JOURNAL OF BACTERIOLOGY, May 1978, p. 446-457
0021-9193/78/0134-0446$02.00/0

Vol. 134, No. 2
Printed in U.S.A.

Genetic Control of Galactokinase Synthesis in *Saccharomyces cerevisiae*: Evidence for Constitutive Expression of the Positive Regulatory Gene *gal4*

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Received for publication 13 September 1977

Temperature-sensitive (ts) mutants for the *gal80* and *gal4* genes of *Saccharomyces cerevisiae* were isolated and characterized. These mutants were classified into two categories; one showed thermolability (TL) and the other showed temperature-sensitive synthesis (TSS) of the respective products. Both the TL and TSS *gal80* mutants are constitutive for galactokinase activity at 35°C and, because they are derived from a dominant super-repressible *GAL80*^{s} mutant, are uninducible at 25°C. Both the TL and TSS *gal4* mutants are galactose negative at 35°C and galactose positive at 25°C. None of the ts *gal4* mutations affected the thermolability of galactokinase activity in cell extracts. Induction of galactokinase activity was studied with these mutants. The results indicate that the *gal80* gene codes for a repressor and the *gal4* gene codes for a positive factor indispensable for the expression of the structural genes or their products. However, striking evidence that the expression of the *gal4* gene is constitutive and not under the control of *gal80* was provided by a kinetic study with the TL *gal4* mutant. The TL *gal4* mutant pregrown in glycerol nutrient medium at 35°C showed a prolonged lag period (35 min) in the induction of galactokinase activity at 25°C, whereas the same mutant pregrown at 25°C showed the same lag period as those observed in the wild-type strain and a revertant clone derived from the TL *gal4* mutant (15 min).

In *Saccharomyces cerevisiae*, the first three enzymes for galactose utilization, galactokinase (EC 2.7.1.6), α-D-galactose-1-phosphate uridyltransferase (EC 2.7.2.12), and uridine diphosphoglucose 4-epimerase (EC 5.1.3.2), are coded for by the *gal1*, *gal7*, and *gal10* genes, respectively (6). These three loci are tightly linked to each other, most probably in the order *gal7-gal10-gal1*, on chromosome II (3). Either a recessive mutation at *gal80* or a dominant mutation at *GAL81* gives rise to constitutive synthesis of the three enzymes pleiotropically (7). Mutation in the *gal4* gene results in the simultaneous loss of all three galactose enzymes (7). The *GAL81* mutation occurs close to the *gal4* gene and results in constitutive production of the enzymes only when the mutation is in the *cis* position to a functional allele of *gal4* (7).

According to the regulatory model proposed by Douglas and Hawthorne for the galactose pathway enzymes in *S. cerevisiae* (7), the *gal80* gene produces a repressor that represses the expression of the *gal4* gene by interacting at the *GAL81* region in the absence of galactose. In the presence of galactose, the repressor is inactivated and the *gal4* gene expresses itself to produce a cytoplasmic product indispensable for the expression of the *gal1*, *gal7*, and *gal10* genes.

That the relationship between *gal80* and *GAL81* is analogous to the repressor-operator relationship was further suggested by the characterization of the super-repressible mutations *GAL80*^{s} (8, 14). It is, however, possible to envisage another regulatory mechanism for the synthesis of galactose catabolic enzymes in which both the *gal4* and *gal80* genes are expressed constitutively and the interaction of these two genes occurs through their cytoplasmic products.

Since detailed kinetic analyses of temperature-sensitive (ts) *lacI* mutations provided valuable information on the repressor in the *lac* system of *Escherichia coli* (9, 10, 15, 17), we attempted to investigate temperature-sensitive regulatory mutants of the galactose system in *S. cerevisiae*. This communication deals with the isolation and characterization of temperature-sensitive mutants for the *gal80* and *gal4* loci. Kinetic studies on the galactokinase induction in these mutants has produced results which are difficult to reconcile with the Douglas and Haw-

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thorne model. The results strongly suggest that the *gal4* gene is expressed constitutively.

MATERIALS AND METHODS

Yeast strains. Yeast strains described in the present communication are listed in Table 1. The genotypes of the *gal4* and *gal10* mutants were confirmed by complementation tests with the standard strains, H-42 (ATCC 26922: a *gal4-1*) from our collection and 100-5B (α *gal10*) from H. C. Douglas, and those of the *gal7* mutants were confirmed by the criteria that they were sensitive to galactose and complemented with and were closely linked to the *gal10* mutation. The *gal10-1 GAL80^s-3* genotype of strain CD2 was determined in the previous study (14) (unpublished data). The genetic symbols used are those proposed by the Nomenclature Committee for Yeast Genetics (16).

Media. General usage and composition of nutrient, minimal, galactose minimal, YPGal (20 g of galactose, 20 g of polypeptone, and 10 g of yeast extract dissolved in and made up to 1 liter with distilled water), EBGal (20 mg of ethidium bromide added per liter of YPGal), YPGly (20 g of glycerol per liter replaced the galactose in YPGal medium), YPGlyGal (20 g of galactose added per liter of YPGly), YPEth (5 g of ethanol per liter replaced the glycerol in YPGly), and YPEthGal (5 g of ethanol and 2 g of galactose replaced the glycerol per liter of YPGly) media were described previously (14) (the amount of galactose in YPGlyGal was increased to 20 g from the 2 g of the original formula [14]). To test the temperature effect on the cells in nongrowing conditions, minimal salt vitamin medium (Burkholder synthetic minimal medium [5] without addition of the carbon and nitrogen sources) was used.

Techniques. The methods for genetic analysis and for galactokinase assay using permeabilized cells to the substrates were as described previously (14).

RESULTS

Construction and characterization of temperature-sensitive *gal80^s* mutants. Since a super-repressible *gal80^s* mutant is unable to grow on galactose minimal medium (8, 14), isolation of a galactose-positive revertant

TABLE 1. *List of yeast strains*

Strain	Genotype[a]	Source
P-28-24C	a wild type	Our stock culture
F10D	α wild type	Our stock culture
1061	a *gal7-1*	Mutant from P-28-24C
1098	a *gal7-2*	Mutant from P-28-24C
1612	α *gal7-1 gal4-2*	Mutant derived from a mutant isolated from 1061
1063	a *gal10-1*	Mutant from P-28-24C
CD2	a *gal10-1 GAL80^s-3*	Mutant from 1063
N61-1A	α *GAL80^s-1*	Segregant from diploid strain N61 (14)
N61-2A	a *GAL80^s-1*	Segregant from diploid strain N61 (14)
G100-3C	a *GAL80^s-1*	Haploid clone obtained by the repeated back cross of N61-4D (a *GAL80^s-1*; a segregant from N61 [14]) to F10D (α wild type)
P612B	α *gal4-2*	Segregant from the 1612 × P-28-24C cross (the *gal4-2* allele is suppressible by *SUP11*)
P612C	a *gal4-2*	Segregant from the 1612 × P-28-24C cross and a sister clone of P612B
PY612A	α *GAL80^s-1 gal4-2*	Segregant from the Y268-2B (14) × P612C cross
CD2-1A	a *GAL80^s-3*	Segregant from the CD2 × F10D cross
CDR2-44	a TSS *gal80^s-3.1*	Mutant from CD2-1A
CDR2-44F-3A	α TSS *gal80^s-3.1*	Segregant from the CDR2-44 × F10D cross
CDR3-4	a TL *gal80^s-3.2*	Mutant from CD2-1A
CDR3-4F-2A	α TL *gal80^s-3.2*	Segregant from the CDR3-4 × F10D cross
N8-2D	a *GAL81-1 gal7-2*	Segregant from the 1098 × 19A2 (14) cross
NR3-23	a *GAL81-1* TSS *gal4-3 gal7-2*	Mutant from N8-2D
NR23-2C	α *GAL81-1* TSS *gal4-3*	Segregant from the NR3-23 × P612B cross
PY8D	α *GAL81-1 gal10-1*	Segregant from the Y268 (14) × 19A2 (14) cross
PYR1-2	α *GAL81-1* TL *gal4-4 gal10-1*	Mutant from PY8D
PYR1-3D	α *GAL81-1* TL *gal4-4*	Segregant from the PYR1-2 × P-28-24C cross
G346-1B	α *GAL81-1* TL *gal4-4*	Segregant from the PYR1-9D (a sister clone of PYR1-3D) × F10D cross
PYN3-3A	a *GAL80^s-1 GAL81-1* TL *gal4-4*	Segregant from the PYR1-3D × N61-2A cross
R104-2A	α TL *gal4-4*	Galactose-negative clone in an ascus showing a 1+:3− segregation on EBGal at 25°C from the PYN3-3A × PY612A cross was crossed with P-28-24C; a temperature-sensitive galactose-positive clone was selected from the segregants of the above cross
R104-2AR	α Gal$^+$	Galactose-positive spontaneous revertant isolated from R104-2A

[a] Genotypes for markers other than the mating type and *gal* were omitted from the table.

due to a secondary mutation at the *gal80* locus is easy. Using this protocol, cells of strain CD2-1A (*GAL80^s-3*) were plated on galactose minimal plates. Colonies appearing on the plate after incubation at 35°C for 4 to 6 days were tested for growth on the same medium at 25°C. Seventeen colonies that could grow at 35°C but not at 25°C were isolated. Galactokinase activity of these isolates was determined by using the cells cultivated in YPGly (uninduced) and YPGlyGal (induced) at 25 or at 35°C and permeabilized for the enzyme substrates as described (14). All of the mutants showed enzyme activity when the cells were cultivated at 35°C, but not at 25°C, regardless of the cultivation medium. Thus, their phenotype is constitutive at 35°C and super-repressible at 25°C.

Two mutants, CDR2-44 (**a**) and CDR3-4 (**a**), were selected from the 17 isolates and crossed with the wild-type strain, F10D (α). The diploids were sporulated, and four-spored asci were dissected. The tetrad segregants were tested for their ability to grow on EBGal medium at 35 and 25°C. All the asci tested, 5 and 11, respectively, showed a 4+:0− segregation at 35°C, whereas they showed a 2+:2− segregation at 25°C. These two mutants were further crossed with the super-repressible *GAL80^s-1* mutant, N61-1A (α). The resulting diploids were subjected to tetrad analysis, and the segregants were tested on EBGal plates. All the asci tested (9 and 10, respectively) showed a 2+:2− segregation and a 0+:4− segregation when the EBGal plates were incubated at 35 and 25°C, respectively. These results clearly indicated that each of the mutations is a single mutation at or close to the *gal80* locus, probably a secondary mutation in *gal80* in addition to the *GAL80^s-3* mutant allele which is originally carried by strain CD21A.

Two haploid segregants, CDR2-44F-3A and CDR3-4F-2A, which could not grow on EBGal at 25°C, were selected from the tetrad segregants derived from the CDR2-44 × F10D and CDR3-4 × F10D crosses, respectively. In diploids prepared by crossing segregants CDR2-44F-3A (α) or CDR3-4F-2A (α) with the wild-type strain, P-28-24C (**a**), galactokinase activity was inducible at both 25 and 35°C, whereas diploids obtained by crossing the same segregants with the super-repressible *GAL80^s-1* mutant, G100-3C (**a**), showed the uninducible phenotype at both temperatures (Table 2). These observations indicate that the temperature-sensitive mutations of mutants CDR2-44F-3A and CFR3-4F-2A are recessive to the wild-type and *GAL80^s-1* alleles. Hence, the genotypes were designated *gal80^s-3.1* and *gal80^s-3.2* for strains CDR2-44F-3A and CDR3-4F-2A, respectively.

These strains were cultivated in YPGly medium at 25°C. When the cultures showed an optical density at 660 nm (OD_{660}) of 0.3, each culture was divided into two parts. The incubation temperature of one part was shifted to 35°C, and the remaining part was kept at 25°C. The time course of the galactokinase appearance was followed by using suspensions of permeabilized cells as enzyme source. In strain CDR3-4F-2A (ts *gal80^s-3.2*), galactokinase activity appeared immediately after the transfer to 35°C, whereas strain CDR2-44F-3A (ts *gal80^s-3.1*) required considerable growth before the appearance of the enzyme activity (Fig. 1). To test the thermolability of the mutant *gal80* products, the mutant cells were cultivated at 25°C in YPGly, and then harvested, washed, and suspended in minimal salt vitamin medium, and the suspension was exposed to 35°C for 60 min. After the heat treatment, the cells were centrifuged, suspended in YPGly medium, and allowed to grow at 25°C. Some galactokinase activity appeared in strain CDR3-4F-2A (ts *gal80^s-3.2*) during the pulse exposure at 35°C. A further abrupt increase of enzyme activity was observed in this

TABLE 2. *Galactokinase activity in strains having various genotypes at the gal80 locus*

Strain	Genotype	Sp act[a]			
		25°C		35°C	
		YPGly	YPGlyGal	YPGly	YPGlyGal
P-28-24C	Wild type	<0.01	5.61	<0.01	2.90
G100-3C	*GAL80^s-1*	<0.01	0.04	<0.01	<0.01
CDR2-44F-3A	TSS *gal80^s-3.1*	<0.01	<0.01	3.61	4.84
CDR2-44F-3A × P-28-24C	TSS *gal80^s-3.1/GAL80+*	0.01	2.18	0.03	4.14
CDR2-44F-3A × G100-3C	TSS *gal80^s-3.1/GAL80^s-1*	<0.01	0.02	<0.01	<0.01
CDR3-4F-2A	TL *gal80^s-3.2*	0.04	0.08	1.36	6.25
CDR3-4F-2A × P-28-24C	TL *gal80^s-3.2/GAL80+*	<0.01	1.15	<0.01	2.58
CDR3-4F-2A × G100-3C	TL *gal80^s-3.2/GAL80^s-1*	<0.01	0.01	<0.01	<0.01

[a] Cells of each strain were grown in YPGly (uninduced) or YPGlyGal (induced) medium at 25 and 35°C with shaking. Galactokinase activity was determined by using permeabilized cells as the enzyme source. Specific activity is expressed as units per $OD_{660\,nm}$ unit of the culture.

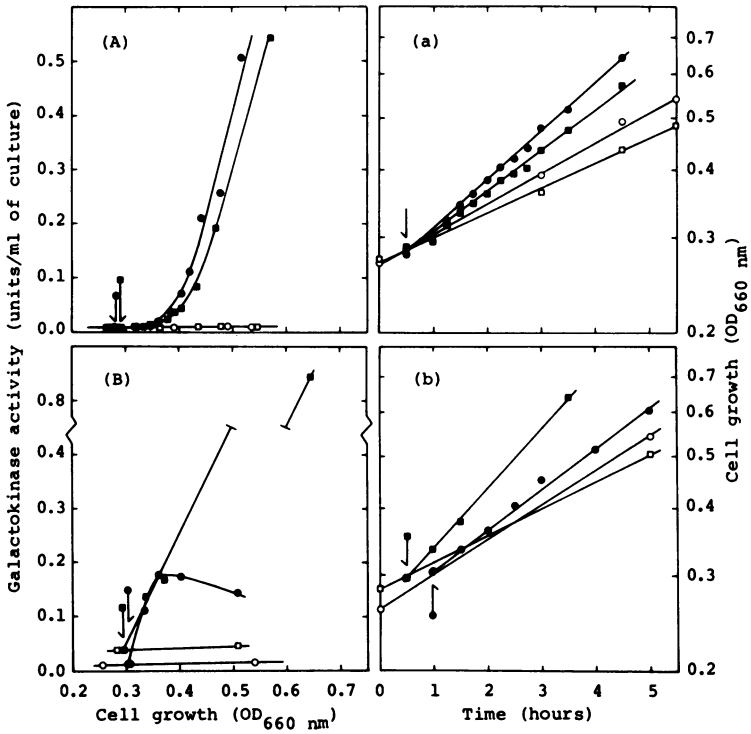

FIG. 1. *Effect of temperature shift on galactokinase synthesis during the cultivation of the temperature-sensitive gal80^s mutants. Cells of the mutants, CDR2-44F-3A (ts gal80^s-3.1) (A and a), and CDR3-4F-2A (ts gal80^s-3.2) (B and b) were shaken at 30°C for 48 h in YPGly. A 0.5-ml portion of each culture was inoculated into 100 ml each of YPGly (○, ●) or YPGlyGal (□, ■) medium and shaken at 25°C. At the time indicated by the arrow, each culture was divided into two parts. One was kept at 25°C (open symbols) while the other was shifted to 35°C (closed symbols), and shaking of the cultures was continued. Galactokinase activity (A and B), as a function of cell growth (a and b; OD_{660} of the culture), was determined by using permeabilized cell suspension as the enzyme source.*

mutant immediately after the transfer to YPGly medium at 25°C, whereas no activity appeared in cells kept at 25°C or in cells of the ts *gal80^s-3.1* mutant (CDR2-44F-3A) similarly treated (Fig. 2). Similar phenomena have been described in ts mutants for β-galactosidase formation in *E. coli* (9, 10, 15, 17). We adopted the terminology used by Sadler and Novick (17): thermolabile (TL) for the ts *gal80^s-3.2* mutation and temperature-sensitive synthesis (TSS) for the ts *gal80^s-3.1* mutation in the *gal80* gene.

However, galactokinase synthesis in strain CDR3-4F-2A (TL *gal80^s-3.2*) ceased within 2 h after the temperature shift to 35°C (Fig. 1). When the same cells were cultivated in YPGlyGal medium, instead of YPGly, at 25°C and the incubation temperature was shifted to 35°C at an appropriate growth phase, they continued to produce the enzyme activity beyond the 2-h period. The same pattern of enzyme synthesis was observed in the other three independent mutants showing the TL phenotype (unpublished data). The reason for this phenomenon is obscure. On the other hand, in the cells of strain CDR2-44F-3A (TSS *gal80^s-3.1*), no essential difference in the lag period was observed in temperature-shift experiments between cells cultivated in YPGly and YPGlyGal medium (Fig. 1).

The results illustrated in Fig. 2 also suggest that the repressor produced by the TSS mutant is not inactivated at 35°C. This possibility was further confirmed by pulse-heating experiments. Cells of strain CDR2-44F-3A (TSS *gal80^s-3.1*) were shaken at 25°C in YPGly medium. When the cell growth reached approximately an OD_{660} of 0.3, the culture was divided into three parts. Cells of the first part were harvested, washed, and suspended in the same amount of minimal salt vitamin medium. The cell suspension was incubated at 35°C and was sampled at 60 and 105 min. Cells were then harvested by centrifugation, suspended in an appropriate amount of YPGly medium to give approximately the same cell concentration as the original culture, and shaken at 35°C for further growth. The second

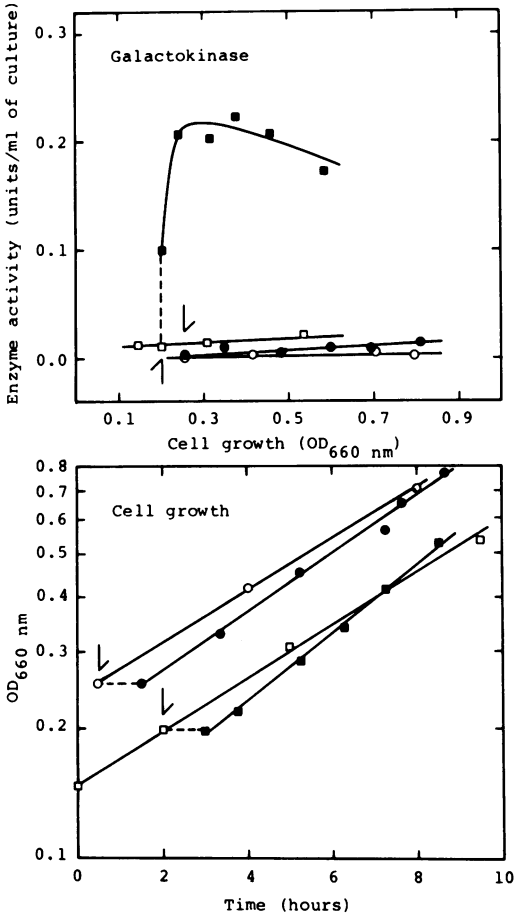

FIG. 2. *Effect of pulse exposure of cells at 35°C in minimal salt vitamin medium on the galactokinase activity in the ts gal80^s mutants. Cells of the mutants, CDR2-44F-3A (ts gal80^s-3.1) and CDR3-4F-2A (ts gal80^s-3.2), were shaken in YPGly medium at 30°C for 48 h. A 0.5-ml portion of each culture was inoculated into 100 ml of fresh YPGly medium and shaken at 25°C. At the time indicated by the arrow, each culture was divided into two parts, and shaking of one was continued at 25°C (open symbols). Cells in the other portion (closed symbols) were harvested by centrifugation at 3,000 × g for 5 min, washed twice, and suspended in minimal salt vitamin medium at or below 25°C. The suspension was shaken at 35°C for 60 min (broken line). The cells were then separated by centrifugation at or below 25°C and suspended in the original volume of fresh YPGly medium prewarmed to 25°C. The suspension was shaken at 25°C. Galactokinase activity was determined as a function of cell growth (OD_{660}) by using cell suspension after the permeabilization of the cells. Data for the pulse-exposed cell suspension were normalized to the original cell density of the culture at the time indicated by the arrow. Symbols: ○ and ●, CDR2-44F-3A (ts gal80^s-3.1); and □ and ■, CDR3-4F-2A (ts gal80^s-3.2).*

part of the culture was shifted directly to 35°C, and the remaining portion of the culture was kept at 25°C. The cells not preheated began to produce galactokinase activity approximately 90 min after the temperature shift (Fig. 3). The cells preheated at 35°C in minimal salt vitamin medium had a 30-min shorter lag period, but no essential differences in the kinetics of the appearance of the enzyme activity were observed between the cells incubated at 35°C for 60 and 105 min. The cells kept at 25°C could not produce galactokinase activity at all. No essential differences in cellular growth were observed between these three different cultures at 35°C. These results indicate that the repressor of strain CDR2-44F-3A (TSS *gal80^s-3.1*) is not thermolabile but is temperature sensitive in its synthesis. The 30-min reduction of the lag period observed in the preheated cells might be due to the dilution of the repressor molecule by its limited extent of turnover during the first 60 min of the treatment, but the exact mechanism is still obscure.

Construction and characterization of temperature-sensitive *gal4* mutants. The respiratory-competent *gal7* and *gal10* mutants are able to grow on YPEth medium but not on YPEthGal medium since they are sensitive to galactose. This galactose sensitivity is relieved either by mutation which eliminates or lowers the galactokinase activity or by a reverse mutation of the *gal7* or *gal10* mutant allele. We attempted isolation of temperature-sensitive *gal4* mutants by this method. We tested more than 100 colonies that appeared on YPEthGal plates incubated at 35°C. However, we found that all of them could complement the standard *gal4-2* mutation by crossing with strains P612B and P612C. We then tried mutant isolation by using strains having the constitutive *GAL81-1* mutant allele (14) in addition to the *gal7* or *gal10* mutation as in the original strain. These strains, N8-2D (**a** *GAL81-1 gal7-2*) and PY8D (α *GAL81-1 gal10-1*), were grown in nutrient medium, washed with sterilized water, and spread on YPEthGal plates to give 10^8 cells per plate. Colonies appearing after 3 to 5 days at 35°C were tested for growth on YPEthGal plates at 25°C. Colonies which failed to grow at 25°C were isolated, since it was expected that a ts *gal4* mutation would relieve the galactose sensitivity due to the *gal7* or *gal10* mutation of the cells at 35°C but not at 25°C. The isolates were purified and crossed with the *gal4-2 GAL7+ GAL10+* strains, P612B (α) or P612C (**a**), to cancel the susceptibility to galactose of the mutants. Each diploid was spotted on two EBGal plates, one of which was incubated at 35°C and

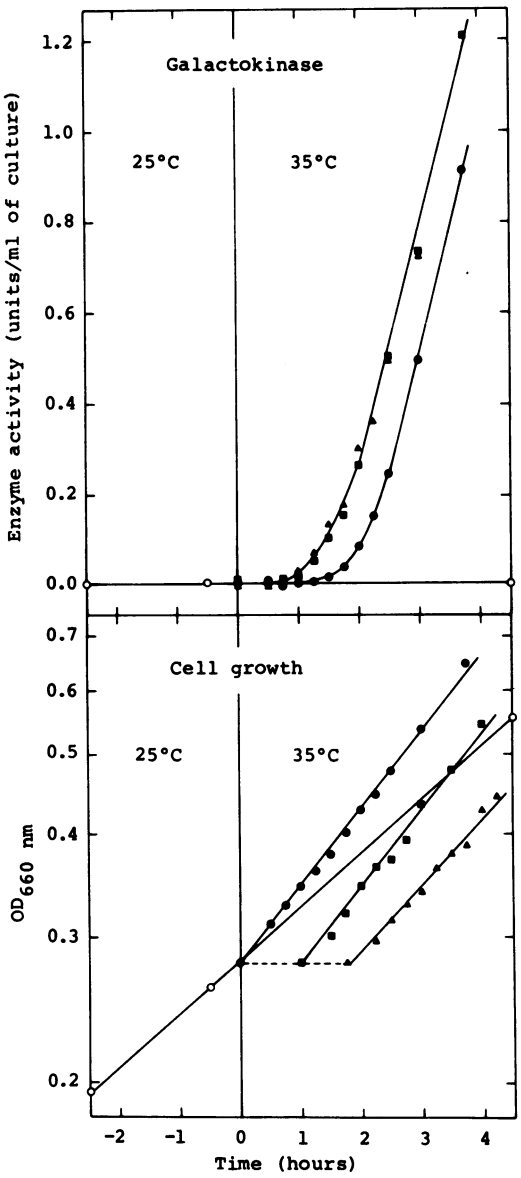

FIG. 3. *Effect of incubation at 35°C in the absence of cell growth on galactokinase synthesis in TSS gal80"-3.1 mutant (CDR2-44F-3A). Cells cultivated in YPGly at 30°C for 48 h were inoculated into fresh YPGly medium and shaken at 25°C as described in the legend for Fig. 1. At the time shown by the vertical line (0 h), the culture was divided into three portions. The cells were harvested from the first part by centrifugation, washed twice with minimal salt vitamin medium at or below 25°C, and suspended in an appropriate volume of the same minimal salt vitamin medium. The washed cell suspension thus obtained was gently shaken at 35°C. The cells harvested after 60 min (■) or 105 min (▲) of incubation at 35°C were then resuspended in YPGly medium prewarmed at 35°C, and the suspensions were again shaken at 35°C. The second part of the culture (●) was directly shifted to 35°C, and the remaining part of the culture (○) was allowed to continue shaking at 25°C. Galactokinase activity was determined after permeabilization of cells. Data for the cells treated at 35°C in the nongrowing conditions were normalized to the original density of the culture when the culture was divided.*

the other of which was incubated at 25°C. Those mutants which could complement the *gal4-2* mutation at 25°C, but not at 35°C, were presumed to have temperature-sensitive *gal4* mutations. Two such mutants, NR3-23 from N8-2D and PYR1-2 from PY8D, were selected, and the respective ts *gal4* mutations were denoted *gal4-3* and *gal4-4*. To confirm this assignment of genotype, strains NR23-2C (α) and PYR1-3D (α), which were selected from the haploid segregants of the NR3-23 (**a**) × P612B (α) and PYR1-2 (α) × P-28-24C (**a**) crosses, respectively, were subjected to further genetic analysis. Since they are insensitive to galactose and temperature sensitive in growth on EBGal medium, they are expected to have the wild-type allele for the *gal7* or *gal10* gene and the *GAL81-1* ts *gal4-3* genotype in NR23-2C and the *GAL81-1* ts *gal4-4* genotype in PYR1-3D. To carry out an allelism test, strains NR23-2C and PYR1-3D were crossed with the **a** *gal4-2* strain, P612C. The diploids were subjected to tetrad analysis, and we observed that all the asci (six asci from the NR23-2C × P612C cross and nine asci from the PYR1-3D × P612C cross) showed a 0+:4− segregation on EBGal medium at 35°C, and a 2+:2− segregation at 25°C.

We examined the effect of pulse exposure to 35°C on the appearance of galactokinase activity in the *GAL81-1* ts *gal4* double mutants. Cells of strains NR23-2C and PYR1-3D were grown at 25°C in YPGly medium. During this primary cultivation, galactokinase activity was produced, because the cells have the *GAL81-1* genotype and the *gal4* mutations are permissive at 25°C. When the cell growth reached an OD_{660} of 0.3, each culture was split into two equal parts. One part was kept at 25°C. The cells of the other part were harvested, washed, and suspended in minimal salt vitamin medium and shaken at 35°C for 60 min to inactivate the *gal4* product if it is TL. Then the treated cells were harvested and suspended in an appropriate amount of YPGly medium for futher growth at 25°C. The galactokinase activity of the cells was slightly reduced (90 to 93% of the original) by this treatment. The *GAL81-1* ts *gal4-3* (NR23-2C) cells began to produce galactokinase activity again

immediately after growth resumed at 25°C, whereas the *GAL81-1* ts *gal4-4* (PYR1-3D) cells exhibited a significant lag period before the activity reappeared (Fig. 4). When these strains were cultivated at 35°C in the same medium (YPGly), no enzyme activity was produced. To test whether the inactivation of the ts *gal4-4* gene product by brief heating is reversible or irreversible, the same cell suspension (PYR1-3D) in minimal salt vitamin medium incubated at 35°C for 60 min as described above was further incubated at 25°C for 120 min. The cells were harvested and suspended in an appropriate amount of YPGly medium to allow cell growth at 25°C. The time course of the appearance of galactokinase activity was investigated. Since almost the same lag period as that in the experiment without the 120-min incubation period at 25°C was observed (Fig. 4), the inactivation of the *gal4-4* product was concluded to be irreversible. These observations strongly suggest that the *gal4* mutation in strain NR23-2C (*gal4-3*) is the TSS type, whereas that in strain PYR1-3D (*gal4-4*) is the TL type.

It was confirmed that the galactokinase activity of cell extracts prepared from the *GAL81-1* TSS *gal4-3* and *GAL81-1* TL *gal4-4* mutants and from a revertant having the *gal4-2 SUP11* genotype and showing the temperature-sensitive galactose-positive phenotype cultivated at permissive temperature has the same thermolability as that from the wild-type cells (unpublished data). Similar observations on the epimerase activity were described by Klar and Halvorson (11).

For kinetic study of galactokinase induction, a TL *gal4* single mutant promised to be extremely interesting. To obtain this mutant type, PYR1-3D (α *GAL81-1* TL *gal4-4*) was crossed with N61-2A (**a** *GAL80^s-1*). The diploid was subjected to tetrad analysis, and the segregants were tested for their ability to grow on EBGal medium at 25 and 35°C. Based on the phenotype of the tetrad clones, we selected a segregant, PYN3-3A, showing the galactose-negative phenotype at 35°C and the galactose-positive phenotype at 25°C from a nonparental ditype tetrad which showed a 2+:2− segregation at 35°C and a 4+:0− segregation at 25°C on EBGal. From the above selection, strain PYN3-3A was expected to have the *GAL80^s-1 GAL81-1* TL *gal4-4* genotype, because the *GAL80^s-1 GAL81-1* genotype gives rise to the galactose-positive phenotype on EBGal (14). Strain PYN3-3A was then crossed with the α *GAL80^s-1 gal4-2* strain, PY612A, and the resultant diploid was subjected to tetrad analysis. Of 181 asci examined, only one ascus showed a 1+:3− segregation, whereas the others showed a 2+:2− segregation at 25°C in the ability to grow on EBGal medium. On the other hand, at 35°C, all the 181 asci showed a 0+:4− segregation on the same medium. The four segregant clones in an ascus showing the 1+:3− segregation at 25°C are expected to have the following genotype: (A) *GAL80^s-1 GAL81-1* TL *gal4-4*, (B) *GAL80^s-1 gal81+* TL *gal4-4*, (C) *GAL80^s-1 GAL81-1 gal4-2*, and (D) *GAL80^s-1 gal81+ gal4-2*. Only the A clone should show the positive phenotype at 25°C for galactose utilization. When those clones showing a negative phenotype on EBGal medium at 25°C were crossed with the wild-type strain, P-28-24C (**a**) or F10D (α), temperature-sensitive clones due to the TL *gal4-4* genotype were expected to have segregated only from the diploid prepared with the B clone. By using the above criteria, we obtained the TL *gal4-4* strain, R104-2A, by the selection of a segregant from the final cross with the B clone.

Kinetics of galactokinase induction in the cells having the TL *gal80^s-3.2* and TL *gal4-4* genotypes. The Douglas and Hawthorne model states that, in the uninduced state, the *gal80* repressor prevents expression of *gal4* which is needed for expression of the *gal7-gal10-gal1* cluster, the structural genes for the galactose pathway enzymes. Inactivation of the *gal80* repressor by the addition of galactose allows synthesis of the *gal4* product which activates the *gal7-gal10-gal1* cluster. This model predicts similar kinetics of enzyme synthesis under the following alternative conditions: (i) induction of the enzymes in wild-type cells by the addition of galactose, (ii) temperature shift of the culture from 25 to 35°C in the TL *gal80^s-3.2* mutant without addition of galactose, and (iii) shift of the incubation temperature from the restrictive to the permissive condition in the TL *gal4-4* mutant with the simultaneous addition of galactose.

The time course of appearance of galactokinase activity in the TL *gal80^s-3.2* mutant after the temperature shift was compared with that in the wild-type cells induced by the addition of galactose. Cells of strain CDR3-4F-2A were shaken at 25°C in YPGly medium. At an OD_{660} of 0.3, the cells were harvested and resuspended in the same amount of fresh YPGly medium prewarmed to 35°C, and the suspension was shaken at 35°C. At appropriate intervals, 1-ml samples were taken into tubes containing 0.5 ml of cycloheximide solution (20 μg/ml), and the cells were harvested immediately by centrifugation. Cycloheximide has no direct effect on the activity of galactokinase, but the treatment is enough to interrupt further synthesis of galactokinase in the cells. Galactokinase activity began to appear 10 min after the temperature

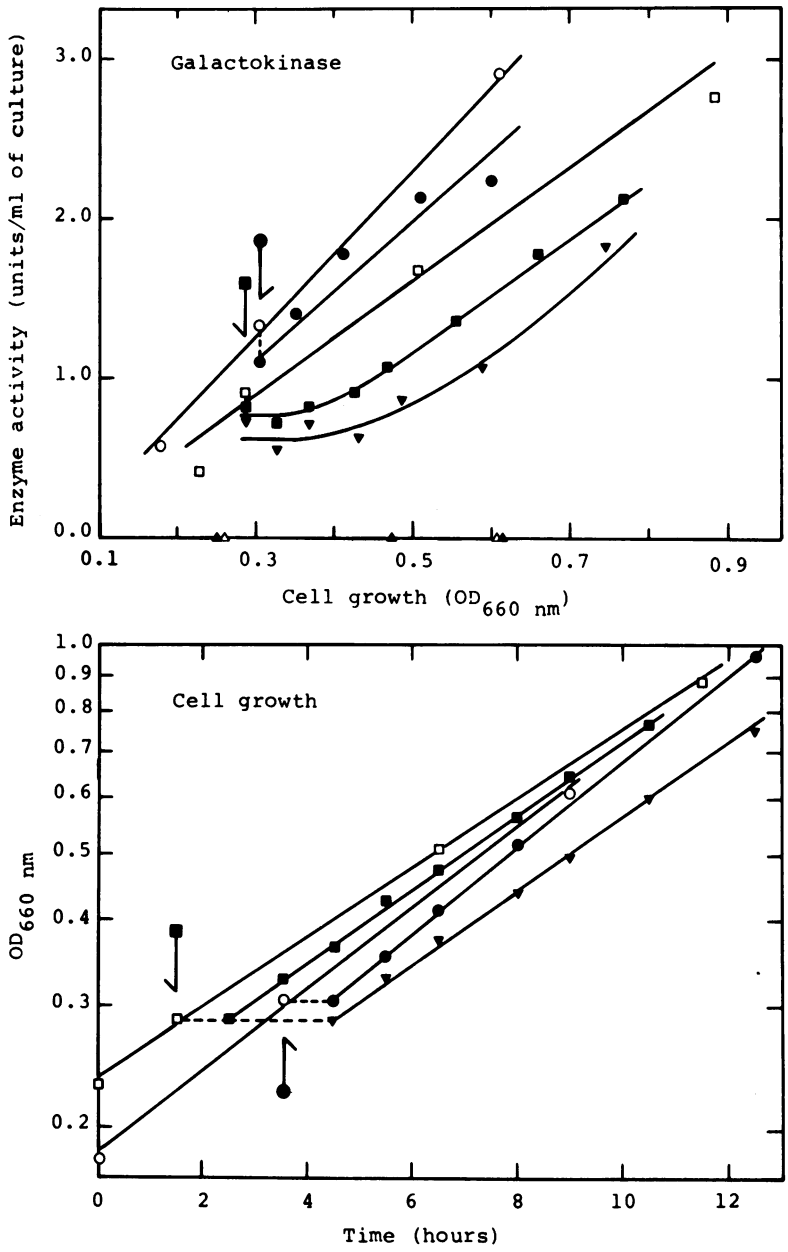

FIG. 4. *Effect of pulse exposure at 35°C in the absence of cell growth on the galactokinase synthesis in the GAL81-1 ts gal4-3 (NR23-2C) and GAL81-1 ts gal4-4 (PYR1-3D) double mutants. Cells grown in YPGly at 30°C for 48 h were inoculated into fresh YPGly medium and shaken at 25°C as described in the legend for Fig. 1. When each culture reached an OD_{660} of about 0.3 as indicated by the arrows, it was divided into two parts. One part* (○ and □) *was kept at 25°C. The cells in the other part* (● and ■) *were harvested, washed, and suspended in minimal salt vitamin medium. The cell suspensions were shaken gently at 35°C for 60 min. After the treatment, the cells were harvested and suspended in YPGly medium, and the whole suspension was shaken at 25°C. A portion* (▼) *of the pulse-exposed cell suspension of strain PYR1-3D (GAL81-1 ts gal4-4) in minimal salt vitamin medium was incubated at 25°C for a further 120 min. The cells were then harvested, suspended in fresh YPGly medium, and shaken at 25°C. Galactokinase activity and cell growth were determined at appropriate intervals. Data were normalized to the original OD_{660} of the culture at the growth phase indicated by the arrows. Symbols:* ○, *NR23-2C at 25°C throughout;* ●, *NR23-2C with pulse exposure at 35°C;* △, *NR23-2C at 35°C throughout;* □, *PYR1-3D at 25°C throughout;* ■, *PYR1-3D with pulse exposure at 35°C;* ▲, *PYR1-3D at 35°C throughout;* ▼, *PYR1-3D with pulse exposure at 35°C for 60 min and subsequently kept at 25°C for 120 min. The growth curves at 35°C throughout were omitted.*

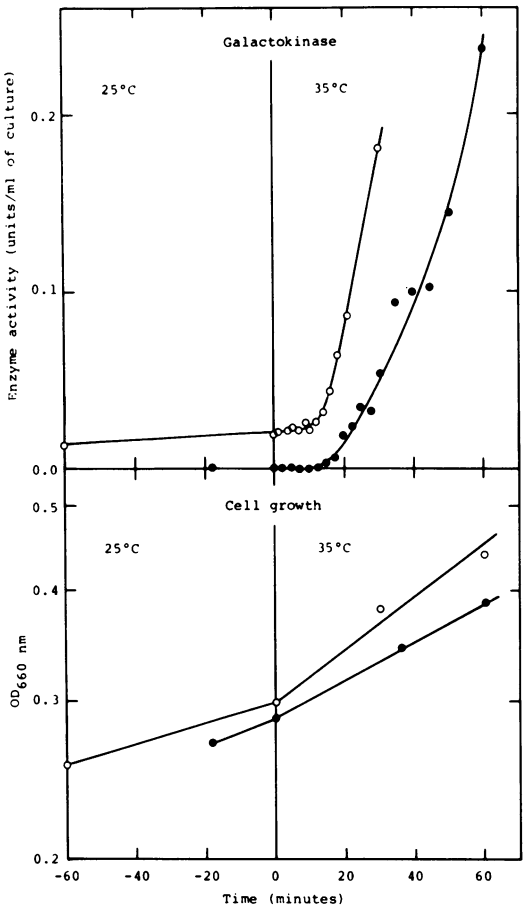

FIG. 5. *Kinetics of galactokinase induction in the TL gal80^{ts} mutant by the shift of incubation temperature. Cells of the TL gal80^{ts}-3.2 mutant (CDR3-4F-2A) and the wild-type strain (P-28-24C) were cultivated in YPGly medium at 30°C for 48 h. A 0.5-ml portion of each culture was inoculated into 100 ml of YPGly medium and shaken at 25°C. When the OD_{660} of the cultures reached approximately 0.3, the cells were harvested by centrifugation at room temperature (20°C) and suspended in the same amount of YPGly medium prewarmed to 35°C for the TL gal80^{ts} mutant or suspended in the same amount of YPGlyGal for the wild-type strain. The suspensions were shaken at 35°C. At appropriate intervals, samples were taken, and further enzyme synthesis was interrupted by cycloheximide treatment. The galactokinase activity was determined with permeabilized cells as the enzyme source. Symbols: ○, strain CDR3-4F-2A (TL gal80^{ts}-3.2); ●, strain P-28-24C (wild type).*

shift (Fig. 5). The lag time observed here was only slightly shorter than that of the wild-type cells, P-28-24C, pregrown at 25°C and then transferred to YPGlyGal medium at 35°C.

Cells of the TL *gal4-4* mutant (R104-2A) and the wild-type strain (P-28-24C) were cultivated in 100 ml of YPGly medium at 25 or 35°C. When the cultures reached approximately an OD_{660} of 0.3, galactose was added to the cultures to give a final concentration of 2%. Immediately after the addition of galactose, the cultures shaken at 35°C were transferred to a water bath at 25°C, and shaking was continued. The temperature of the medium was shifted to 25°C within 2 min by this procedure. The cultures pregrown at 25°C were kept at the same temperature after the addition of galactose. Samples (1 ml) were taken from the cultures at an appropriate interval and were added to 0.5 ml of cycloheximide solution (20 μg/ml) to interrupt further synthesis of galactokinase. The cultures of both strains, R104-2A and P-28-24C, showed linear growth for at least 4 h from the addition of galactose at almost the same growth rate under the conditions described above (Fig. 6). The galactokinase activity appeared with a lag of approximately 15 min in the wild-type cells irrespective of the precultivation temperature (Fig. 6). The lag period of the wild-type cells (Fig. 5) was approximately 10 min, whereas that in the present experiment with cells of the same wild-type strain was 15 min. This difference is probably due to the difference in the incubation temperature, i.e., 35 and 25°C, at the time of enzyme induction. On the other hand, cells of the TL *gal4-4* mutant pregrown at 35°C began to produce the enzyme activity approximately 35 min after the addition of galactose, whereas the same mutant cells pregrown at 25°C showed almost the same lag period as the wild-type cells (Fig. 6). A revertant clone, R104-2AR, showing the galactose-positive phenotype at 35°C as well as at 25°C on EBGal plate, was isolated from strain R104-2A spontaneously. Data of the same experiment with this revertant could be plotted on the corresponding curves of the wild-type strain (Fig. 6). The revertant cells showed a slightly higher growth rate than strain R104-2A, but the difference was negligibly small for kinetic study. Hence, the lag of approximately 35 min observed in strain R104-2A is due to the TL *gal4-4* mutation.

The Douglas and Hawthorne model (7), further implies that the *GAL81* mutation is an operator mutation which causes constitutive synthesis of the *gal4* product. The observations in the cultivation of the *GAL81-1* TL *gal4-4* double mutants in YPGly medium at 25°C (Fig. 4 and 7) do not conflict with this view as the mutants showed constitutive synthesis of galactokinase activity. When the double mutants were cultivated at 35°C, no galactokinase activity was produced in the same medium due to the TL *gal4-4* mutation. The incubation temperature was then shifted to 25°C, and the same culture produced the enzyme activity after a lag of 35 min or more irrespective of whether galac-

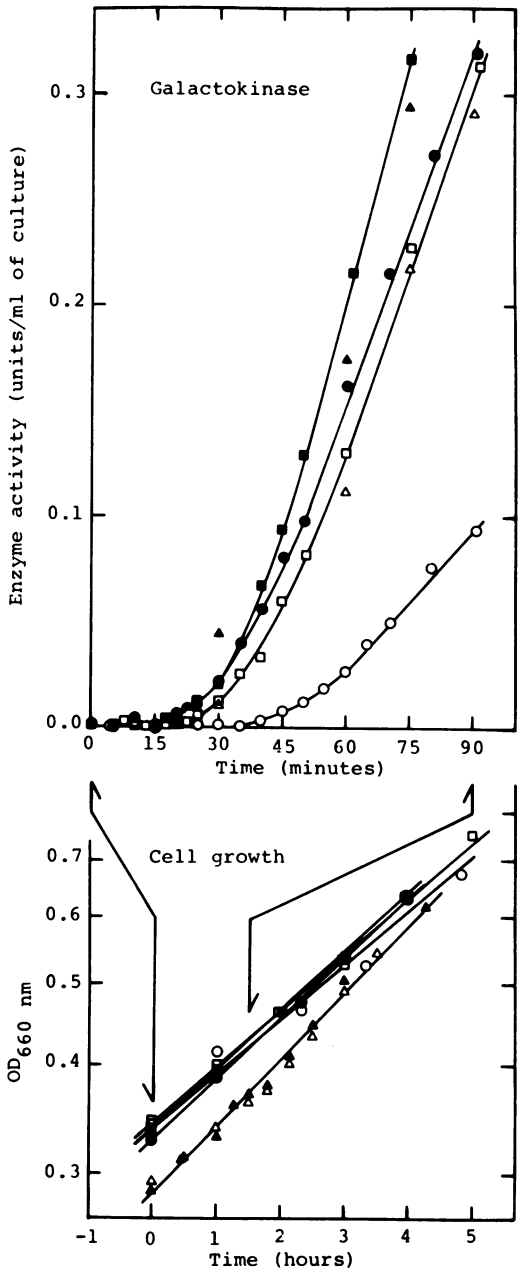

FIG. 6. *Effect of pregrowth temperature on the induction period for galactokinase synthesis in the TL gal4 mutant. Cells of the TL gal4-4 mutant (R104-2A), the Gal⁺ revertant (R104-2AR), obtained from R104-2A, and the wild-type strain (P-28-24C) were cultivated in YPGly medium at 30°C for 48 h. A 0.5-ml portion of each culture was inoculated into 100 ml of YPGly medium. The cultures were shaken at 25°C (closed symbols) or at 35°C (open symbols). When the cell density of the cultures reached approximately an OD_{660} of 0.3, 5 ml of a concentrated galactose solution (42%) in water was added to the cultures to give a final galactose concentration of 2%. Simultaneously with the addition of galactose, the cultures shaken at 35°C were transferred to a water bath at 25°C, and shaking was continued. At appropriate intervals, samples were taken, and further enzyme synthesis was interrupted by cycloheximide treatment. The galactokinase activity was determined with permeabilized cells as the enzyme source. Symbols: strain R104-2A (TL gal4-4) cultivated at 25°C (●) and at 35°C (○); strain R104-2AR (the Gal⁺ revertant from R104-2A) cultivated at 25°C (▲) and at 35°C (△); and strain P-28-24C (wild type) cultivated at 25°C (■) and at 35°C (□).*

tose was added simultaneously with the temperature shift (Fig. 7). The lag period observed in this experiment is essentially the same length as that of the *gal4-4* single mutant pregrown at 35°C and is significantly longer than that observed in the wild-type strain or in the *gal4-4* single mutant pregrown at 25°C (15 min; Fig. 6). The facts observed in experiments illustrated in Fig. 6 and 7 are irreconcilable with the behavior predicted by the Douglas and Hawthorne model, suggesting that revision of the model is necessary.

DISCUSSION

In the *lac* system of *E. coli*, two types of temperature-sensitive *lacI* mutants have been identified. One is thermolabile, and β-galactosidase is induced by pulse-heating of the mutant cells in buffer at 45°C for 30 min (10). The other type shows temperature-sensitive synthesis of the repressor, and the enzyme activity can not be induced by pulse heating, but only after growth at a higher temperature (43°C) (15, 17). We have obtained corresponding TL and TSS mutations in the *gal4* locus and also analogous mutations showing the super-repressible versus constitutive phenotypes in the *gal80* locus of *S. cerevisiae*. The observation that the super-repressible mutations, *gal80ˢ-3.1* and *gal80ˢ-3.2*, were recessive to the wild-type allele (Table 2) suggests a subunit structure for the *gal80* product. If the wild-type and the *gal80ˢ* repressor molecules were independent in their function in the cytoplasm, the *gal80ˢ* product would be dominant over the wild-type product in the presence of galactose. When the cells of the TL *gal80ˢ-3.2* mutant were pulse-exposed to 35°C in the minimal salt vitamin medium, galactokinase activity appeared during the heating without an increase of cell density. This fact agrees with the model in which the *gal80* product is a repressor (7). The internal pools of carbon and nitrogen sources in the cells might be enough to support the residual synthesis of galactokinase, if the repressor was destroyed by heating. In

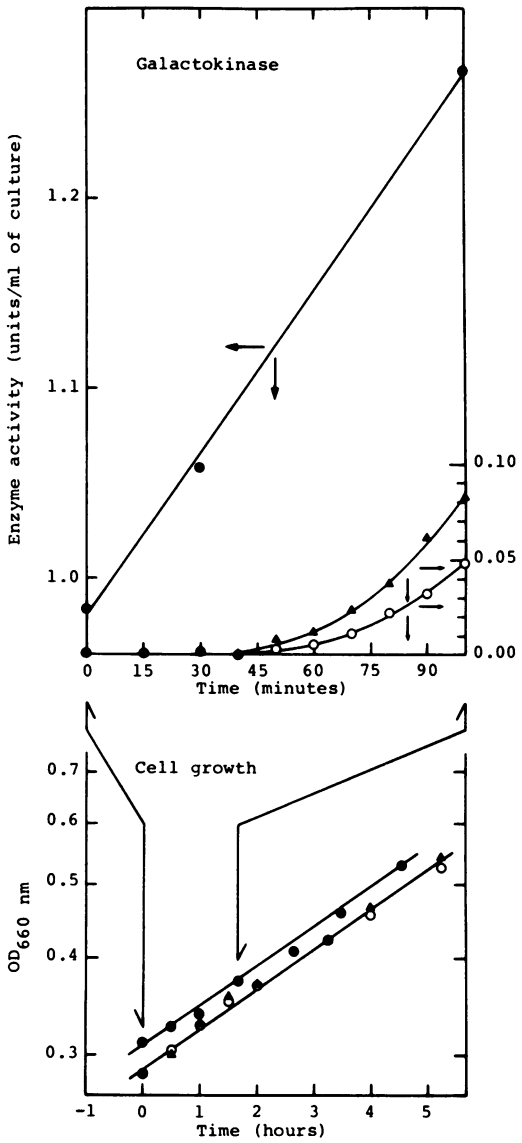

FIG. 7. *Effect of pregrowth temperature on the appearance of galactokinase activity in the GAL81-1 TL gal4-4 double mutant. Strain G346-1B was grown in YPGly medium at 30°C for 48 h. A 0.5-ml portion of the culture was inoculated into 100 ml of YPGly medium, which was shaken at 35°C* (○ and ▲) *or at 25°C* (●). *When the cultures incubated at 35°C reached approximately an* OD_{660} *of 0.3, they were transferred to a water bath at 25°C, and shaking was continued. Simultaneously with the temperature shift, to one culture* (▲) *was added 5 ml of a concentrated galactose solution (42%) in water to give a final galactose concentration of 2% and to the other* (○) *was added 5 ml of sterilized water. The remaining culture* (●) *was shaken at 25°C throughout, without addition of galactose solution or water. Galactokinase activity and cell growth were determined after the temperature shift or after the culture reached approximately an* OD_{660} *of 0.3 for the culture incubated at 25°C throughout. Decrements of the* OD_{660} *values in the cultures pregrown at 35°C were caused by the addition of the galactose solution* (▲) *or water* (○).

spite of this residual synthesis, we may conclude that the *gal4-4* product is thermolabile because incubation at 35°C under nongrowing conditions results in loss of the ability to produce galactokinase activity and that the synthesis of the *gal4-3* product is temperature sensitive because pulse exposure at 35°C has no immediate effect on the enzyme synthesis after shift-down of incubation temperature (Fig. 4).

The induction kinetics of enzyme synthesis in yeast have been studied in several systems. Adams (1) reported that galactokinase was induced 6 to 8 min after the addition of galactose at 30°C in wild-type cells grown on lactose-Wickerham medium. The same author (2) showed that α-glucosidase activity was induced in glucose-grown cells of a wild-type strain within 20 min at 30°C when the washed cells were suspended in nutrient medium containing maltose. In the urea degradation system, Bossinger and Cooper (4) showed a 12-min lag period for the induction of allophanate hydrolase activity at 22°C. In the present study, the induction period for galactokinase activity in the TL *gal80*^{s}*-3.2* strain on shifting the incubation temperature up to 35°C was approximately 10 min, almost the same as in wild-type cells to which galactose was added at 35°C. However, in the TL *gal4-4* mutant, the lag in induction by galactose addition at the permissive temperature to cells pregrown at the nonpermissive temperature was appreciably longer than that in the same mutant pregrown at the permissive temperature and in the wild-type cells (Fig. 6). This experiment was repeated successfully with strain R104-2A and with other strains of the same genotype. This fact strongly suggests that the de novo synthesis of the *gal4* product is required in the TL *gal4-4* cells cultivated at 35°C for the expression of the structural genes, whereas the wild-type cells and those cultivated at 25°C might have sufficient *gal4* product for the enzyme induction. This argument implies that the *gal4* gene is expressed before the addition of galactose to the medium.

Several alternative possibilities have been proposed for the function of the *gal4* gene product. It may be an activator or a common subunit of the galactose pathway enzymes (7), or it may be required for the aggregation of the three galactose-pathway enzymes in the cytoplasm (18). However, Klar and Halvorson (11) have suggested that the product is not a common

subunit of the three enzymes, based on the observation that the epimerase activity in the cell extracts of a ts *gal4* mutant did not differ in thermolability from that of the wild-type strain. The same authors (12) suggested that the *gal4* product might be concerned in a positive control mechanism for the expression of the structural genes, as they observed no clear dosage effect of the *gal4* gene on the level of the epimerase activity. The observations described in the present communication support their idea that the *gal4* gene is concerned in a positive control for the expression of the structural genes, but the details of the mechanism are still obscure. These data, along with the observation described in a previous publication (14) which indicates specific relationships between the *gal80* and *GAL81* genes, support the direct interaction of the *gal4* product with the *gal80* product, the reputed repressor molecule, in the cytoplasm. Similar direct interaction between regulatory proteins has been proposed in the case of the *phoB* and *phoR* products for the regulation of alkaline phosphatase synthesis in *E. coli* (13). It is possible to infer that the activity of the *gal4* product is affected by the *gal80* product through a cytoplasmic interaction. The *GAL81* site might not be an operator for the *gal4* gene but may code for an affinity site of the *gal4* product for the *gal80* product. The observation that the *GAL81-1* TL *gal4-4* double mutant pregrown at 35°C showed the prolonged lag period irrespective of the addition of galactose (Fig. 7) is consistent with this idea. The fact that the TL *gal4-4* mutation could be separated from the *GAL81* mutation suggests that polypeptide regions coded by *gal4-4* and *GAL81-1* exert relatively independent functions. To examine this possibility, fine mapping of the *GAL81* and *gal4* mutants is now under way.

ACKNOWLEDGMENTS

We thank R. B. Wickner for his critical reading of this manuscript.

This study was supported by grant no. 048164 to Y. Oshima from the Scientific Research Fund of the Ministry of Education, Japan.

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