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Two Differentially Regulated mRNAs with Different 5′ Ends Encode Secreted and Intracellular Forms of Yeast Invertase

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Summary

The *SUC2* gene of yeast (Saccharomyces) encodes two forms of invertase: a secreted, glycosylated form, the synthesis of which is regulated by glucose repression, and an intracellular, nonglycosylated enzyme that is produced constitutively. The *SUC2* gene has been cloned and shown to encode two RNAs (1.8 and 1.9 kb) that differ at their 5′ ends. The stable level of the larger RNA is regulated by glucose; the level of the smaller RNA is not. A correspondence between the presence of the 1.9 kb RNA and the secreted invertase, and between the 1.8 kb RNA and the intracellular invertase, was observed in glucose-repressed and -derepressed wild-type cells. In addition, cells carrying a mutation at the *SNF1* locus fail to derepress synthesis of the secreted invertase and also fail to produce stable 1.9 kb RNA during growth in low glucose. Glucose regulation of invertase synthesis thus is exerted, at least in part, at the RNA level. A naturally silent allele (*suc2°*) of the *SUC2* locus that does not direct the synthesis of active invertase was found to produce both the 1.8 and 1.9 kb RNAs under normal regulation by glucose. A model is proposed to account for the synthesis and regulation of the two forms of invertase: the larger, regulated mRNA contains the initiation codon for the signal sequence required for synthesis of the secreted, glycosylated form of invertase; the smaller, constitutively transcribed mRNA begins within the coding region of the signal sequence, resulting in synthesis of the intracellular enzyme.

Introduction

Yeast strains (Saccharomyces) carrying any one of the unlinked *SUC*$^+$ genes (*SUC1–SUC7;* Winge and Roberts, 1952; Mortimer and Hawthorne, 1969; Carlson et al., 1980, 1981b) produce the sucrose-cleaving enzyme invertase. *SUC*$^+$ yeast strains make two forms of invertase: a secreted, glycosylated enzyme and an intracellular, nonglycosylated enzyme. Biochemical and genetic evidence indicates that the *SUC* genes are each structural genes for both forms of invertase (Hackel, 1975; Grossmann and Zimmermann, 1979; Carlson et al., 1981a; Rodriguez et al., 1981). The protein moieties of the two forms are very similar, each consisting of a dimer of approximately 60,000 dalton subunits (Gascon and Lampen, 1968; Trimble and Maley, 1977), but no precursor–product relation has been demonstrated (Lampen et al., 1972; Gallili and Lampen, 1977). The level of the secreted, glycosylated form is regulated by glucose repression; changes of 100 to 1000 fold in response to glucose regulation have been reported in various strains (Gascon and Ottolenghi, 1967; Gascon and Lampen, 1968; Ottolenghi, 1971). The glycosylated form accounts for most of the invertase activity in derepressed cells. The intracellular, nonglycosylated invertase is made at low levels, which do not change significantly with changes in glucose concentration. One of our primary goals was to clarify the relation between the regulated synthesis of the secreted invertase and the constitutive synthesis of the intracellular enzyme.

We recently reported a genetic analysis of sucrose utilization in the Saccharomyces cerevisiae strain S288C (*SUC2*$^+$). We found sucrose-nonfermenting mutants with lesions at the *SUC2* locus and also at an apparent regulatory locus, *SNF1*. The *suc2* mutants produce no invertase; *snf1* mutants produce the nonglycosylated invertase but are unable to derepress the glycosylated invertase and several other glucose-repressible enzymes (Carlson et al., 1981a). We also characterized a silent *SUC2* gene, a naturally occurring negative allele called *suc2°*, which for unknown reasons is unable to direct the synthesis of active invertase (Carlson et al., 1980, 1981b).

We describe the molecular cloning of the *SUC2* gene and use of the cloned DNA to characterize the structure of the *SUC2* gene and its RNA products. Regulation of *SUC2* gene expression at the RNA level, in response to the concentration of glucose in the medium, was studied in wild-type, *snf1* mutants and *suc2°* strains. Very recently, a report appeared suggesting that *SUC* loci encode distinct RNAs for cytoplasmic and secreted invertase (Perlman and Halvorson, 1981); using in vitro translation methods, these authors obtained evidence suggesting that *SUC* loci might produce three regulated mRNAs. We show directly that two mRNAs are transcribed from the *SUC2* gene, only one of which is regulated by glucose.

Results

Molecular Cloning of *SUC2* DNA

To obtain the DNA encoding *SUC2,* we constructed a recombinant plasmid library including cloned DNA segments representative of the genome of S. cerevisiae strain S288C (which carries only a single active *SUC* gene, at the *SUC2* locus) and then selected plasmids carrying cloned *SUC2* DNA by virtue of their ability to complement a *suc2*$^-$ mutation in yeast. In anticipation of the need to identify with certainty the complementing cloned gene as *SUC2,* however, the library was constructed from DNA of a strain isogenic to S288C but carrying a *suc2* amber mutation. A

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cloned *suc2-am* gene could then be distinguished by its phenotype (that is, dependence on an amber suppressor) from any other (hypothetical) gene capable of conferring the ability to ferment sucrose.

We constructed a recombinant plasmid library using as a vector the plasmid YEp24 (see Figure 1; Botstein et al., 1979), which replicates in both Escherichia coli and yeast, confers ampicillin resistance upon bacteria and complements the *ura3* mutation in yeast and the *pyrF* mutation in bacteria. Total genomic DNA from yeast strain DBY939 (*suc2-215am*) was partially digested with restriction endonuclease Sau 3AI, and fragments larger than 10 kb were recovered and inserted by ligation into the Bam HI site of YEp24, as described in the Experimental Procedures. The resulting recombinant DNA molecules were used to transform E. coli to ampicillin resistance in three experiments, yielding three libraries, each containing about 7000 independent transformants.

Selection for plasmids containing the *suc2-am* gene was carried out in yeast. Recombinant plasmid DNA prepared from each library was used to transform yeast strain DBY981 (*a suc2-432 ura3-52 his4-539am lys2-801am SUP-am*) to uracil prototrophy. Transformants carrying a plasmid capable of complementing the *suc2-432* missense mutation in the presence of the amber suppressor were then selected by their ability to ferment sucrose. Fifteen sucrose-fermenting transformants were chosen for further analysis.

The recombinant plasmids carried by the 15 yeast transformants were transferred again to bacteria (see Experimental Procedures), and plasmid DNAs were prepared for restriction site analysis. Gel electrophoresis of DNAs digested with either Sau 3AI or Hind III and Eco RI revealed that the inserted yeast DNA segments in all of the plasmids gave rise to common DNA fragments (data not shown). Seven different plasmids were recovered from the three libraries. Several restriction sites were mapped to determine the extent of overlap among the seven cloned yeast DNA segments, as shown in Figure 1. All the plasmids include a common 4 kb region. These data indicate that the plasmids contain overlapping DNA segments derived from the same chromosomal locus.

To confirm that the DNA obtained is in fact *SUC2* DNA, a further experiment was undertaken to test whether the ability of the cloned gene to complement a *suc2* mutation is dependent on the presence of an amber suppressor, as would be expected for a cloned *suc2-am* allele. The seven different plasmid DNAs were used to transform yeast strain DBY981 and also DBY982 (*α suc2-432 ura3-52 his4-539am lys2-801am*) to uracil prototrophy. Transformants of DBY981 (*SUP-am*) fermented sucrose, as before, but transformants of DBY982, which carries no amber suppressor, did not ferment sucrose. His^+ revertants of DBY982 transformed with pRB55 were selected.

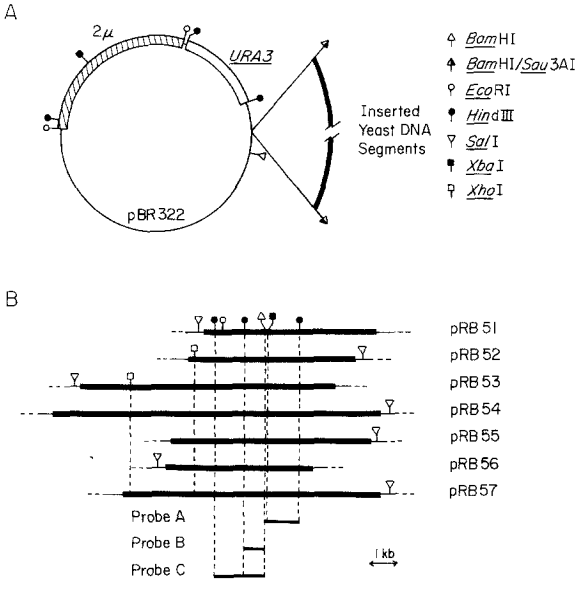

Figure 1. Restriction Maps of Recombinant Plasmids Carrying the *SUC2* Gene

(A) Structure of recombinant plasmids. Thin line: the pBR322 DNA in the YEp24 vector (Botstein et al., 1979). Hatched bar: the 2.2 kb Eco RI fragment of yeast 2μ (Form B) DNA. Open bar: the 1.1 kb Hind III fragment from the yeast *URA3*$^+$ gene. Solid bar: the yeast DNA fragments, produced by partial digestion with Sau 3AI, that are inserted at the Bam HI site of YEp24.

(B) Maps of yeast DNA segments carrying the *SUC2* gene. Solid bar: yeast DNA. Thin lines: vector DNA (not shown to scale). The Sal I site of YEp24 is shown to indicate the orientation of the yeast DNA with respect to the vector DNA. The orientation and extent of overlap of the different cloned yeast DNA segments was determined by electrophoretic analysis, on agarose gels, of the fragments produced by digestion of the plasmids with Xba I, Xba I and Sal I, or Xba I and Xho I. YEp24 contains single Xba I and Sal I sites (see [A]). The Bam HI, Hind III and Eco RI sites in the common 4 kb region are also shown; these sites were mapped by digestion with enzymes singly and in pairs, and also by partial digestion of DNA end-labeled with ^{32}P at the Bam HI site (Smith and Birnstiel, 1976). Recent data (M. Carlson, S. Kustu, P. Grisafi and D. Botstein, unpublished data) suggest that there are two closely spaced (<50 bp apart) Hind III sites, rather than one site, 0.8 kb to the left of the Bam HI site. The yeast DNA segments in probes A, B and C are indicated; these were used as probes in gel-transfer experiments described in the text.

Two independent revertants that were also now Lys^+ (indicating that they acquired an amber suppressor) turned out also to be sucrose-fermenting.

Subcloning Fragments of *suc2-am* DNA

Three Bam HI–Hind III restriction fragments derived from one of the *suc2*-carrying recombinant plasmids (pRB52) were subcloned in the vector pBR322 (Bolivar et al., 1977) to construct plasmid subclones A, B and C (Figure 1; see Experimental Procedures). These fragments span the central part of the 4 kb region known to include the *suc2-215* gene, and were expected to contain part or all of the gene. Use of the subclones as hybridization probes in gel-transfer hybridization experiments (Southern, 1975) with total yeast DNA confirmed that these subclones exhibit

sequence homology to the known *SUC* loci (*SUC1–SUC5, SUC7;* Carlson et al., 1980). These subclones were then used as probes to detect homologous RNAs, with the intent of both identifying RNAs homologous to *SUC2* (*SUC* RNAs) and mapping the *SUC2* gene on the cloned DNA segments.

Two *SUC* RNAs in Cells Synthesizing Two Forms of Invertase

Poly(A)-containing RNA was prepared from glucose-repressed and -derepressed yeast strains, as described in Experimental Procedures. The RNA samples were treated with glyoxal and dimethylsulfoxide (McMaster and Carmichael, 1977), subjected to electrophoresis on an agarose gel and transferred to diazotized paper (Alwine et al., 1977). RNAs homologous to the subcloned fragments were detected by hybridization to plasmid DNA made radioactive by nick translation. Figure 2 (lane a) shows that two RNA species (1.9, 1.8 kb) were detected in glucose-derepressed cells of wild-type strain DBY782 (*SNF1*$^+$ *SUC2*$^+$) with probe B (see Figure 1). Only the smaller RNA was detected in glucose-repressed cells of this strain (Figure 2 lane b). The number of RNA species correlates with the forms of invertase present in these cells; derepressed cells synthesize both forms of invertase, and repressed cells contain only the nonglycosylated enzyme. The intensity of the 1.8 kb band is approximately the same in both lanes; approximately equal amounts of RNA were loaded in each lane. The 1.9 kb RNA is present in derepressed cells at higher levels than the 1.8 kb RNA. Both RNA species are also homologous to probe A (see Figure 1), indicating that the transcriptional unit spans the Bam HI site. The size of these RNAs, their transcription from a region central to the 4 kb segment known to include *SUC2* and the correlation between the levels of these RNAs and the levels of the two forms of invertase in glucose-regulated cells suggest that these RNA species are transcriptional products of the *SUC2* gene. Further mapping of the transcriptional unit (see below) shows that the 4 kb region could not include another transcriptional unit large enough to encode invertase, since a 60,000 dalton protein requires a coding sequence of approximately 1.6 kb.

SNF1 Locus Affects *SUC* RNA Levels

The *snf1* mutants isolated previously (Carlson et al., 1981a) are unable to derepress synthesis of the glycosylated invertase, but they produce the nonglycosylated enzyme normally in glucose-repressed and -derepressed cells. To determine whether the *snf1* mutation affects *SUC2* gene expression at the RNA level, *SUC* RNAs present in glucose-repressed and -derepressed DBY934 (*SUC2*$^+$ *snf1-28*) were examined as described above. Figure 2 (lanes c and d) shows that this *snf1* mutant contains the 1.8 kb *SUC* RNA, but fails to produce the 1.9 kb species under

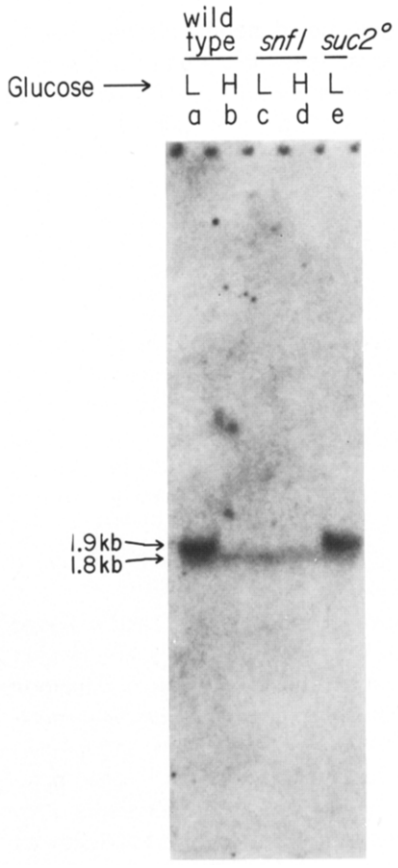

Figure 2. Glucose Regulation of *SUC* RNAs in Wild-Type and Mutant Yeast Strains

Poly(A)-containing RNA was prepared from glucose-repressed and -derepressed yeast strains and analyzed by gel-transfer hybridization as described in Experimental Procedures. RNAs 1.8 and 1.9 kb in size were detected by autoradiography; sites were determined by comparison with ribosomal RNAs (1.7 kb and 3.4 kb; Philippsen et al., 1978). Probe B (see Figure 1) was used in this experiment; probe A gave identical results. With probe C, a 2.2 kb RNA was detected, in addition to the 1.8 and 1.9 kb RNAs (data not shown). RNA samples were prepared from cells as follows: (lane a) wild-type DBY782, grown in low glucose (L); (lane b) DBY782, grown in high glucose (H); (lane c) DBY934 (*snf1*), low glucose; (lane d) DBY934, high glucose; (lane e) DBY1046 (*suc2*°), low glucose.

derepressing conditions. These data indicate that the *SNF1* product is needed for synthesis of the larger *SUC* RNA, and also strengthen the correlation between the presence in a yeast cell of the 1.8 and 1.9 kb RNAs and the nonglycosylated and glycosylated invertases, respectively.

Structure of *SUC* RNAs and Map of Transcriptional Unit

The S1-nuclease-protection mapping technique of Berk and Sharp (1977), as modified by Weaver and Weissman (1979), was employed to determine the structure of the two *SUC* RNA species and to map the *SUC2* transcriptional unit on the cloned DNA (Figure 3). The single Bam HI site in plasmid pRB58, a plasmid with a functional *SUC2*$^+$ gene derived from pRB55 (see below), is located between subcloned DNA frag-

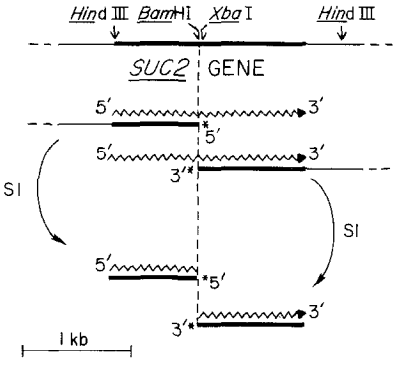

Figure 3. S1 Mapping of *SUC* RNAs

The S1 mapping experiments described in the text are outlined schematically in the diagram. The map of the *SUC2* transcriptional unit (solid bar) on the cloned yeast DNA is shown (see also Figure 1), and the direction of transcription is indicated by the polarity of the *SUC* RNA (wavy line). Asterisks: ^{32}P-labeled DNA ends. Curved arrows: treatment with S1 endonuclease.

ments that hybridize to *SUC* RNA. It thus seemed likely that *SUC* RNA would hybridize to, and protect from S1 nuclease digestion, the sequences immediately flanking this Bam HI site. We cleaved the plasmid pRB58 DNA with Bam HI endonuclease, and labeled the 5′ ends at the restriction site with ^{32}P using polynucleotide kinase, as described in Experimental Procedures. The labeled DNA was denatured and allowed to hybridize to poly(A)-containing RNA prepared from glucose-repressed or -derepressed cells of wild-type strain DBY782; samples were then digested with S1 endonuclease to degrade single-stranded DNA not protected by RNA in a DNA–RNA duplex structure (Berk and Sharp, 1977; see Experimental Procedures). Samples were then subjected to electrophoresis on an alkaline–agarose gel (McDonnell et al., 1977), and the protected DNA fragments retaining radioactively labeled 5′ ends were detected by autoradiography. The size of the protected fragment is a measure of the contiguous DNA sequence, extending from the Bam HI site towards the 5′ end of the RNA, that is protected by RNA. Figure 4A shows that a 0.83 kb fragment is protected by RNA from glucose-derepressed cells, but not by RNA from glucose-repressed cells. RNA from cells grown under both conditions protected fragments approximately 0.76 kb in size; the contrast between the sharp 0.83 kb band and the relatively diffuse 0.76 kb band suggests that the fragments composing the lower band are heterogeneous in size. The difference in intensity of the lower band between lanes b and c of Figure 4A is the result of the use of more RNA from repressed cells (lane c) than from derepressed cells (lane b) in this experiment. These data show that contiguous DNA sequences homologous to the glucose-regulated 1.9 kb *SUC* RNA species extend 0.83 kb from the Bam HI site towards the 5′ end of the RNA (see Figure 3). Similarly, contiguous DNA sequences homologous to the unregulated 1.8 kb RNA extend approximately 0.76

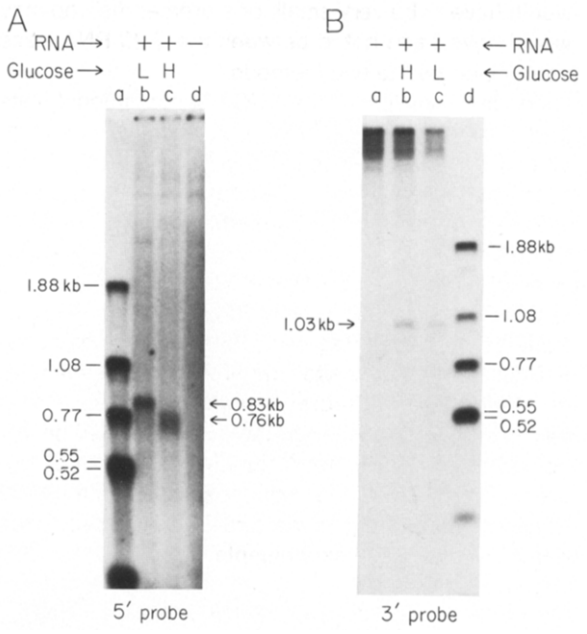

Figure 4. Two *SUC* RNAs Differ at Their 5′ Ends

S1-nuclease-protection experiments were carried out with 5′-end-labeled probe (A) and 3′-end-labeled probe (B) as described in the text. Autoradiographs are shown.

(A) (Lane a) Radioactively labeled marker fragments of SV40 DNA digested with Hinf I. (Lane b) DNA fragments (0.83 kb and approximately 0.76 kb) protected by poly(A)-containing RNA prepared from cells grown in low glucose (L; derepressed). (Lane c) DNA fragments (approximately 0.76 kb) protected by poly(A)-containing RNA prepared from cells grown in high glucose (H; repressed). (Lane d) Control sample, showing no protection of DNA in the absence of added yeast RNA.

(B) (Lane a) Control sample, with no added yeast RNA. (Lane b) DNA fragments (1.03 kb) protected by poly(A)-containing RNA from cells grown in high glucose (H). (Lane c) DNA fragments (1.03 kb) protected by poly(A)-containing RNA from cells grown in low glucose (L). (Lane d) Marker Hinf I fragments of SV40 DNA.

kb, but the 5′ end of homologous RNA appears heterogeneous.

To map the extent of RNA sequences from the Bam HI site towards the 3′ end of the RNAs, we performed an analogous experiment using as a probe Bam HI–cleaved pRB58 DNA radioactively labeled at the 3′ ends by Micrococcus luteus DNA polymerase I (see Experimental Procedures). Figure 4B shows that RNA from glucose-repressed and -derepressed cells protected 3′-end-labeled fragments of the same size, 1.03 kb. Thus the 3′ ends of botn *SUC* RNAs extend 1.03 kb from the Bam HI site.

The sizes of the protected fragments in these two experiments add up to 1.86 and 1.79 kb, which agree with the sizes determined by RNA gel-transfer hybridizations for the two *SUC* RNAs. This agreement indicates that the *SUC2* gene contains no intervening sequence near the middle of the gene. Although intervening sequences close to the 5′ or 3′ ends of either *SUC* RNA would not have been detected by this S1-nuclease-protection study, any additional exons

would have to be very small, or a greater discrepancy would have been noted between the *SUC* RNA sizes determined by the two methods.

The map position of the *SUC2* transcriptional units on the cloned DNA segment was at this point ambiguous with respect to whether the 5′ ends lie 0.83 and 0.76 kb from the Bam HI site on the same or opposite side as the Xba I site. We determined the orientation by digesting the labeled DNA probes with Xba I prior to hybridization. Figure 5A shows that Xba I digestion of the 5′-end-labeled probe did not remove the label from the protected fragment; thus the 5′ ends of the RNAs lie on the opposite side of the Bam HI site from the Xba I site. Conversely, Figure 5B shows that following Xba I digestion of the 3′-end-labeled probe, no end-labeled protected fragment was detected. Figure 3 summarizes the map position of the *SUC2* transcriptional unit and the direction of transcription deduced from these experiments.

Regulation of Expression of *SUC2* Gene on a Plasmid Vector

The *suc2-am* gene cloned in the multicopy plasmid vector YEp24 is expressed to a sufficient extent in an amber-suppressing yeast strain to permit selection for the cloned gene on the basis of function. For the purpose of investigating the regulation of expression of the cloned gene, however, it was convenient to study the *SUC2*$^+$ rather than the *suc2-am* allele. We obtained a recombinant plasmid carrying *SUC2*$^+$ by selecting sucrose-fermenting progeny of strain DBY982 (*suc2-432*) transformed with pRB55 (*suc2-215am URA3*$^+$). Sucrose-fermenting progeny could arise in several ways: reversion of the chromosomal *suc2-432* mutation; reversion of the *suc2-215am* mutation on one or more copies of the plasmid; acquisition of an amber suppressor; or recombination or gene conversion involving the chromosomal and episomal genes and yielding a *SUC*$^+$ allele at either site (S. C. Falco and D. Botstein, personal communication). It is likely that most of the Suc$^+$ progeny recovered were generated by the latter mechanism because the frequency of Suc$^+$ colonies was roughly 100-fold greater than would have been expected from simple reversion; *suc2-215* and *suc2-432* are at opposite ends of the *SUC2* genetic map (Carlson et al., 1981a). Two independently arising strains carrying episomal *SUC*$^+$ alleles were identified by their frequent segregation of sucrose-nonfermenting progeny. Repeated colony purification yielded in each case a yeast clone carrying a homogeneous population of *SUC*$^+$ plasmids, as judged by the consistent cosegregation of the *SUC*$^+$ and *URA3*$^+$ markers. The two independent *SUC*$^+$ plasmids are called pRB58 and pRB60. A final purification of each *SUC*$^+$ plasmid was accomplished by passage through bacteria; yeast DNA prepared from each strain was used to transform E. coli, and then plasmid DNA from the bacterial transformants was used to transform yeast DBY982 again (see Experimental Procedures).

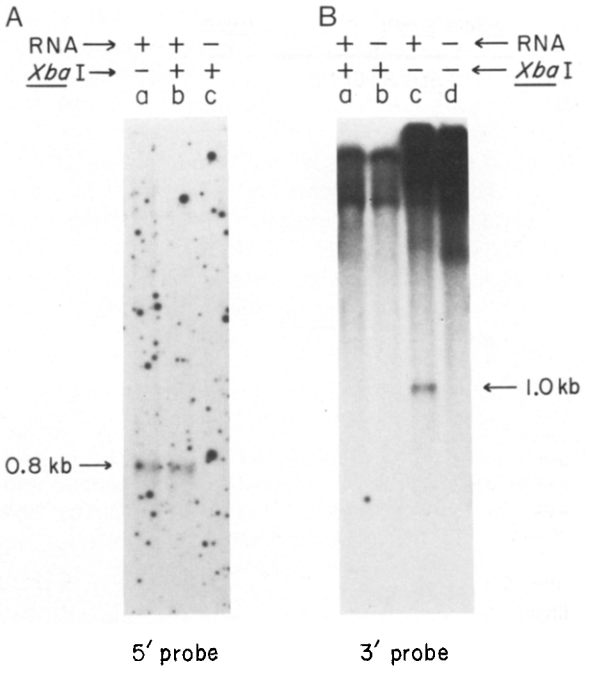

Figure 5. Direction of Transcription of the *SUC2* Gene

S1-nuclease-protection experiments were performed with 5′-end-labeled probe (A) and 3′-end-labeled probe (B). Where Xba I digestion is indicated (+), these probes were cleaved with Xba I, extracted with phenol and precipitated with ethanol. The digestion products were then used in S1 protection experiments. Poly(A)-containing RNA prepared from DBY782 cells grown in low glucose (derepressed) was used.

(A) Treatment with Xba I (lane b) did not remove radioactive label from the 5′ end of the protected 0.8 kb fragment. Control samples contained undigested probe and yeast RNA (lane a) or Xba I–digested probe and no added yeast RNA (lane c).

(B) No 1.0 kb protected fragment was detected following Xba I digestion of 3′-end-labeled probe (lane a). Control samples contained Xba I–digested probe and no added yeast RNA (lane b), undigested probe and yeast RNA (lane c) or undigested probe and no added yeast RNA (lane d).

DBY982 cells carrying pRB58 or pRB60 were grown under glucose-repressing and -derepressing conditions and then assayed for glycosylated and nonglycosylated invertase by a gel assay method (Gabriel and Wang, 1969; Carlson et al., 1981a). Both forms were present in derepressed cells, and synthesis of the glycosylated form was regulated by glucose (data not shown). The control of expression of the plasmid *SUC2* gene by the *SNF1* locus was tested by genetic analysis. DBY1047 (*snf1-31 ura3-52*) was crossed to DBY982 (pRB58) and DBY982 (pRB60) to construct *SNF1*$^+$/*snf1-31 ura3-52*/*ura3-52* diploids carrying each of the *SUC2*$^+$ *URA3*$^+$ plasmids. The diploids were sporulated on media selective for the *URA3*$^+$ plasmid markers and subjected to tetrad analysis. Tetrads in which all spores harbored the *SUC2*$^+$ *URA3*$^+$ plasmid were identified by the Ura$^+$ phenotype

of the four spores. We recovered ten such tetrads carrying pRB58 and three carrying pRB60. These tetrads displayed 2 Suc^+:2 Suc^- segregations. All Suc^- spores were unable to utilize glycerol as a carbon source, a phenotype of the *snf1-31* mutation (Carlson et al., 1981a); all Suc^+ spores were able to do so. These data indicate that the *snf1-31* mutation prevents phenotypic expression of episomal copies of $SUC2^+$. Thus the location of *SUC2* on a plasmid and its presence in multiple copies per cell do not appear to affect regulation of its expression by glucose or the *SNF1* locus.

The Silent *suc2°* Gene Expresses *SUC* RNAs

A naturally occurring negative allele at the *SUC2* locus, called *suc2°*, has previously been shown to be a silent *SUC* gene (or pseudogene) by genetic and physical analysis (Carlson et al., 1980, 1981b). The *suc2°* allele contains sequences homologous to $SUC2^+$, but does not direct synthesis of active invertase or confer a sucrose-fermenting phenotype. Possible explanations for this failure were that the gene is not expressed or that *suc2°* encodes a defective invertase. We investigated the possibility that *suc2°* is defective in transcription by looking for *SUC* RNAs in a strain carrying the *suc2°* allele but no active SUC^+ allele or other silent *suc°* allele. The strain chosen, DBY1046 (*suc2°*), is congenic to S288C (Carlson et al., 1981b). Poly(A)-containing RNA was prepared from glucose-repressed and -derepressed cells and analyzed for the presence of *SUC* RNAs as before. Figure 6 shows that 1.8 and 1.9 kb *SUC* RNAs were detected in derepressed cells, and that only the 1.8 kb species was found in repressed cells. Although subtle differences in RNA structure from that of wild-type may have escaped detection, no gross defect in synthesis or regulation of *suc2°* RNA is apparent.

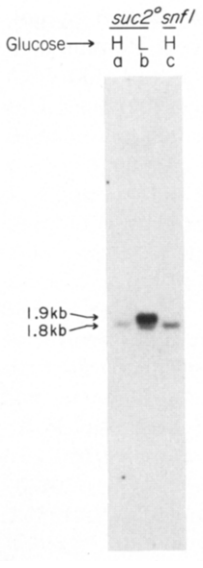

Figure 6. The Silent *suc2°* Gene Produces Normally Regulated *SUC* RNAs

Poly(A)-containing RNAs were prepared from DBY1046 (*suc2°*) cells grown in high (H) glucose (lane a); DBY1046 cells grown in low (L) glucose (lane b); and, as a marker, DBY934 (*snf1*) cells grown in high (H) glucose (lane c). The RNAs were analyzed by gel-transfer hybridization, as described in Experimental Procedures, with probe C. See also Figure 2 (lane e) for evidence that the RNAs from the *suc2°* gene are the same size as those from the $SUC2^+$ gene.

Discussion

We have cloned the *SUC2* gene by complementation in yeast. To be sure that the complementing gene was indeed *SUC2* and not another gene, we used an amber $suc2^-$ allele and showed that the complementation depended on the presence of a suppressor of amber mutations. The cloned *SUC2* DNA was used to characterize *SUC* RNA species present in cells growing in excess glucose (where invertase synthesis is repressed) and limiting glucose (where invertase synthesis is derepressed). Two *SUC* RNA species (1.8 and 1.9 kb in size) were detected by gel-transfer hybridization in derepressed cells, which synthesize the secreted, glycosylated form of invertase as well as the intracellular, nonglycosylated form. The region that is common to plasmids capable of complementing a $suc2^-$ defect is about 4 kb long. The only RNAs that are large enough to encode invertase and that lie entirely within this region are the 1.8 and 1.9 kb RNAs, which, as discussed below, are the same except for their 5′ ends. Only the smaller (1.8 kb) RNA was found in repressed cells, which synthesize only the intracellular, nonglycoslyated invertase.

These data are consistent with the idea that glucose regulation of the amount of the glycosylated form of invertase occurs, at least in part, at the level of RNA synthesis or stability: the presence or absence of the secreted enzyme correlates with the presence or absence of the larger (1.9 kb) *SUC* RNA. This conclusion differs from that drawn by Chu and Maley (1980), who suggested, on the basis of in vitro translation assays, that changes in RNA levels could not account for glucose regulation of invertase. Although our gel-transfer experiments were not quantitative, the 1.9 kb RNA was clearly more abundant than the 1.8 kb RNA in the derepressed cells; this difference corresponds nicely with the greater amounts of the secreted, glycosylated invertase found in these cells. Our conclusions also differ from those of Perlman and Halvorson (1981), who suggested that *SUC* genes encode three mRNAs, all three of which are subject to glucose regulation.

We recently identified a locus (*SNF1*) that appears to be involved in glucose regulation of invertase; *snf1* mutations have pleiotropic effects on utilization of a variety of carbon sources (Carlson et al., 1981a). The *snf1* mutants synthesize the intracellular, nonglycosylated form of invertase normally, but fail to synthesize the secreted, glycosylated form when dere-

pressed. Analysis of the *SUC* RNA content by gel-transfer hybridization showed that *snf1* mutants contain the 1.8 kb RNA, but fail to make the 1.9 kb RNA even under conditions of glucose derepression. This result strengthens the correlation between the presence of the larger *SUC* RNA and the synthesis of secreted, glycosylated invertase, and indicates that the *SNF1* gene product exerts its effect in glucose regulation at the level of RNA synthesis or stability.

Regulation of the amount of invertase mRNA appears to be exerted through a mechanism that is insensitive to the number of copies of the structural gene in the cell, since the expression of a *SUC2* gene on a multicopy plasmid is normally regulated, except that the amount of invertase (of both forms) is increased. Interestingly, the silent, naturally occurring *suc2°* gene (Carlson et al., 1981b) shows normal synthesis and regulation of the 1.8 and 1.9 kb mRNAs. Thus the defect in invertase expression from the *suc2°* gene is posttranscriptional.

We studied the structural relation of the 1.8 and 1.9 kb RNAs using the S1 nuclease mapping technique (Berk and Sharp, 1977; Weaver and Weissman, 1979), and found that the two RNAs differ only at their 5′ ends. Both are transcribed in the same direction from the same region, but the longer RNA contains extra contiguous material at its 5′ end. No intervening sequences were detected in either RNA, although it should be noted that our experiments cannot exclude the possibility of an intervening sequence with a small mRNA-coding region (exon) very close to an end of either of the *SUC* RNAs. We have, however, excluded the idea that the larger RNA consists of the smaller RNA with extra RNA spliced onto it from a position farther in the 5′ direction.

Thus our results suggest the simple idea that the *SUC2* RNAs are the mRNAs for the two forms of invertase. The smaller RNA might, in this view, encode the nonglycosylated, intracellular enzyme, and the larger RNA might then encode the secreted, glycosylated enzyme. Our results on the differential regulation of the two RNAs are entirely consistent with this view.

We have recently begun sequence analysis of DNA flanking a Hind III site near the 5′ end of the *SUC2* gene. When our preliminary sequence is compared with the partial amino acid sequence of the amino terminus of invertase (Perlman et al., 1982) an almost perfect match is obtained, confirming that the *SUC2* gene is indeed a structural gene for invertase. The Hind III site that we used maps in the region carried by the 1.9 kb RNA, but not present in at least the bulk of the somewhat heterogeneous 1.8 kb RNA. The amino acid sequence at this point resembles a "signal" sequence of the kind found in many secreted proteins in both procaryotes and eucaryotes that is translated but is then removed during secretion (Davis and Tai, 1980). Perlman and Halvorson (1981) have obtained evidence that invertase mRNAs, when translated in vitro, encode precursor forms that can be processed and secreted in the presence of dog pancreas membranes, as well as a shorter form that might correspond to the intracellular invertase. They first suggested that multiple invertase mRNAs exist that differentially encode intracellular and secreted invertase.

All of these results, when taken together with the results described here, suggest a model for synthesis and regulation of invertase (Figure 7). According to this model, the 1.9 kb RNA we have described encodes the entire sequence of the precursor of secreted invertase, and translation of this RNA begins with the methionine codon at the beginning of the putative signal sequence. The 1.8 kb RNA, according to this simple model, begins beyond the Hind III site within the signal sequence region and lacks the start of translation used for the 1.9 kb RNA. Translation of the shorter RNA would begin at the next initiation codon, which in our preliminary sequence (and that of Perlman et al., 1982) is found just one codon beyond the point at which Perlman and coworkers found processing of the putative secreted-invertase precursor in vitro.

This model easily accounts for the known facts about the relation of intracellular and secreted invertases: they are encoded (at least in part) by the same structural gene, but apparently do not exhibit a precursor–product relation (Lampen et al., 1972; Gallili and Lampen, 1977). The two enzymes are found in different cell compartments simply because one has a signal sequence whereas the other does not. This would be consistent with the idea that the signal sequence is essential for secretion and glycosylation of some eucaryotic proteins.

The proposed model requires an additional feature, however, to bring it into concordance with our results concerning regulation of the two forms of invertase. The simplest idea is that the two RNAs are differentially regulated at the level of transcription. On this theory, two promoter sequences are present; one responds to no signal related to glucose repression and results in the production of the 1.8 kb RNA and thereby of intracellular invertase, while the other pro-

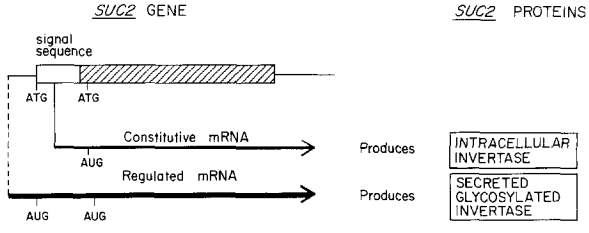

Figure 7. Model for the Synthesis and Regulation of Invertase

The *SUC2* structural gene is represented by a bar that is hatched except in the coding region for the signal sequence. Arrows: RNA transcripts, pointing in the direction of the 3′ end. Initiation codons are indicated. See text for explanation.

moter responds to glucose regulation (including the *SNF1* product) and results in the synthesis of the longer 1.9 kb RNA and thereby of secreted, glycosylated invertase. Only the larger RNA need be regulated. Models in which stability of the RNA (rather than synthesis) is regulated are not excluded, although any such model must provide that the mechanism of differential degradation recognize the 5′ end of the 1.9 kb *SUC* RNA specifically. Yet other models involving differential RNA processing are not excluded either, although any model involving splicing must take into account our experimental finding that any intervening sequences must be very close to the ends of the *SUC* RNAs.

Differential regulation through the production of different mRNAs from a single gene has been observed before. The earliest case was the repressor protein of bacteriophage lambda, which is transcribed from different promoters under different regulation during establishment and maintenance of lysogeny (Reichardt and Kaiser, 1971). Young et al. (1981) have shown that a single mouse α-amylase gene encodes two tissue-specific mRNAs that differ only in 5′ noncoding sequences. The secreted and membrane-bound forms of immunoglobulin μ chain are encoded by two developmentally regulated mRNAs that differ only in 3′ coding and noncoding sequences and are produced from transcripts of a single μ gene (Alt et al., 1980; Early et al., 1980; Rogers et al., 1980).

Experimental Procedures

Strains and Genetic Methods

Genetic techniques and media have been described (Carlson et al., 1981a). All strains are isogenic or congenic to S. cerevisiae strain S288C. Alleles have been described (Carlson et al., 1981a); *ura3-52* was obtained from F. Lacroute. Yeast strains DBY782 (*α SUC2⁺ ade2-101*), DBY934 (*a snf1-28 SUC2⁺ his4-619*) and DBY939 (*α suc2-215am ade2-101*) were derived from S288C by mutation. DBY981 (*a suc2-432 ura3-52 his4-539am lys2-801am SUP-am*) and DBY982 (*α suc2-432 ura3-52 his4-539am lys2-801am*) were segregants of the same cross and have predominantly the S288C genetic background. The *SUP-am* marker is a spontaneous mutation that suppresses *his4-539, lys2-801* and *suc2-215* (Carlson et al., 1981a). DBY1046 (*α suc2° his4-619*) is the product of 13 crosses to S288C-derived strains (Carlson et al., 1981b). The genotype of DBY1047 is *a snf1-31 SUC2⁺ ura3-52 ade2-101 his4-539*.

We selected His⁺ revertants of DBY982 transformed with pRB55 by plating on minimal medium supplemented with lysine, a growth condition that also selects for maintenance of the *URA3⁺* marker on the plasmid. We selected sucrose-fermenting progeny of strain DBY982 carrying pRB55 by spreading single colonies on YEP-sucrose and incubating anaerobically at 30°C (Carlson et al., 1981b).

Restriction Enzymes and Gel Electrophoresis of DNA

All restriction enzymes were purchased from New England BioLabs. Agarose gel electrophoresis of DNA fragments was carried out in 89 mM Tris–HCl, 89 mM boric acid, 2.5 mM EDTA (pH 8.3) (Peacock and Dingman, 1968).

Construction of Yeast Recombinant Plasmid Library

Total genomic DNA was isolated from DBY939 (*suc2-215am*) by a modification of the method of Cryer et al. (1975). DNA (450 μg) was partially digested with endonuclease Sau 3AI; aliquots were removed at several time points and extracted with phenol. The resulting DNA fragments were fractionated by centrifugation in 10%–40% sucrose gradients in 1 M NaCl, 20 mM Tris–HCl (pH 8), 10 mM EDTA. The size of the DNA fragments in each fraction was determined by agarose gel electrophoresis. Fragments 10 kb or larger were recovered and concentrated by ethanol precipitation. Fragments were then inserted into the Bam HI site of plasmid YEp24 (Botstein et al., 1979) as follows. YEp24 DNA was cleaved with Bam HI endonuclease, treated with calf intestinal phosphatase (Boehringer Mannheim) to prevent later self-ligation, extracted with phenol and precipitated with ethanol. This vector DNA was then ligated to the yeast DNA fragments (mass ratios of 2:1 or 1:1) with T4 DNA ligase (New England BioLabs) in 50 mM Tris–HCl (pH 7.8), 10 mM $MgCl_2$, 20 mM dithiothreitol, 1 mM ATP at 12°C overnight. The ligated DNA was used to transform E. coli HB101 (Boyer and Roulland-Dussoix, 1969) to ampicillin resistance. Transformation was effected essentially as described by Carlson and Brutlag (1977), except that cells were spread on LB plates containing 100 μg/ml ampicillin, rather than plated in soft agar, to maximize uniformity in colony size. Three separate libraries, each containing about 7000 independent transformants, were recovered. Approximately 90% of the transformants were sensitive to tetracycline at levels to which YEp24 confers resistance.

Selection of Plasmids Carrying the *suc2-am* Gene

Plasmid DNA was prepared from each library by cesium chloride–ethidium bromide equilibrium centrifugation and was used to transform (Hinnen et al., 1978) yeast strain DBY981 to uracil prototrophy. We recovered transformants from the regeneration agar by passing the agar through a syringe and eluting cells into water. We selected transformants carrying a plasmid able to complement *suc2-432* in the amber-suppressing strain DBY981 by spreading cells on YEP-sucrose and incubating anaerobically (Carlson et al., 1981a). Fourteen sucrose-fermenting colonies, including several from each library, were chosen for further study; colonies derived from the same library were not necessarily independent. It also proved possible to select directly for sucrose-fermenting transformants by plating the transformed spheroplasts in regeneration agar containing sucrose on YEP-sucrose medium. An additional colony obtained in this way was also analyzed (and yielded pRB51). These 15 transformants were purified by single-colony isolation, and total genomic DNA was prepared from each by a modification of the procedures described by Cameron et al. (1977). These DNAs were used to transform E. coli HB101 to ampicillin resistance, thereby transferring the recombinant plasmid into bacteria. Plasmid DNAs were prepared from bacteria by a rapid method (Rambach and Hogness, 1977) for use in restriction site analysis and transformation of yeast. Four plasmids were found to contain additional 2μ DNA sequences besides those of YEp24, presumably as a result of recombinational events occurring in yeast.

Construction of Subclones

pRB52 DNA purified on a cesium chloride–ethidium bromide density gradient was digested simultaneously with Bam HI, Sal I and Xho I. The Bam HI–Sal I and Bam HI–Xho I fragments were separated by agarose gel electrophoresis and recovered by electrophoretic elution (Carlson and Brutlag, 1978). The fragments were purified by hydroxyapatite chromatography, dialyzed and concentrated by ethanol precipitation. The fragments were then partially digested with Hind III and ligated, as described above, to the purified Bam HI–Hind III vector fragment of pBR322 (Bolivar et al., 1977). The ligated DNAs were used to transform E. coli HB101 to ampicillin resistance. Plasmids pRB59, pRB117 and pRB118 were recovered and are here referred to as subclones A, B and C. Their structures were determined by restriction site analysis (see Figure 1).

Preparation of Yeast RNA

Glucose-repressed and -derepressed yeast cells were grown as before (Carlson et al., 1981a), except that the growth period following the glucose shift was only 1 hr. Cells (100 ml) were harvested by centrifugation in the cold. To the cell pellets were added 8 ml of cold breaking buffer (0.5 M NaCl, 0.2 M Tris–HCl [pH 7.5], 0.01 M EDTA),

8 g of acid-washed glass beads (0.45–0.50 mm diameter) and diethyloxydiformate to a concentration of 0.1%. Immediately following addition of 4 ml of phenol, chloroform and isoamyl alcohol (25:24:1 [v/v]), cells were broken by vigorous vortexing. Repeated extractions with phenol, chloroform, isoamyl alcohol and chloroform, isoamyl alcohol were performed, and then total nucleic acid (2–3 mg) was precipitated with ethanol. Poly(A)-containing RNA was purified from the nucleic acid by chromatography on poly(U)-Sepharose (Pharmacia) (Lindberg and Persson, 1974). Samples were heated in 10 mM sodium phosphate (pH 6.7) for 3 min at 68°C and quickly chilled. RNA was loaded on the column in binding buffer (25% formamide, 0.7 M NaCl, 10 mM sodium phosphate [pH 6.7], 10 mM EDTA). The column was washed with 50% formamide, 0.5 M NaCl, 10 mM sodium phosphate (pH 6.7), 10 mM EDTA, 0.1% sarkosyl. Poly(A)-containing RNA was eluted with 95% formamide, 10 mM sodium phosphate (pH 6.7), 10 mM EDTA, 0.1% sarkosyl. Because *SUC* RNAs are nearly the same size as ribosomal RNA, the chromatography was repeated a second time. RNA was concentrated by ethanol precipitation.

Gel Electrophoresis of RNA, Transfer to Diazotized Paper and Hybridization

RNA samples were precipitated with ethanol, treated with glyoxal and dimethylsulfoxide (McMaster and Carmichael, 1977) and subjected to electrophoresis on 1.2% agarose (SeaKem) in 10 mM sodium phosphate (pH 6.7). RNAs were transferred from the gel by the method of Alwine et al. (1977), as modified by Alwine et al. (1980), to diazophenylthioether paper, prepared according to the method of B. Seed (unpublished procedure). Pretreatment of the paper and hybridization were carried out as described by Wahl et al. (1979) with dextran sulfate. Radioactively labeled probes were prepared by nick translation of plasmid DNAs (Rigby et al., 1977). Following hybridization, the paper was washed in 50% formamide, 0.75 mM NaCl, 75 mM trisodium citrate, 20 mM sodium phosphate (pH 6.7), 0.1% SDS at 42°C, and then allowed to expose Kodak XR-5 film at −70°C with DuPont Lightning Plus screens.

S1 Mapping

Plasmid pRB58 DNA was cleaved with Bam HI endonuclease, extracted with phenol and precipitated with ethanol. For 5′ end labeling, DNA was dephosphorylated with calf intestinal phosphatase and 5′-phosphorylated with T4 polynucleotide kinase (Boehringer Mannheim) and γ-^{32}P-ATP (New England Nuclear) according to the method of Maxam and Gilbert (1980). For 3′ end labeling, α-^{32}P-dGTP was incorported with M. luteus DNA polymerase I (Miles) (Shortle and Nathans, 1978). S1 mapping was performed essentially as described by Berk and Sharp (1977). The optimal temperature for hybridization (43°C) and the amount of S1 endonuclease (Miles) added were determined empirically. E. coli tRNA (Boehringer Mannheim) was used as carrier. Alkaline gel electrophoresis (McDonnell et al., 1977) was performed on 1.5% agarose, and autoradiography of the dried gel was performed as described above.

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