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Isolation of *Saccharomyces cerevisiae* Mutants Constitutive for Invertase Synthesis

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A new method for detecting invertase activity in *Saccharomyces cerevisiae* colonies was used to screen for mutants resistant to catabolite repression of invertase. Mutations causing the highest level of derepression were located in two previously identified genes, *cyc8* and *tupl*. Several of the *cyc8* mutations, notably *cyc8-10* and *cyc8-11*, were temperature dependent, repressed at 23°C, and derepressed at 37°C. The kinetics of derepression of invertase mRNA in *cyc8-10* cells shifted from 23 to 37°C was determined by Northern blots. Invertase mRNA was detectable at 5 min after the shift, with kinetics of accumulation very similar to that of wild-type cells shifted from high-glucose to low-glucose medium. Assays of representative enzymes showed that many but not all glucose-repressible enzymes are derepressed in both *cyc8* and *tupl* mutants. *cyc8* and *tupl* appear to be the major negative regulatory genes controlling catabolite repression in yeasts.

Saccharomyces cerevisiae cells grown on glucose have much lower levels of many enzymes in comparison with cells grown on alternate carbon sources such as acetate or glycerol. Similar differences are noted between different phases of batch growth on glucose, with these same enzymes becoming derepressed as the glucose in the medium is exhausted. This phenomenon is known as carbon catabolite repression, or glucose repression. Since control is known to occur in many cases at the level of transcription, catabolite repression provides a prime example of coordinate regulation of a large battery of genes.

Invertase, the cell wall enzyme which hydrolyzes sucrose to glucose and fructose, is well-suited for the study of catabolite repression since it is repressed over 100-fold by glucose. In contrast to most other catabolite-repressible enzymes in yeasts, invertase is regulated exclusively by glucose repression. Two types of mutants affected in the regulation of invertase synthesis have been isolated. Mutations in any of six *snf* genes prevent derepression of invertase synthesis and thus identify elements of positive control (4, 18). Mutations causing constitutive synthesis of invertase, and thus defective in negative control, were isolated by selecting for growth on the trisaccharide raffinose in the presence of 2-deoxyglucose (30). The selected mutants were derepressed for invertase as well as several other enzymes including maltase. Carlson et al. (3) isolated suppressors of the *snf1* mutation, which prevents derepression of glucose-repressible genes. Mutations at one of the suppressor genes, *ssn6*, cause constitutive synthesis of invertase and other repressible enzymes.

Since mutant screens relying upon selective methods may yield only a subset of the possible derepressed mutants, nonselective mutant screens may be necessary to identify additional types of mutants. Montencourt et al. (14) isolated a mutant strain, FH4C, by screening colonies for glucose-repression-resistant invertase synthesis. Because of its very high level of invertase production, FH4C has been used to purify invertase (26) and to study its regulation (20). Unfortunately, genetic analysis of FH4C has been impossible owing to its poor mating ability.

This paper describes the first genetic analysis of yeast mutants isolated directly on the basis of their derepressed invertase synthesis. To screen large numbers of mutants, I developed a new method for detecting invertase activity in yeast colonies, based on the method for detecting activity in polyacrylamide gels (8). Yeast colonies on agar plates are transferred to dry Whatman no. 3 filters (9.0-cm diameter) by pressing the filters onto the plates. A second dry filter is stapled to the first filter to make a sandwich with the yeast colonies in the interior. The filter sandwich is placed into a Buchner funnel on a vacuum filtration flask and rinsed by vacuum with 100 ml of H_2O to remove any glucose or other reducing sugars which would interfere with the assay. The filters are then wetted with a solution of 5% sucrose in 10 mM sodium acetate (pH 4.6) and incubated for 3 to 5 min at room temperature, and each is transferred to a separate plastic petri dish floating in a 50°C water bath. A 3-ml portion of 0.1% triphenyltetrazolium chloride in 0.5 N NaOH is added, and after the red color develops in invertase-positive colonies (1 to 2 min), the filters are removed and blotted to remove excess liquid.

For the isolation of mutants, two *S. cerevisiae* strains, AH22 (*Mata can1 leu2 his4*) and RTY10 (*MATα trp1*), were mutagenized with ethyl methanesulfonate as described previously (27). AH22 and RTY10 are both of S288c genetic background and are thus *SUC2* and *mal*^{0} (capable of fermenting sucrose but not maltose). The mutagenized cells were diluted and spread onto YEP (1% yeast extract, 2% Bacto-Peptone [Difco Laboratories, Detroit, Mich.], and 2% agar) containing 10% glucose. To include possible temperature-sensitive mutants, the plates were incubated at 23°C for 2 days and then at 37°C overnight. Invertase activity was determined by the filter assay, and colonies with the highest activity were streaked for isolation of single colonies and then retested by the filter assay after growth at 23 and 37°C.

Six mutants were derived from AH22, and nine from RTY10, for a total of 15 mutants from 12,000 screened colonies, or a yield of 0.121%. Figure 1 illustrates the genetic analysis of one of these mutants utilizing the invertase filter assay. The mutant strain has much higher invertase activity

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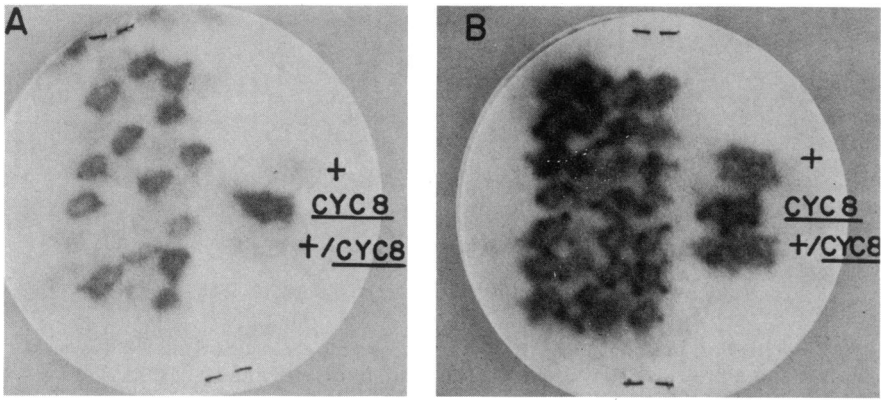

FIG. 1. Filter assay of invertase activity. The two parents from the cross between the wild-type DBY963 (*MATa his4 lys2*) (+) and the constitutive mutant RTY82 (*MATα trp1 cyc8-16*) (*cyc8*), the diploid, and spores from seven tetrads were grown on plates containing YEP plus 2% glucose and then replica plated onto YEP plus 10% glucose (A) and YEP plus 2% sucrose (B). After overnight growth at 30°C, invertase activity was detected on paper filters as described in the text.

than the wild-type strain on YEP plus 10% glucose. The heterozygous diploid has invertase activity similar to that of the wild-type parent, demonstrating that the mutant allele is recessive. In each of the seven tetrads from the sporulated diploid there are two repressed and two derepressed spores, indicating that a single mutation is responsible for derepression. Since all of the strains are derepressed on the plate containing 2% sucrose, the mutant is evidently deficient in the glucose repression of invertase synthesis.

The mutant screen was designed to include temperature-sensitive mutants incapable of growing at 37°C but derepressed at this temperature for sufficient time to produce invertase. Indeed, several of the original mutant isolates did not grow at 37°C (RTY66 and RTY68). However, after outcrossing, the constitutive mutations could be separated from secondary mutations causing temperature-sensitive growth, and further experiments were carried out on the derived non-temperature-sensitive strains.

To assign the mutations to complementation groups, all of the mutants derived from AH22 were crossed with the mutants derived from RTY10. An analysis of invertase derepression in the diploids and meiotic segregants produced a consistent assignment of the mutations to four genes. Of the 15 mutations originally obtained, 12 belonged to *cyc8* and 1 to *tup1* (both previously identified genes; see below), and 1 each belonged to *inc2* and *inc3* (for invertase constitutive; their chromosomal location and possible identity with other known genes has not been determined).

The identity of the mutations in the *cyc8* and *tup1* strains was suggested by their secondary phenotypes. Strains containing *tup1-100* are extremely flocculent, and *cyc8* strains have various degrees of flocculence. The *tup1-100* mutation was originally isolated in a *MATa* strain. When *MATα tup1-100* strains were constructed, they exhibited very unusual morphologies, previously described as "self-shmooing" (12), and mated at a low frequency with both *MATa* and *MATα* strains. Mutations in a single gene known as *tup1* (28), *umr7* (12), *flk1* (23), and *cyc9* (22) have been shown to produce a range of pleiotropic effects, including permeability to thymidine monophosphate (28), flocculent growth (23), deficient carbon catabolite repression (23), and morphological aberration and bisexual mating in *MATα* strains (12, 24, 28).

To determine their relationship with previously characterized genes, representative mutants were crossed with *cyc8-1* and *cyc9-1* strains (22). Complementation was assessed by measuring invertase activity and sporulation ability. As predicted by its pleiotropic effects, the putative *tup1-100* allele complemented the *cyc8-1* mutation but not the *cyc9-1* mutation. The *cyc8-13* mutation complemented *cyc9-1* but not *cyc8-1*. Thus, the complementation tests directly confirmed the tentative classifications of the mutants as *tup1* and *cyc8*. As a further confirmation of their identity, the map distances of the *tup1-100* and *cyc8* mutations to *thr4* and *lys2*, respectively, were very close to the published values (data not shown) (16, 22).

The degree of derepression in the mutants was measured by quantitative invertase assays of log-phase cells grown at three different temperatures, 23, 30, and 37°C. The repressed wild-type cells had activities of about 1.0 U/10^9 cells (Table 1). The mutants had maximum activities ranging from 12 to 62 U/10^9 cells, with the exception of RTY87, RTY89, and RTY94. Derepression of invertase activity in RTY89 and RTY94 was evident on plates containing 10% glucose grown at 23°C, but not at 30 or 37°C. In quantitative assays RTY89 and RTY94 showed large variations between experiments. Assays of cells from different stages of growth indicated that RTY89 and RTY94 became derepressed only after the glucose concentration had reached a low level, but before wild-type cells were derepressed (data not shown). In contrast, the other mutants were derepressed at all glucose concentrations. Moreover, RTY89 and RTY94 had maximal activity at 23°C, while most of the other mutants had the highest activity at 37°C. In fact, several of the mutants (RTY100 and RTY102) were almost completely repressed at 23°C, but were strongly derepressed at 37°C.

The kinetics of derepression of strains containing these temperature-dependent mutations was measured after shifting the growth temperature from 23 to 37°C. A wild-type strain (RTY10) at 23°C and the *cyc8-10* strain (RTY99) maintained at 23°C remained repressed, while the mutant strain was rapidly derepressed after the shift to 37°C. Nearly identical results were obtained with another temperature-dependent mutant, RTY102 (*cyc8-11*).

To examine the derepression process more closely, the kinetics of invertase mRNA accumulation was determined by Northern blots (Fig. 2). For purposes of comparison, invertase mRNA in a wild-type strain shifted to low-glucose medium was also determined. Wild-type and mutant cells were grown in YEP plus 2% glucose at 23°C. The wild-type

cells were shifted to low-glucose medium at 37°C, and the *cyc8-10* mutant was shifted to 37°C but maintained in high glucose. Derepression of invertase mRNA in the wild-type cells was evident at 5 min after the shift and reached a maximum at about 15 min. Synthesis was apparently shut off at this time, and little message remained at 40 min. Very similar kinetics were observed for the *cyc8-10* mutant after the temperature shift, with invertase mRNA not detected at 23°C. Higher levels of invertase mRNA were attained in the mutant than in the derepressed wild-type, even though the former was maintained in high-glucose medium. This experiment demonstrates that the *CYC8* gene product directly or indirectly controls invertase derepression at the level of mRNA.

It has been reported previously that a temperature shift from 23 to 37°C produces a transitory derepression of invertase synthesis in wild-type yeasts, detected as either enzyme activity (15) or mRNA (21). However, no direct comparisons were made of the magnitude of the response produced by temperature shock and glucose deprivation. The wild-type strain RTY10 when shifted from 23 to 37°C in high-glucose medium produced a small amount of invertase mRNA which was detectable at 10 min but disappeared at 20 min (Fig. 2). The amount made was much less than in the derepressed mutant or in wild-type cells under derepressed conditions.

The rapid decrease in invertase mRNA levels beginning 15 min after the temperature shift is probably due to the heat shock response, which inhibits the transcription of most genes except those specific for heat shock proteins (13). Subsequent experiments following invertase mRNA up to 2.5 h after the temperature shift have shown that invertase mRNA synthesis resumes after 1 h, but mRNA levels never attain the 15-min maximum. Invertase assays of these cultures demonstrated a direct relationship between the invertase mRNA levels and increases in invertase activity (data not shown).

TABLE 1. Invertase activities of mutants

Strain[a]	Mutation	Invertase activity (U/10^9 cells)[b] at:		
		23°C	30°C	37°C
RTY110	*tup1-100*	8.6	8.2	17
RTY100	*cyc8-10*	2.3	5.7	23
RTY102	*cyc8-11*	2.3	7.8	23
RTY118	*cyc8-12*	14	5.6	32
RTY104	*cyc8-13*	4.0	61	22
RTY80	*cyc8-14*	4.8	3.5	42
RTY81	*cyc8-15*	21	23	43
RTY82	*cyc8-16*	17	12	29
RTY87	*cyc8-17*	2.7	0.87	3.4
RTY90	*cyc8-18*	8.9	4.6	12
RTY91	*cyc8-19*	9.7	4.8	14
RTY92	*cyc8-20*	62	31	40
RTY93	*cyc8-21*	15	25	32
RTY89	*inc2-1*	2.4	1.5	0.43
RTY94	*inc3-1*	2.7	1.1	1.1
RTY10	Wild type	0.94	0.55	0.68

[a] The *tup1-100* and *cyc8-10* through *cyc8-14* mutants were originally isolated from AH22, and the *inc2-1*, *inc3-1*, and *cyc8-15* through *cyc8-21* mutants were isolated from RTY10. RTY99, RTY100, RTY102, RTY104, and RTY118 were segregants from crosses between the original mutant isolates and RTY10. RTY110 was derived by isolating *tup1* segregants from successive crosses to DBY782 (*MATα ade2*) and 699-8C (*MATa leu2 ade2-1 his4-712 ura3-1 can1-100*).

[b] Cultures were grown overnight at 23°C in YEP plus 2% glucose, diluted to an A_{600} of 0.1 (about 1.2×10^6 cells per ml), and then grown in YEP plus 2% glucose at the three temperatures indicated. Cells were harvested in the mid-log phase (A_{600} of 0.6 to 1.0), and invertase assays were performed on intact cells essentially as described by Goldstein and Lampen (9). Cell concentrations were calculated from a standard curve of A_{600} versus a direct count of viable cells. Cells were vortexed in 25 mM EDTA before measuring absorbance to disperse cell aggregates present in the *tup1* and *cyc8* mutant cultures. Enzyme activities are given as International Units (micromoles of substrate hydrolyzed per minute).

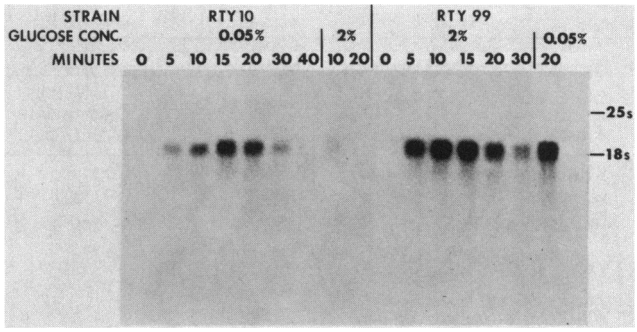

FIG. 2. Invertase mRNA accumulation during derepression in mutant and wild-type cells. The kinetics of derepression in wild-type cells shifted to low-glucose medium and in *cyc8-10* mutant cells shifted to 37°C were compared. Both RTY10 (*MATα trp1*) and RTY99 (*MATα trp1 cyc8-10*) were grown at 23°C in YEP plus 2% glucose to the early log phase. The cells were collected on Millipore nitrocellulose filters and then suspended in YEP plus 2% glucose or YEP plus 0.05% glucose medium, which was prewarmed to 37°C. After time intervals of further growth at 37°C, 10-ml samples were chilled in dry ice-ethanol and centrifuged. RNA was extracted by suspending the cell pellets in 50 μl of LETS buffer (0.1 M Tris hydrochloride [pH 7.5], 0.1 M LiCl, 1 mM EDTA) and vortexing with glass beads. The homogenate was extracted twice with phenol-chloroform-isoamyl alcohol (25:24:1, vol/vol) and twice with chloroform. The nucleic acids were precipitated overnight at −20°C after adding 2 volumes of ethanol, washed with 70% ethanol, dried, and dissolved in water. Total cellular RNA (4 μg) from each time point was run on a 1% agarose–formaldehyde gel containing MOPS [3-(*N*-morpholino)propanesulfonic acid] buffer (5), transferred to nitrocellulose, and hybridized by the method of Thomas (25). A 5-kilobase *Bam*HI-*Sal*I restriction fragment from plasmid pRB58 (2) containing the 3′ half of the invertase gene was nick translated and used as the hybridization probe. The positions of the 25S and 18S yeast rRNAs are shown for size comparison.

Many different enzyme pathways are repressed in yeast cells growing on glucose. The degree of repression of representative enzymes from different pathways was determined in *cyc8* and *tup1* mutants to answer two questions: (i) do *cyc8* and *tup1* affect all glucose-repressible enzymes; and (ii) do they affect the same group of enzymes?

The various enzymes were assayed in homogenates prepared from wild-type and mutant cells grown to the mid-exponential phase and wild-type cells grown to the early stationary phase which should be released from glucose repression (Table 2). All of the glycosidases assayed (invertase, maltase, and α-methylglucosidase) were derepressed in the *cyc8* and *tup1* mutants (the chromogenic substrate *p*-nitrophenyl-α-glucoside is hydrolyzed by both maltase and α-methylglucosidase [19] and provides a more sensitive assay than the substrates maltose or α-methylglucoside). These enzymes were previously reported to be derepressed in a *tup1* mutant (23). The activity of carboxypeptidase Y, a vacuolar enzyme, was about three times higher in the wild-type cells in the stationary phase than in those in the logarithmic phase, but was not derepressed in the mutants. Repression of succinate dehydrogenase was not detected in this experiment, and activities in the *cyc8* and *tup1* mutants were about half of the wild-type

TABLE 2. Various enzyme activities in mutants

Yeast strain	Enzyme assayed[a]							
	Invertase (U/mg)	Maltase (U/mg)	α-Methyl-glucosidase (mU/mg)	PNPGase (μU/mg)	Carboxypep-tidase Y (μU/mg)	Succinate dehydrogenase (mU/mg)	NADH:cytochrome *c* oxidoreductase (mU/mg)	Isocitrate lyase (U/mg)
RTY10 (wild type)								
Log phase[b]	0.29	0	0	0.98	0.14	102	3.3	0
Stationary phase	0.93	0	0	7.4	0.35	118	8.4	3.8
RTY92 (*cyc8-20*; log phase)	3.6	0.45	0.73	82	0.15	50	12	1.4
RTY110 (*tup1-100*; log phase)	1.5	1.5	2.8	233	0.11	44	11	1.5

[a] Maltase and α-methylglucosidase were assayed by modifying the invertase assay of Goldstein and Lampen (9) so that maltose and α-methylglucoside served as substrates, and the reaction was carried out in 50 mM sodium phosphate buffer, pH 7.0. α-Glucosidase activity was assayed with *p*-nitrophenyl-α-glucoside at a final concentration of 0.2 mg/ml in 50 mM sodium phosphate (pH 7.0) as described by Needleman et al. (17). The following enzymes were assayed as described previously: carboxypeptidase Y (11); succinate dehydrogenase (6); NADH: cytochrome *c* oxidoreductase (10); and isocitrate lyase (G. H. Dixon and H. L. Kornberg, Biochem J. 72:3p, 1959). Protein concentrations were determined by the dye-binding method of Bradford (1) with bovine serum albumin as the standard. PNPGase, *p*-Nitrophenyl-α-glucosidase.

[b] Cells were grown in YEP plus 2% glucose medium at 30°C and harvested in the exponential phase (A_{600} = 0.6 to 1.0) or the early stationary phase (A_{600} of 1:5 dilution = 1.0).

level. This contrasts with a previous report (23) of partial derepression in an *flk1* mutant (= *tup1*). The activity of NADH:cytochrome *c* oxidoreductase, part of the mitochondrial complex involved in electron transport, was derepressed roughly threefold in the mutants. No isocitrate lyase activity was detected in the repressed wild-type cells, and activities in the *tup1* and *cyc8* mutants was about half that of stationary-phase cells. In summary, the *tup1* and *cyc8* mutants are derepressed for the glycosidases invertase, maltase, and α-methylglucosidase, for NADH:cytochrome *c* oxidoreductase, and for isocitrate lyase of the glyoxylic acid cycle. They appear to have a similar range of action, affecting many but not all glucose-repressible enzymes.

This screen for mutants with invertase synthesis resistant to glucose repression yielded mutations in four genes: 12 *cyc8*, 1 *tup1*, 1 *inc2*, and 1 *inc3*. The mutations producing the highest levels of derepression belonged to the previously identified genes *tup1* and *cyc8*. The *inc2* and *inc3* mutants were more difficult to analyze and were not systematically tested with known genes. They are similar to the *CAT1-2d* and *cat2-1* mutations (29) in that they become derepressed at intermediate glucose concentrations but are completely repressed at high concentrations.

Although it has been shown previously that both *cyc8* and *tup1* mutants are derepressed for invertase synthesis, mutations in these genes have not been identified in screens for mutants with this phenotype. The only previous report of a nonselective screen described the invertase-hyperproducing mutant FH4C, which shares with *cyc8* and *tup1* other properties including general derepression and clumpiness (14). FH4C proved refractory to genetic analysis, and the genetic basis for derepression was not determined. A screen for constitutive mutants selected for their resistance to 2-deoxyglucose yielded mutations primarily in two genes, *HEX1* and *HEX2* (30). The nature of the *hex2* mutations is unclear, but *HEX1* has been shown to be identical to *HXK2*, the structural gene for hexokinase PII (7). Significantly, no *cyc8* or *tup1* mutants were isolated, implying that mutants resistant to deoxyglucose represent only a subset of mutants constitutive for invertase. *cyc8* mutants have been isolated by selecting for suppressors of *snf1*, which prevents normal derepression of invertase (3).

The mutant screen was designed to include temperature-sensitive mutations in genes essential for cell viability. Although several of the original mutant isolates did not grow at 37°C, all of the mutations were recovered in strains viable at 37°C after outcrossing. These results suggest that this screen is capable of isolating temperature-sensitive mutations which might have been found in a larger collection of mutants. Both *tup1* and *cyc8* mutations have numerous pleiotropic effects, and the consequence of a complete lack of function of either of these genes is unknown. Several of the *cyc8* mutations isolated are expressed at 37°C but not at 23°C, suggesting that the mutant gene product is at least partially inactive at the higher temperature. These temperature-dependent mutations could be useful for analyzing the functional role of *CYC8*.

Catabolite repression is controlled by both positive and negative regulatory genes. The *snf* mutations which block derepression identify the positive control elements. The *CYC8* and *TUP1* genes are negative regulators, since mutations in these genes result in constitutive expression of catabolite-repressible genes. Although their phenotypes are analogous to classical repressor mutants in bacteria, present evidence suggests that they act indirectly by interfering with positive activation (18). Characterization of the *CYC8* and *TUP1* genes and gene products may be necessary to determine their role in regulating transcription.

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