking the secreted invertase but producing normal levels of the intracellular enzyme were unable to ferment sucrose or raffinose. The biological function of this form of invertase is not obvious.

It is interesting to note that the upstream region that is essential for *SUC2* gene expression is conserved in another structural gene for invertase, *SUC7*. Like the *SUC2* gene, *SUC7* encodes two forms of invertase, and synthesis of the secreted form is regulated by glucose repression. Comparison of the 5′ noncoding sequences of the two genes revealed good homology extending from the structural gene upstream to position −240. Homology was also found between the sequences extending from −504 to −360, including a region from −451 to −395 exhibiting 80% homology (L. Sarokin, R. Taussig, and M. Carlson, unpublished data).

Upstream regions required for regulated expression of other glucose-repressible yeast genes have been identified. Beier and Young (1) reported that deletion of a region upstream from the TATAA box of the *ADR2* gene, which encodes alcohol dehydrogenase I, resulted in constitutive expression. Guarente et al. (11) have characterized an upstream activation site (UAS) of *CYC1*, the structural gene for iso-1-cytochrome *c*, that mediates both catabolite repression and heme regulation of gene expression.

ACKNOWLEDGMENTS

This work was supported by grant NP-358A from the American Cancer Society and by Public Health Service grant GM32065 from the National Institutes of Health to M.C. L.S. is a predoctoral trainee supported by Public Health Service training grant GM07088 from the National Institutes of Health.

We thank C. N. Chang for communication of results before publication.

LITERATURE CITED

1. **Beier, D. R., and E. T. Young.** 1982. Characterization of a regulatory region upstream of the *ADR2* locus of *S. cerevisiae*. Nature (London) **300**:724–728.
2. **Botstein, D., S. C. Falco, S. E. Stewart, M. Brennan, S. Scherer, D. T. Stinchcomb, K. Struhl, and R. W. Davis.** 1979. Sterile host yeasts (SHY): a eukaryotic system of biological containment for recombinant DNA experiments. Gene **8**:17–24.
3. **Carlson, M., and D. Botstein.** 1982. Two differentially regulated mRNAs with different 5′ ends encode secreted and intracellular forms of yeast invertase. Cell **28**:145–154.
4. **Carlson, M., B. C. Osmond, and D. Botstein.** 1981. Mutants of

yeast defective in sucrose utilization. Genetics **98**:25–40.
5. **Carlson, M., B. C. Osmond, L. Neigeborn, and D. Botstein.** 1984. A suppressor of *snf1* mutations causes constitutive high-level invertase synthesis in yeast. Genetics **107**:19–32.
6. **Carlson, M., R. Taussig, S. Kustu, and D. Botstein.** 1983. The secreted form of invertase in *Saccharomyces cerevisiae* is synthesized from mRNA encoding a signal sequence. Mol. Cell. Biol. **3**:439–447.
7. **Celenza, J. L., and M. Carlson.** 1984. Cloning and genetic mapping of *SNF1*, a gene required for expression of glucose-repressible genes in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **4**:49–53.
8. **Davis, R. W., M. Thomas, J. Cameron, T. P. St. John, S. Scherer, and R. A. Padgett.** 1980. Rapid DNA isolations for enzymatic and hybridization analysis. Methods Enzymol. **65**:404–411.
9. **Gabriel, O., and S.-F. Wang.** 1969. Determination of enzymatic activity in polyacrylamide gels. I. Enzymes catalyzing the conversion of nonreducing substrates to reducing products. Anal. Biochem. **27**:545–554.
10. **Goldstein, A., and J. O. Lampen.** 1975. β-D-Fructofuranoside fructohydrolase from yeast. Methods Enzymol. **42**:504–511.
11. **Guarente, L., B. Lalonde, P. Gifford, and E. Alani.** 1984. Distinctly regulated tandem upstream activation sites mediate catabolite repression of the *CYC1* gene of S. cerevisiae. Cell **36**:503–511.
12. **Hinnen, A., J. B. Hicks, and G. R. Fink.** 1978. Transformation of yeast. Proc. Natl. Acad. Sci. U.S.A. **75**:1929–1933.
13. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
14. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65**:499–560.
15. **Perlman, D., H. O. Halvorson, and L. E. Cannon.** 1982. Presecretory and cytoplasmic invertase polypeptides encoded by distinct mRNAs derived from the same structural gene differ by a signal sequence. Proc. Natl. Acad. Sci. U.S.A. **79**:781–785.
16. **Rigby, P. W. J., M. Dieckmann, C. Rhodes, and P. Berg.** 1977. Labeling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. J. Mol. Biol. **113**:237–251.
17. **Scherer, S., and R. W. Davis.** 1979. Replacement of chromosome segments with altered DNA sequences constructed *in vitro*. Proc. Natl. Acad. Sci. U.S.A. **76**:4951–4955.
18. **Sherman, F., G. R. Fink, and C. W. Lawrence.** 1978. Laboratory manual for a course, methods in yeast genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
19. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
20. **Taussig, R., and M. Carlson.** 1983. Nucleotide sequence of the yeast *SUC2* gene for invertase. Nucleic Acids Res. **11**:1943–1954.
21. **Vogelstein, B., and D. Gillespie.** 1979. Preparative and analytical purification of DNA from agarose. Proc. Natl. Acad. Sci. U.S.A. **76**:615–619.
22. **Winston, F., F. Chumley, and G. R. Fink.** 1983. Eviction and transplacement of mutant genes in yeast. Methods Enzymol. **101**:211–228.