Analysis of the *GAL3* Signal Transduction Pathway Activating *GAL4* Protein-Dependent Transcription in *Saccharomyces cerevisiae*

P. Jayadeva Bhat, Dahlkyun Oh[1] and James E. Hopper

Department of Biological Chemistry, The Milton S. Hershey Medical Center, The Graduate Program in Genetics and The Cell and Molecular Biology Program, The Pennsylvania State University, Hershey, Pennsylvania 17033

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ABSTRACT

The *Saccharomyces cerevisiae GAL/MEL* regulon genes are normally induced within minutes of galactose addition, but *gal3* mutants exhibit a 3–5-day induction lag. We have discovered that this long-term adaptation (LTA) phenotype conferred by *gal3* is complemented by multiple copies of the *GAL1* gene. Based on this result and the striking similarity between the *GAL3* and *GAL1* protein sequences we attempted to detect galactokinase activity that might be associated with the *GAL3* protein. By both *in vivo* and *in vitro* tests the *GAL3* gene product does not appear to catalyze a galactokinase-like reaction. In complementary experiments, *Escherichia coli* galactokinase expressed in yeast was shown to complement the *gal1* but not the *gal3* mutation. Thus, the complementation activity provided by *GAL1* is not likely due to galactokinase activity, but rather due to a distinct *GAL3*-like activity. Overall, the results indicate that *GAL1* encodes a bifunctional protein. In related experiments we tested for function of the LTA induction pathway in *gal3* cells deficient for other gene functions. It has been known for some time that *gal3gal1*, *gal3gal7*, *gal3gal10*, and *gal3* rho– are incapable of induction. We constructed isogenic haploid strains bearing the *gal3* mutation in combination with either *gal15* or *pgi1* mutations: the *gal15* and *pgi1* blocks are not specific for the galactose pathway in contrast to the *gal1*, *gal7* and *gal10* blocks. The *gal3gal5* and *gal3pgi1* double mutants were not inducible, whereas both the *gal5* and *pgi1* single mutants were inducible. We conclude that, in addition to the *GAL3*-like activity of *GAL1*, functions beyond the galactose-specific *GAL1*, *GAL7* and *GAL10* enzymes are required for the LTA induction pathway.

IN yeast, the *GAL4* and *GAL80* proteins interact, providing a switch that determines the transcriptional state of the galactose/melibiose (*GAL/MEL*) regulon genes (JOHNSTON 1987). The *GAL4* protein is a DNA binding transcriptional activator that is absolutely required for the activation of the *GAL/MEL* regulon genes (HOPPER, BROACH and ROWE 1978; ST. JOHN and DAVIS 1979; POST-BEITTENMILLER, HAMILTON and HOPPER 1984; GINIGER, VARNUM and PTASHNE 1985). The *GAL80* protein blocks the transcriptional activation function of *GAL4* in the absence of galactose (TORCHIA *et al.* 1984) by direct physical interaction with *GAL4* protein (LUE *et al.* 1987). Addition of galactose to such uninduced cells elicits a *GAL3*-mediated signal which, in turn, relieves the *GAL80* block and results in subsequent rapid induction of *GAL/MEL* gene transcription (TORCHIA and HOPPER 1986).

Considerable effort has recently been directed toward elucidating how *GAL4* activates transcription. Less attention has been directed toward elucidating the *GAL3*-mediated signal, which triggers an alteration of the *GAL80/GAL4* interplay and the activation of *GAL4*. This may be due to the disparate and complex phenotypes exhibited by cells bearing a *gal3* mutation. In otherwise wild type and respiratory-sufficient cells, the *gal3* mutation results in a unique biological phenotype known as long-term adaptation (LTA) (WINGE and ROBERTS 1948). These mutants display an induction lag of 2–5 days in response to the addition of galactose to the medium. The induction lag in wild-type cells is several minutes (WINGE and ROBERTS 1948; SPIEGELMAN, SUSSMAN and PINSKA 1950; KEW and DOUGLAS 1976; TORCHIA and HOPPER 1986). If the *gal3* cells are also defective in any one of the Leloir pathway enzymes [galactokinase (*GAL1*), UDPgalactose epimerase (*GAL10*), or galactose-1-P uridyl transferase (*GAL7*)[(BROACH 1979) or defective in respiratory competence (rho$^-$) (DOUGLAS and PELROY 1963), they cannot be induced at all. It has also been established that *gal3gal1* and *gal3gal10* double mutant cells can neither maintain the induced state nor initiate induction (TORCHIA and HOPPER 1986; NOGI 1986). Overall these observations suggest that in the absence of *GAL3* function some activity of the Leloir pathway together with a mitochondrial activity is required for induction.

The noninducible phenotype of *gal3gal1*,

[1] Present address: Laboratory of Animal Behavior, Rockefeller University, New York, New York 10021.

gal3gal10, *gal3gal7*, and *gal*3-rho$^-$ cells is suppressed by mutations at the *GAL80* and *GAL4* loci that cause constitutive expression of the *GAL/MEL* regulon genes (DOUGLAS and HAWTHORNE 1966; TORCHIA and HOPPER 1986). Moreover, an increase in the dosage of *GAL4* relative to *GAL80*, that leads to constitutive expression in wild-type cells produces the constitutive phenotype in *gal3gal1gal7* cells as well (TORCHIA and HOPPER 1986). The fact that conditions producing constitutivity in *GAL3* cells also bypass the induction block in these doubly compromised *gal3* cells suggests that *GAL3* sends a signal to either the *GAL80* or *GAL4* protein, changing their interaction and activating the *GAL4* protein (TORCHIA and HOPPER 1986; NOGI 1986).

Two hypotheses have been proposed concerning the nature of the *GAL3*-mediated signal. On the basis of an analysis of the deinduction kinetics displayed by *gal3* cells, TSUYUMU and ADAMS (1973, 1974) proposed that the *GAL3* protein is required for the production of an endogenous coinducer. UDPglucose was cited as a logical candidate for such a coinducer. According to the model of TSUYUMU and ADAMS (1974), the eventual induction of *gal3* strains (*i.e.*, the LTA phenotype) is due either to the residual activity of the mutant *GAL3* enzyme or to the activity of a distinct, biosynthetic pathway UDPglucose pyrophosphorylase isozyme. New observations have made this notion unlikely. First, *GAL3* deletion mutants also exhibit the LTA phenotype (TORCHIA and HOPPER 1986); this argues that residual activity of the *gal3* mutant protein is not responsible for the eventual induction. Second, our assays of isogenic *GAL3*-deletion and *GAL3* overproducing yeast reveal no difference in the level of UDPglucose pyrophosphorylase (P. J. BHAT and J. E. HOPPER, unpublished data).

On the basis of the noninducibility of *gal3gal1*, *gal3gal10*, and *gal3gal7* double mutants, BROACH (1979) postulated that the *GAL3* protein converts galactose to an inducer molecule which is a normal intermediate of galactose metabolism. The BROACH model provides quite a different explanation for the LTA phenotype: in the absence of *GAL3*, low basal levels of the galactose enzymes in uninduced cells convert galactose to the inducer. In this view, the autocatalytic nature of induction in *gal3* cells eventually leads to a threshold level of inducer required for induction. There have been no reported observations inconsistent with this view.

Recently the complete DNA sequence of the *GAL3* gene was reported (BAJWA, TORCHIA and HOPPER 1988). The derived *GAL3* protein sequence exhibits striking similarity to the yeast *GAL1* specified galactokinase and significant similarity to the *Escherichia coli galK* galactokinase (BAJWA, TORCHIA and HOPPER 1988). On the basis of these data we initiated new studies aimed at elucidating the nature of the early signal transduction events that trigger *GAL/MEL* gene transcription activation. Here we provide evidence that the yeast *GAL1* gene but not the *E. coli galK* gene functionally complements a *GAL3* defect *in vivo*. In contrast, the *GAL3* gene does not complement a *GAL1* defect *in vivo* nor does it appear to specify detectable galactokinase activity. We also provide evidence suggesting that the alternate induction pathway (LTA) operating in *GAL3*-deficient cells extends at least up to, and most probably through, the phosphoglucose isomerase reaction catalyzed by the product of the *PGI1* gene. We discuss the implications of our results for models of the LTA induction pathway.

MATERIALS AND METHODS

Strains, growth conditions and genetic methods: The yeast strains used in this study are listed in Table 1. YEP media contained 1% (w/v) bactopeptone, 0.5% (w/v) yeast extract and 25 mg of adenine per liter. Synthetic complete medium was prepared as described by HOPPER, BROACH and ROWE 1978). Carbon sources were sterilized separately and added to YEP or synthetic complete media to final concentrations of 2% (w/v) glucose (Glu), 2% (w/v) galactose (Gal), or 3% (v/v) glycerol plus 2% (w/v) potassium lactate (pH 5.7) (G/L). Yeast strains carrying plasmids were maintained in synthetic complete medium lacking either uracil (Ura$^-$) or leucine (Leu$^-$) depending on the selectable marker present in the plasmid. The bacterial strain used to propagate plasmids was *E. coli* strain HB101 (BOYER and ROULLAND-DUSSOIX 1969). Yeast and *E. coli* transformations were done as described elsewhere (ITO *et al.* 1983; DAGERT and EHRLICH 1979, respectively). Haploid yeast cells of required mating type were obtained by utilizing the HO gene induced mating type switching (RUSSEL *et al.* 1986), as previously described (OH and HOPPER 1990). Yeast strains of required genetic background were obtained by disrupting the appropriate gene loci as described by ROTHSTEIN (1983). Strains disrupted for two genes were obtained either by sequential gene disruptions or by crossing singly disrupted strains and screening the isolated tetrads to identify a double disruption segregant. Standard genetic techniques were performed as described elsewhere (MORTIMER and HAWTHORNE 1966).

Preparation and analysis of plasmid DNA, genomic DNA and RNA: Plasmid DNA from *E. coli* was isolated using the alkaline lysis method (BIRNBOIM and DOLY 1979). Genomic DNA for Southern analysis was isolated according to the method of DAVIS *et al.* (1980). Total RNA for northern analysis was isolated according to the method of MCNEIL and SMITH (1986). ^{32}P-labeled DNA probe was prepared using the random primer labeling kit obtained from Pharmacia Fine Chemicals. Conditions for blot transfers of DNA or RNA to GeneScreen Plus membranes and blot hybridizations were performed according to manufacturer's recommendation (New England Nuclear).

Plasmids and constructions: YEp24 was provided by D. BOTSTEIN (BOTSTEIN *et al.* 1979). Plasmid pMP 555 consists of the vector YEp24 containing the *MEL1* gene (POST-BEITTENMILLER, HAMILTON and HOPPER 1984). Plasmid pJK-1 consists of vector YEp24 carrying at its *Bam*HI site a 6.4-kb *Sau*3A yeast genomic fragment encompassing the entire *GAL7* gene (J. HIRSCHMAN and J. HOPPER, unpublished results). Plasmid pJK-7 consists of YEp24 containing at its *Bam*HI site a yeast 6.5-kb *Sau*3A genomic fragment

TABLE 1

List of strains

Name	Genotype	Source
21R	a *leu2-3, leu2-112 ura3-52 ade1 ile MEL1*	S. A. JOHNSON
21R-2D	a *leu2-3, leu2-112 ura3-52 ade1 ile MEL1 gal2D::LEU2*	This study
21R-3D	a *leu2-3, leu2-112 ura3-52 ade1 ile MEL1 GAL3::LEU2*	T. TORCHIA
21R-4D	a *leu2-3, leu2-112 ura3-52 ade1 ile MEL1 gal4D::LEU2*	L. M. MYLIN
21Rpgi-1D	a *leu2-3, leu2-112 ura3-52 ade1 ile MEL1 pgi1D::URA3*	This study
21R-80D	a *leu2-3, leu2-112 ura3-52 ade1 ile MEL1 gal80D*	T. TORCHIA
21R-5UD	a *leu2-3, leu2-112 ura3-52 ade1 ile MEL1 gal5D::URA3*	D. OH
21R-3D.2D	a *leu2-3, leu2-112 ura3-52 ade1 ile MEL1 GAL3::LEU2 gal2D::LEU2*	This study
21R-3D.5UD	a *leu2-3, leu2-112 ura3-52 ade1 ile MEL1 GAL3::LEU2 gal5D::URA3*	This study
21R-3D.pgi-1D	a *leu2-3, leu2-112 ura3-52 ade1 ile MEL1 GAL3::LEU2 pgi1D::URA3*	This study
YM147	a *gal$^{\Delta}$-152 ura3-52 trp1-289*	MARK JOHNSTON

encompassing the entire *GAL7* gene in YEp24 (J. KIRSCHMAN and J. HOPPER, unpublished results). Plasmid pClu1 consists of vector YEp13 carrying at its *Bam*HI site a 11.5-kb *Sau*3A yeast genomic fragment encompassing the entire *GAL1-GAL10-GAL7* cluster (J. KIRSCHMAN and J. HOPPER, unpublished results). Plasmid pJKdvr1-10 consists of a 0.9-kb yeast genomic fragment encompassing the *GAL1-10* intergenic region (ST. JOHN and DAVIS 1981) cloned into the *Eco*RI site of YEp24 (J. KIRSCHMAN and J. HOPPER, unpublished results). The pATH1 vector used for producing the trpE-GAL3 fusion protein has been described (DIECKMANN and TZAGOLOFF 1985). Plasmid YCpR2 containing *CYC1::galK* fusion was provided by RICHARD ZITOMER and has been described (RYMOND *et al.* 1983). Plasmid pTUG4 containing the *GAL2* gene has been described (TSCHOPP *et al.* 1986). Plasmid pGD1040, which carries the yeast *PGI1* gene gapped by *Sal*I and *Bgl*II and disrupted with the 2.6-kb *Sal*I/*Bgl*II *LEU2* gene from YEp13, was obtained from Zymogenetics Inc., 2121 N. 35th street, Seattle, WA 98103. The *Sal*I-*Bgl*II deletion removed the *PGI1* coding region and 933 bp of 5′ flanking and approximately 1000 bp of 3′ flanking region (see sequence in TEKAMP-OLSON, NAJARIAN and BURKE 1988).

The plasmid used for the disruption of *GAL2* was constructed as follows. The 3.0-kb *Bgl*II *LEU2* fragment isolated from vector YEp13 was ligated into pTUG4 at the unique *Bam*HI site located at 873 bp 3′ from the ATG of *GAL2* gene to yield plasmid pBDK2. In order to destroy the 5′ coding region and a part of the noncoding region, plasmid pBDK2 was cut by *Xho*I (within the *LEU2* gene) and partially with *Acc*I. The ends of the large fragment were rendered blunt and subsequently ligated to recircularize. The DNA was transformed into *E. coli* and plasmids from a number of transformants were screened by restriction analysis to identify a plasmid bearing an *Acc*I/*Xho*I junction (the *Acc*I *site* located at 123 bp 5′ to the ATG). One such plasmid referred to as pBDK2-B lacked 123 bp of the 5′ noncoding region of *GAL2* gene and 291 amino acids of the N-terminal part of the *GAL2* protein (refer to the *GAL2* sequence in SZKUTNICKA *et al.* 1989). This plasmid was subjected to complete digestion with *Hin*dIII and partial digestion with *Eco*RI to produce a 4.2-kb fragment that was used to transform yeast for disruption of the genomic *GAL2* locus.

The plasmid used for the disruption of the chromosomal phosphoglucose isomerase locus was constructed from plasmid pGD1040. pGD1040 was cut with *Sal*I and *Bgl*II to remove the *LEU2* fragment. The plasmid ends were rendered blunt with Klenow and ligated to a blunt ended 1.1-kb *Hin*dIII *URA3* fragment isolated from YEp24. The resulting plasmid, pGIΔURA, was digested with *Bam*HI and *Eco*RI, and the DNA was used to transform yeast strains 21R and 21R-3D to Ura$^+$. The transformants were selected on Ura$^-$ plates containing 2% fructose (w/v) and 0.05% glucose (w/v). The Ura$^+$ transformants obtained in the above step were then screened for their inability to grow on glucose as sole carbon source by replica plating to Ura$^-$ glucose plates. Transformants that could not grow on glucose as the sole carbon source were tested for glucose toxicity by replica plating to YEP plates containing 2% fructose and glucose at concentrations varying from 0.01 to 0.1% (w/v). All transformants incapable of utilizing glucose as the sole carbon source exhibited glucose toxicity above 0.05% (w/v) glucose. The plasmid used for the expression of trpE-GAL3 fusion protein was constructed as follows. The 0.64-kb *Bgl*II-*Xho*I fragment from PT1-3B (TORCHIA and HOPPER 1986) encompassing a part of the coding region of *GAL3* was isolated and ligated to a *Bam*HI-*Xho*I cut pATH1 vector. The confirmed DNA construct predicts that the resulting trpE-GAL3 fusion protein contains the amino terminal 320 residues of component one of anthranilite synthetase followed by 13 amino acids from junction sequence within the multiple cloning site of the vector and 196 amino acids from the *GAL3* coding sequence.

Enzyme assays: Yeast cell extracts for the estimation of enzyme activities were prepared by the glass bead cell disruption method (BOSTIAN *et al.* 1980). Determination of galactokinase (BLUME and BEUTLER 1975), α-galactosidase (Post-Beittenmiller, Hamilton and HOPPER 1984) and phosphoglucose isomerase enzyme activity (LEE 1982) have been described elsewhere. Protein content was estimated using the Bio-Rad protein dye reagent. Due to the frequent occurrence of constitutive mutants for *GAL/MEL* regulon expression within the 21Rpgi-1D and 21R-3D.pgi-1D populations, it was necessary to adopt a plate assay method for α-galactosidase. One hundred single colonies of 21Rpgi-1D and 21R-3D.pgi-1D were isolated on YEPG/L plates after growing them in YEPG/L broth. The single colonies thus obtained were patched on to YEPG/L plates, then replica plated to YEPG/L plates (to identify constitutive mutants) and to YEPGal/G/L plates (to identify inducible colonies). A chromogenic overlay assay for α-galactosidase activity in whole cells on plates was carried out every 12 hr over a period of 7 days (POST-BEITTENMILLER, HAMILTON and HOPPER 1984).

Production of antiserum to trpE-GAL3 fusion protein and Western blot analysis: The pATH1 trpE-GAL3 fusion plasmid was used to express the trpE-GAL3 fusion protein in *E. coli* according to general methods described elsewhere (SPINDLER, ROSSER and BERK 1984). The cells were harvested and lysed using 3 mg/ml solution of lysozyme in 50

mM Tris-HCl (pH 7.4) and 5 mM EDTA. The insoluble protein was recovered (KLEID *et al.* 1981) and solubilized in sodium dodecyl sulfate (SDS) sample buffer (LAEMMLI 1970) by heating at 100° for 5 min. The solubilized fusion protein was purified using preparative SDS-polyacrylamide gel electrophoresis (PAGE) (5% stacking and 7.5% separating gel). The apparent molecular mass of the trpE-GAL3 fusion protein is 60 kD. This is in good agreement with the molecular mass predicted from the DNA fusion construct. Approximately 500 μg of the fusion protein was injected into rabbits with adjuvant obtained from RIBI Immunochem Research Inc, P.O. Box 1409, Hamilton MT 59840. Affer 4 weeks rabbits were injected again, and after 1 week following the booster, blood was withdrawn and serum was collected.

Western blot analysis was carried out as follows. Twenty five milliters of 2×10^7 cells/ml were harvested and washed once with ice cold water. The pellets were resuspended in 700 μl of 0.01 M Tris-HCl buffer (pH 8.0) containing 1 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin and 1 μM pepstatin. Cell extracts obtained by the glass bead disruption method were centrifuged for 3–5 sec in an Eppendorf to remove unbroken cells. The supernatant was made 1× in SDS sample buffer (LAEMMLI 1970) and heated at 100° for 5 min. A 15-μl aliquot of this extract was subjected to electrophoresis on 10% acrylamide gels. Proteins were then transferred to nitrocellulose and processed further (MYLIN, BHAT and HOPPER 1989). An equal amount of protein was loaded in each of the lanes. Staining with Coomassie brilliant blue R was carried out to confirm that each lane received an equivalent amount of protein.

RESULTS

The *GAL3* protein does not appear to have galactokinase activity: The *GAL3* protein of *Saccharomyces cerevisiae* contains three distinct regions of striking similarity to regions within the galactokinase of *Saccharomyces carlsbergensis* (BAJWA, TORCHIA and HOPPER 1988). The N-terminal region from amino acid 46–180 of *GAL3* in *S. cerevisiae* and from amino acid 52–188 of the *GAL1* protein in *S. carlsbergensis* exhibits 80% similarity. The amino acids 242–250 of *GAL3* and 249–257 of *GAL1* are identical. The third region, which is 97% similar, extends from amino acid 361 to 405 of *GAL3* and from amino acid 365 to 409 of *GAL1*. In view of this *GAL1/GAL3* similarity we tested for *GAL3* associated galactokinase-like activity.

Extracts were prepared from yeast that carry multiple copies of the *GAL3* gene but which lack the *GAL1* gene. Strain YM147 (*gal1*$^{\Delta}$) was transformed with pT1–3B (TORCHIA and HOPPER 1986) which carries wild-type *GAL3* gene under its own promoter and with pJK-1(*GAL1*) or with YEp24 as controls. Galactokinase activity could be detected in extracts of strain YM147 transformed with pJK-1 but not in extracts of cells carrying either YEp24 or pT1–3B (Table 2). In the event that a *GAL3* specified galactokinase-like activity might be very low or involve a cosubstrate other than ATP, we tested UTP and GTP as well as ATP as co-substrates in the galactokinase assay using both crude cell extracts and a 9000-fold purified *GAL3* protein fraction (P. J. BHAT and E. MATTES, unpublished data). No *GAL3*- associated activity was detected in these experiments.

TABLE 2

Determination of galactokinase activity in YM147 harboring YEp24, pT1-3B and pJK-1

Strain	Plasmid	Carbon source: Glycerol/lactate	Carbon source: Galactose/glycerol/lactate
YM147	pJK-1	<0.01	477
	pJK-1	<0.01	446
YM147	pT1-3B	<0.01	<0.01
	pT1-3B	<0.01	<0.01
YM147	YEp24	<0.01	<0.01
	YEp24	<0.01	<0.01

Transformants of YM147 with the indicated plasmids were grown in Ura$^-$ G/L medium. When the cell density reached 2×10^7 cells/ml, one half of the culture received galactose to a final concentration of 2% (w/v) and the other half received the same volume of water after which the cells were allowed to grow for another hour. Cells were then harvested and processed for the determination of galactokinase activity as mentioned in MATERIALS AND METHODS. Galactokinase activity is expressed as nmoles of galactose phosphorylated per mg protein per minute. Two independent transformants from each group were used for this experiment.

In addition to the above *in vitro* tests we also carried out an *in vivo* test. We tested whether multiple copies of *GAL3* could complement a *gal1* mutant for growth on galactose. The rationale was predicated on the possibility that our *in vitro* assay conditions were not appropriate for detecting a *GAL3*-associated galactokinase activity. We reasoned that an *in vivo* complementation test might work if indeed *GAL3* does have galactokinase-like activity. To test *GAL3* for *in vivo* complementation of *gal1* it was necessary to use *GAL3* on a multicopy plasmid since a single dose of *GAL3* in a *GAL1* deficient cell is not sufficient to allow growth on galactose. Therefore, pT1–3B (TORCHIA and HOPPER 1986) was transformed into the yeast strain YMI47 (*gal1*$^{\Delta}$), and the Ura$^+$ transformants were scored for galactose utilization by plating on YEPGal plates. The pT1–3B transformants clearly did not utilize galactose as the sole carbon source on plates or in broth while the same strain transformed with pJK-1 did utilize galactose as the sole carbon source.

In order to confirm that the *GAL3* protein was overproduced in cells bearing pT1–3B we carried out western immunoblot analysis using antiserum raised against *GAL3* protein (see MATERIALS AND METHODS). The criteria used to establish that the antiserum produced against the trpE-GAL3 fusion protein reacts specifically with the *GAL3* protein in yeast extracts were as follows: (1) The antiserum reacts with a polypeptide having a molecular mass of 49 kD which is in good agreement with the molecular mass predicted from the deduced amino acid sequence of *GAL3* (shown by the arrow in Figure 1A). (2) The polypep-

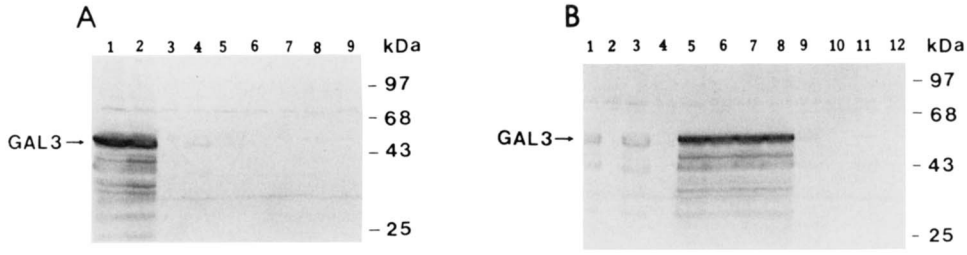

FIGURE 1.—Western blot analysis of *GAL3* protein. A, Western blot analysis of the cell extracts made from 21R bearing YEp24 and 21R-3D bearing YEp24 or PT13-B. Cell extracts made from the above strains grown in Ura⁻ G/L, Gal/G/L and Glu/G/L were subjected to electrophoresis and transferred to nitrocellulose which was then probed with antiserum raised against the trpE-GAL3 fusion protein at a dilution of 1:300 as mentioned in MATERIALS AND METHODS. All the lanes received 25 μg of total protein. Lanes 1, 2 and 3 are loaded with extracts made from 21R-3D strain bearing pT1–3B grown in Gal/G/L, G/L and Glu/G/L, respectively. Lanes 4, 5 and 6 are loaded with extracts made from 21R bearing YEP24 grown in Gal/G/L, G/L and Glu/G/L, respectively. Lanes 7, 8 and 9 are extracts from 21R-3D bearing YEp24 grown in Gal/G/L, G/L and Glu/G/L, respectively. B, Western blot analysis of *GAL3* protein in extracts obtained from YM147 harboring different plasmids. Two independent transformants of YM147 harboring pJK-1, pT1–3B and YEp24 were grown in Ura⁻ G/L medium. When the cell density reached 2×10^7 cells/ml, galactose to a final concentration of 2% (w/v) was added to one half of the culture and the other half received the same volume of water. The cells were then allowed to grow for another hour. Cells were then harvested and extracts prepared from them were subjected to Western blot analysis as mentioned in MATERIALS AND METHODS. Extract obtained from YM147 harboring pJK-1 grown in Gal/G/L (lanes 1 and 3), G/L (lanes 2 and 4). Extract obtained from YM147 harboring pT1–3B grown in Gal/G/L (lanes 5 and 7), G/L (lanes 6 and 8). Extracts obtained from YM147 harboring YEp24 grown in Gal/G/L (lanes 9 and 11), G/L (lanes 10 and 12).

tide recognized by the antiserum is not detected in extracts obtained from an isogenic strain deleted for *GAL3* (compare lane 4 and lane 7, Figure 1A). (3) The carbon responsiveness follows the profile for the *GAL3* transcript (BAJWA, TORCHIA and HOPPER 1988); the detected polypeptide is present at higher levels in galactose grown cells (lane 4, Figure 1A) than in G/L-grown cells (lane 5, Figure 1A) and is not detectable in glucose-grown cells (lane 3, Figure 1A). (4) Preimmune serum does not recognize the polypeptide (data not shown). The Western blot analysis carried out using the above antiserum indicated that the *GAL3* protein was markedly overproduced in transformants bearing pTI-3B (Figure 1B). Thus, the results of both our *in vitro* and *in vivo* experiments indicate that the *GAL3* protein, despite its homology to both yeast and *E. coli* galactokinases does not appear to have galactokinase activity.

***GAL1* complements *gal3* for rapid induction:** Since *GAL3* did not appear to specify a galactokinase-like activity, the structural motifs in common between the galactokinase and the *GAL3* proteins might define the induction function of *GAL3*. We addressed this possibility by testing whether overproduction of the *GAL1* gene product reduces the induction lag caused by the *GAL3* defect. *GAL1* protein overproduction was considered necessary since single copy *GAL1* does not complement the *GAL3* defect. To overproduce the *GAL1* protein we used *GAL1* carried on multicopy vectors YEp24 and YEp13 which attain levels of 7 to 10 copies per cell (ZAKAIN and SCOTT 1982). For the first experiment, strain 21R-3D was transformed with the plasmid pClu1 (YEp13 carrying *GAL1-GAL10-GAL7*) or YEp13 alone, and the induction lag was assessed by monitoring α-galactosidase production on plates. The results of this experiment indicated that there was a two- to threefold reduction in the LTA lag in pClu1-bearing cells but not YEp13-bearing cells (data not shown). To establish whether this induction effect resided exclusively in *GAL1* or elsewhere within the *GAL1-GAL10-GAL7* cluster, we transformed 21R-3D with pJK-1(*GAL1*), pJK-7(*GAL7*), YEp24 bearing the *GAL1-GAL10* intergenic region, or YEp24 alone. The transformants were grown in Ura⁻ G/L medium (noninducing nonrepressing carbon source) to midlog phase and galactose was added, and *GAL/MEL* induction was monitored by following α-galactosidase activity. As illustrated in Figure 2, the results indicate that when in excess the *GAL1* protein reduced the lag conferred by the *GAL3* deletion from 50 hr to about 4.5 hr. In contrast, cells bearing either the *GAL7* plasmid or the *GAL1/10* intergenic region plasmid or the vector alone show the characteristic *gal3* LTA phenotype. Southern blot analysis showed that the plasmid copy number was similar in all of these transformants (data not shown).

In order to distinguish between the galactokinase and a *GAL3*-like activity of the *GAL1* protein, we transformed 21R-3D with single copy (CEN) vector bearing a *CYC1::galK* cassette to express *E. coli* galactokinase (RYMOND *et al.* 1983). 21R-3D was also transformed with the vector lacking *galK* coding region. If galactokinase activity of *GAL1* is responsible for the reduction in the lag, then the *E. coli* galactokinase should produce the same effect. The *E. coli* galactokinase activity was determined in the above transformants. Cells bearing the complete cassette expressed galactokinase at a level of 20% of the fully induced wild type yeast while 21R-3D bearing the vector alone had no detectable galactokinase activity. Determina-

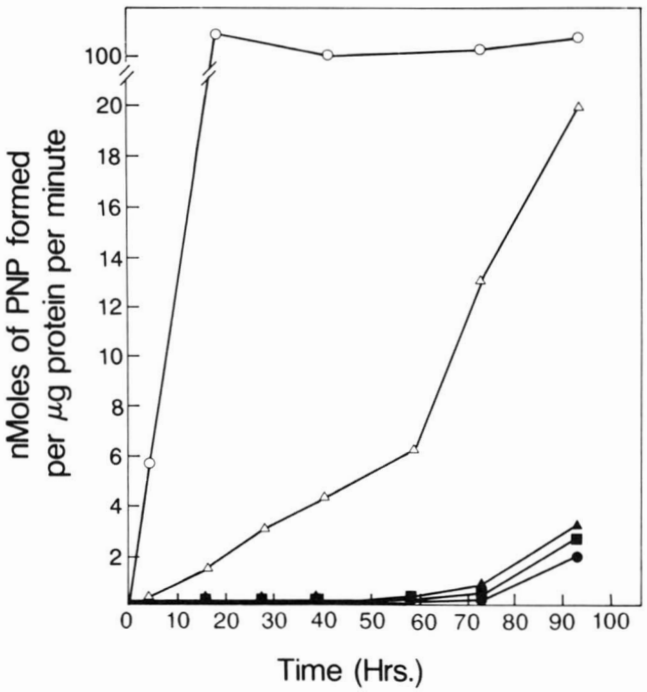

FIGURE 2.—α-Galactosidase activity in 21R bearing YEp24 and 21R-3D bearing YEp24 or pJK-1 or pJK-7 or YEp24 bearing GAL (1–10) divergent region. The above transformants were inoculated into Ura⁻ G/L medium. When the cell density reached 2×10^7 cells/ml galactose to a final concentration of 2% (w/v) was added and samples were collected at indicated time points. Zero time point samples were collected just before the addition of galactose. During the experiment cell cultures were maintained at midlog phase by dilution into fresh prewarmed media. α-Galactosidase activity was determined in all the samples as mentioned in MATERIALS AND METHODS. 21R transformed with YEp24 (○), 21R-3D transformed with pJK-1 (△), 21R-3D transformed with pJK-7 (■), 21R-3D transformed with (1–10) divergent region (▲) and 21R-3D transformed with YEp24(●).

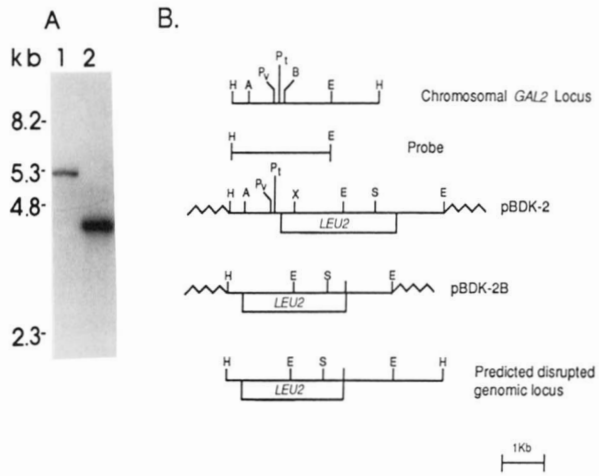

FIGURE 3.—Southern analysis of genomic DNA from wild-type and *GAL2* disrupted strains. Yeast genomic DNA was digested with *Hin*dIII, subjected to electrophoresis in 0.8% agarose gel, transferred to GeneScreen membrane (NEN) and hybridized with ^{32}P-labeled (labeling by the random primer procedure) 2.45-kb *Hin*dIII-*Eco*RI fragment of *GAL2* gene isolated from pTUG4. A, Lane 1 represents *GAL2* disrupted pattern while lane 2 represents wild-type pattern. *Bgl*II-*Hin*dIII digested ^{32}P-labeled DNA was used as the molecular weight standard. B, Relevant restriction map of the chromosomal *GAL2* locus, pBDK-2 and pBDK-2B. The restriction enzyme sites are: A, *Acc*I; B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; Pt, *Pst*I; Pv, *Pvu*II; S, *Sal*I; X, *Xho*I. Wavy lines represent vector sequences and straight lines represent chromosomal sequences.

tion of α-galactosidase in these transformants over a period of 20 hr after the addition of galactose indicated that *E. coli* galactokinase did not reduce the lag (data not shown).

***GAL5* and *PGI1*, but not *GAL2*, are required for LTA induction:** At least the *GAL1*, *GAL7* and *GAL10* functions are required for the LTA induction pathway as shown by BROACH (1979). Our results suggest that *GAL1* might specify the rate-limiting step in the LTA induction pathway. According to BROACH's hypothesis, the LTA induction pathway is activated by a normal intermediate in galactose metabolism, and this intermediate is the inducer produced so effectively by *GAL3*. To identify this postulated intermediate, we wanted to establish more precisely where in normal galactose metabolism it arises. We first set out to determine whether functions immediately preceding and/or following the *GAL1*, *GAL7*, and *GAL10* specified steps are required for the LTA induction pathway.

The *GAL2* gene specifies a galactose permease which catalyzes the first step unique to galactose metabolism (DOUGLAS and CONDIE 1954; CIRILLO 1968). The *GAL1* specified galactokinase reaction immediately follows. Mutants defective at the *GAL2* locus have been shown to be galactose negative when exogenous galactose levels are low (DOUGLAS and CONDIE 1954). Based on their analyses of deinduction kinetics, TSUYUMU and ADAMS (1973) implicated *GAL2* in the inducibility of the *GAL/MEL* regulon. In light of our results, we viewed it prudent to test directly for *GAL2* involvement in induction. Since the molecular nature of the *gal2* alleles used in any of the previous studies is unknown, we thought it essential to test for the effect of a null mutation at the *GAL2* locus either alone or in combination with a null mutation at the *GAL3* locus. We therefore constructed a chromosomal deletion of the *GAL2* gene in strains 21R and 21R-3D as described in MATERIALS AND METHODS. An *in vitro* constructed deletion mutation (see MATERIALS AND METHODS) was used to disrupt the genomic *GAL2* locus (ROTHSTEIN 1983), and the disruption was confirmed by Southern analysis (Figure 3). *Hin*dIII-digested DNA from the wild-type strain shows a 3.8-kb band while DNA from the disrupted strain shows a 5.3-kb band when probed with a *GAL2* fragment. This difference is consistent with the *in vitro* constructed null mutation.

Northern analysis was performed to provide further evidence that the *GAL2* gene was indeed disrupted. The transcript identified as *GAL2* was constitutively expressed in a strain deleted for *gal80*, was induced

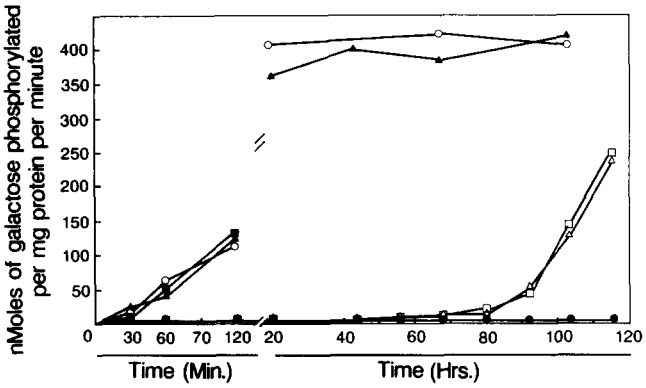

FIGURE 4.—Kinetics of induction of galactokinase in 21R and its mutant derivatives. Cells were inoculated into YEP G/L medium and when the cell density reached 2×10^7/cells/ml galactose was added to a final concentration of 2% (w/v). Cell aliquots were collected at different time points thereafter as indicated. Just before the addition of galactose zero time point samples were collected. During the experiment, cell cultures were maintained at midlog phase by dilution into fresh prewarmed media. 21R (○), 21R-2D (▲), 21R-3D (□), 21R-5UD (■), and 21R -3D.5UD (●) and 21R-3D.2D (△).

by the addition of galactose to wild-type strain and was not detectable in a mutant strain deleted for either *GAL2* or *GAL4*. Nor was the transcript detectable in the wild-type strain grown in either glycerol-lactic acid or glucose (data not shown). The disruption strain exhibited the classical *gal2* mutant phenotype in that it did not grow on YEP plates containing less than 0.5% (w/v) galactose, whereas the isogenic nondisruption strain did grow. Thus, on the basis of a variety of tests, the disruption strain constructed is functionally disrupted for *GAL2* as predicted by the known *GAL2* DNA sequence (SZKUTNICKA *et al.* 1989).

Figure 4 illustrates the induction kinetics of galactokinase upon addition of galactose to strains growing in a noninducing nonrepressing carbon source (YEPG/L). Strains 21R and 21R-2D exhibited rapid induction upon galactose addition. Strains 21R-3D and 21R-3D.2D exhibited similar induction kinetics indicating that the disruption of the *GAL2* gene in 21R-3D strain does not alter the abnormally slow pattern of induction caused by lack of *GAL3* function. We conclude that disruption of *GAL2* either alone or in combination with the disruption of *GAL3* does not affect the inducibility of the *GAL/MEL* regulon.

We next determined the effect of blocking galactose metabolism at the phosphoglucomutase step, the step immediately following the reaction catalyzed by the *GAL7* specified galactose-1-P uridyl transferase. Phosphoglucomutase (*GAL5* or *PGM2*), which interconverts glucose-1-phosphate and glucose-6-phosphate, was recently cloned in our laboratory and was disrupted in 21R to create 21R-5UD (OH and HOPPER 1990). Although the disruption of *GAL5* leads to very poor growth on galactose, the induction of the system was not affected (OH and HOPPER 1990). By disruption of the *GAL3* locus in strain 21R-5UD (see MATERIALS AND METHODS) we obtained strain 21R-3D.5UD. The induction kinetics of galactokinase in the isogenic series were determined after galactose addition over a period of 120 hr (Figure 4). Although 21R-5UD induced as rapidly as the wild-type isogenic strain, 21R, strain 21R-3D.5UD did not induce at all. As established previously (OH and HOPPER 1990) 21R-5UD is toxified upon addition of galactose, and hence induction was determined over the course of 2 hr. In this experiment, strain 21R-3D showed the classical LTA phenotype. Induction of *GAL1* (galactokinase) in 21R occurred normally.

Finding that the *GAL5GAL3* double deficiency produces the noninducible phenotype prompted us to test the effect of blocking the phosphoglucose isomerase reaction (the step immediately following PGM in the conversion of galactose to pyruvate). Phosphoglucose isomerase (also referred to as glucose phosphate isomerase EC 5.3.1.9) catalyzes the interconversion of glucose-6-phosphate and fructose-6-phosphate, and is specified by a single locus, the *PGI1* locus, in *S. cerevisiae* (MAITRA and LOBO 1977; AGUILERA and ZIMMERMANN 1986). *PGI1* has been cloned and sequenced (KAWASAKI and FRAENKEL 1982; AGUILERA and ZIMMERMANN 1986; TEKAMP-OLSON, NAJARIAN and BURKE 1988). It has been established that cells defective at *PGI1* cannot utilize glucose as a sole carbon source (MAITRA 1971; CLIFTON, WEINSTOCK and FRAENKEL 1978; CIRIACY and BRIETENBACH 1979; AGUILERA 1988). *PGI1* mutants can grow on fructose provided that a low amount of glucose not exceeding a toxicity threshold level of 0.05% is available (CIRIACY and BREITENBACH 1979; AGUILERA 1988).

To test the inducibility of a *gal3 pgi1* double disruption strain in the 21R genetic background, *pgi1* and *gal3 pgi1* double disruptions were constructed in 21R as described in MATERIALS AND METHODS. The disruption at the *PGI1* locus was confirmed by Southern analysis. Genomic DNA from 21R or from 21R-3D was digested with *Hin*dIII and probed with a 3.0-kb *Bam*HI-*Eco*RI fragment isolated from pGIΔURA. Two bands, one of approximately 4.5 kb and one approximately 8.0 kb, were observed (Figure 5). The genomic DNA isolated from the disrupted strains was subjected to a similar analysis. As expected, the size of the corresponding fragments was reduced. These deletion mutants had no detectable phosphoglucose isomerase enzyme activity (Table 3). Experiments were performed to determine the inducibility of the *GAL/MEL* regulon. We monitored α-galactosidase (see MATERIALS AND METHODS) in 100 colonies of each of the strains, 21Rpgi-1D and 21R-3D.pgi-1D, over a 7-day period following replica plating to G/L or Gal/G/L plates. Strain 21Rpgi-1D was induced normally on the Gal/G/L plates while 21R-3Dpgi-1D was not

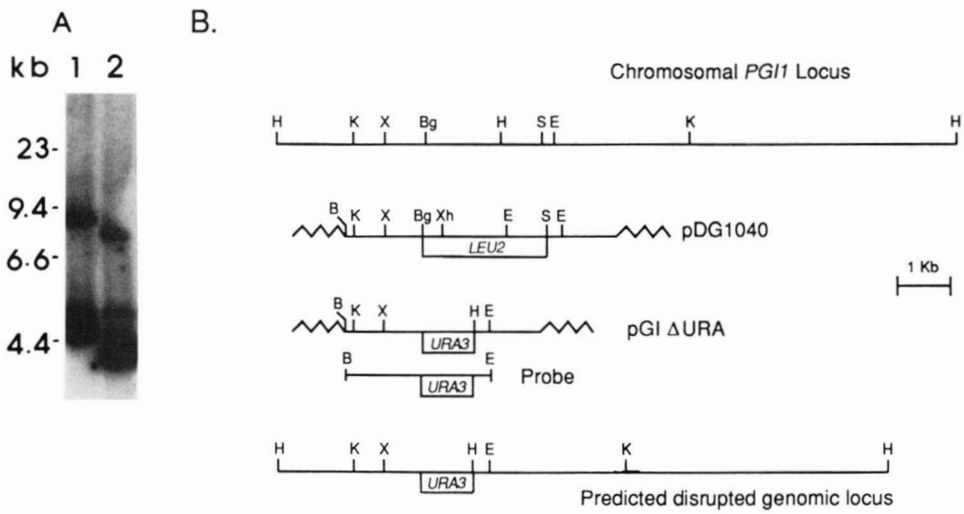

FIGURE 5.—Southern analysis of genomic DNA from 21R and *PGI1* disrupted strain. DNA isolated from the above strains was digested with *Hin*dIII, subjected to electrophoresis in 0.8% agarose gel, transferred to Genescreen membranes (NEN) and probed with the ^{32}P-labeled 3.0-kb *Bam*HI-*Eco*RI fragment isolated from pGIΔURA (A). Lane 1 represents wild-type pattern while lane 2 represents *PGI1* disrupted pattern. *Hin*dIII digested ^{32}P-labeled DNA was used as the molecular weight standard. B, Relevant restriction sites of the chromosomal *PGI1* locus and the *in vitro* constructed deletion mutation. The restriction enzyme sites are: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; *H*, *Hin*dIII; K, *Kpn*I; S, *Sal*I; X, *Xba*I; Xh, *Xho*I. Wavy lines represent vector sequences and straight lines represent chromosomal sequences.

TABLE 3

Effect of disruption of *PGI1* locus on phosphoglucose isomerase enzyme activity

Strain	Milliunits/mg protein
21R	3000
21R pgi-1D	<0.1
21R pgi-1D	<0.1
21R-3D	2900
21R-3D.pgi-1D	<0.1
21R-3D.pgi-1D	<0.1

21R, 21R-3D, 21Rpgi-1D and 21R-3D.pgi-1D were grown to mid-log in YEPG/L medium. (Yeast strains disrupted for PGI1 grow in YEPG/L medium in the absence of exogenously added glucose.) Cells were then harvested and phosphoglucose isomerase enzyme activity was determined as mentioned in MATERIALS AND METHODS. One milliunit of enzyme activity is defined as the amount of enzyme that catalyses the conversion of one nanomole of fructose 6-phosphate to glucose 6-phosphate per minute. Two independent strains disrupted for *PGI1* locus from both 21R and 21R-3D background were analyzed.

induced. Consistent with the induction in 21Rpgi-1D but not 21R3D.pgi-1D, we observed a toxic effect of galactose on the former but not the latter.

DISCUSSION

A galactose-triggered signal alters the physical interplay between the *GAL80* and *GAL4* proteins, resulting in *GAL4* protein-dependent transcriptional activation. The nature and mode of action of this signal must be elucidated in order to understand the precise mechanism of transcriptional induction.

In this work we present new data characterizing the signal. We propose that the *gal3* complementing activity of *GAL1* does not involve its galactokinase activity. We suggest this on the basis of the following considerations. First, the similarity between *GAL1* and *GAL3* is more extensive than that between *GAL1* and the *E. coli galK*, and considerable *GAL1/GAL3* homology lies outside of the region of *GAL1/galK* similarity (BAJWA, TORCHIA and HOPPER 1988). Given that the *GAL1* and *galK* galactokinases catalyze the same reaction, the wider similarity between *GAL1* and *GAL3* suggests a common motif beyond that required for the galactokinase activity. This argument predicts that the *E. coli* galactokinase produced in yeast should not complement the *gal3* LTA phenotype. Our experiments with the *E. coli galK* gene in yeast confirm this prediction.

The second consideration is the discovery that excess *GAL1* dramatically reduces the induction lag conferred by the *GAL3* deficiency. This takes on particular significance in light of the fact that by either *in vitro* or *in vivo* assays we detect no galactokinase activity associated with *GAL3*, though our results do not preclude the possibility that the *GAL3* protein has a galactokinase activity.

In view of the *GAL1/GAL3* similarity and our results suggesting that *GAL3* does not specify a galactokinase activity, a further consideration is the relative sizes of the *GAL1*, *galK* and *GAL3* encoded polypeptides. The *GAL1* protein is 146 amino acids longer than the *galK* protein (CITRON and DONELSON 1984), and yet the *galK* protein functions as a galactokinase in yeast (RYMOND *et al.* 1983). Also the *GAL1* product is 102 amino acids longer than the *GAL3* product. We imagine that the additional information content specified by *GAL1* is somehow required to confer a *GAL3*-like activity. Isolation of mutants that affect the *GAL1* galactokinase activity but not its *gal3*-complementing activity would confirm this idea.

We do not yet understand the action of the *GAL3* and *GAL1* proteins during induction. Based on the noninducibility of *gal1gal3*, *gal7gal3* and *gal10gal3*

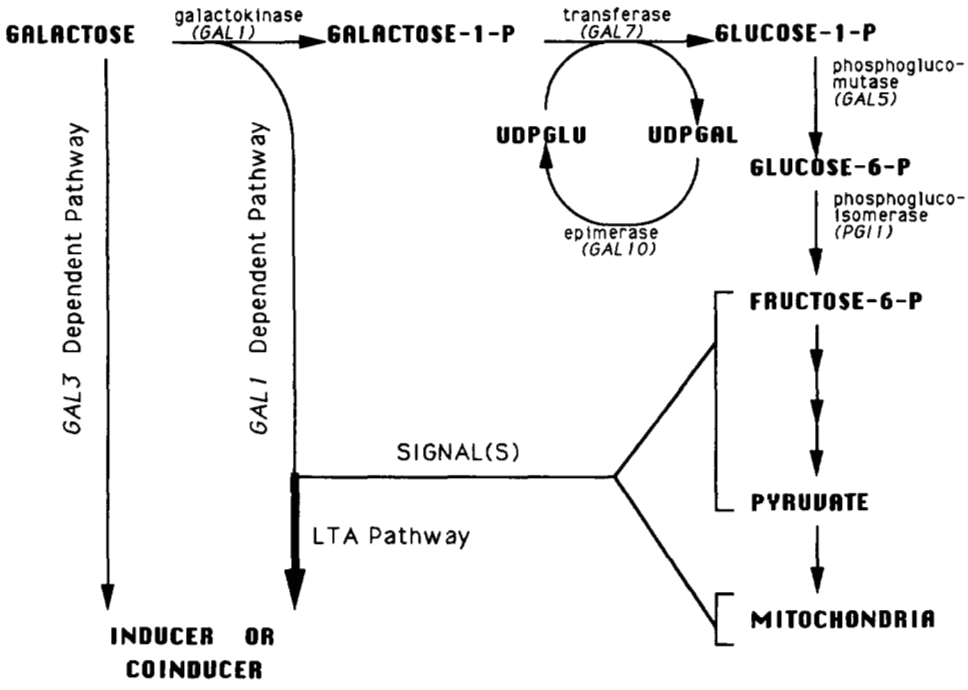

FIGURE 6.—A model for the initiation of induction in a *gal3* strain. In a *gal3* strain the *GAL3*-like activity of the *GAL1* protein converts galactose to an inducer or coinducer. However, this pathway requires that the entire pathway of galactose catabolism and mitochondrial functions are intact. The *GAL3* signal transduction pathway does not have such requirements.

cells, BROACH (1979) proposed that the inducer is a normal intermediate of galactose metabolism. We have tested *gal3* in combination with metabolic blocks beyond the specific galactose pathway; finding that both *gal3gal5* and *gal3pgi1* cells display the noninducible phenotype necessitates a reconsideration of possible models of inducer formation. If the LTA phenotype depends exclusively on the *GAL1-GAL7-GAL10* series of reactions (classical galactose pathway) for the production of an inducer, our results indicate that sufficient production of such an inducer is somehow prevented by blocks beyond this pathway (*e.g.*, *gal5* and *pgi1*). On the other hand, the inducer could arise as a consequence of activities subsequent to the *PGI1*-catalyzed step. However, this possibility is difficult to reconcile with the striking sequence similarity between the *GAL1* and *GAL3* proteins and our discovery of *GAL3*-like activity associated with *GAL1*. These results dictate that at least one event leading to inducer formation in the LTA condition arises directly from an activity of the *GAL1* protein.

Clearly then, an activity associated with the *GAL1* protein as well as an additional function distal to the classical galactose pathway appears to be necessary for the LTA pathway induction. Mitochondrial function has been implicated in the induction mechanism. Respiratory incompetent *gal3* cells cannot be induced (DOUGLAS and PELROY 1963), but *gal3* cells that are induced while respiratory competent are able to maintain the induced state when made respiratory deficient (TSUYUMU and ADAMS 1973). Thus, the induction pathway characteristic of *gal3* respiratory competent cells (LTA pathway) requires mitochondrial function for initiating but not for maintaining the induced state. We favor a model in which this mitochondrial function signals the energy state of the cell to the *GAL1* gene or protein (Figure 6). According to this model the induction block in *gal3gal5* and *gal3pgi1* cells is due to the lack of this energy dependent signal. In *gal3* cells any block in the yield of energy from galactose will block induction; thus any block in the galactose pathway, the glycolytic pathway, or mitochondrial function will render a *gal3* cell noninducible.

How might the system work in consideration of both the normal (*GAL3*) and the LTA (*gal3*) induction pathways? The *GAL3* protein is present in cells grown on glycerol/lactic acid (BAJWA, TORCHIA and HOPPER 1986); that is, sufficient quantities of *GAL3* protein are available to produce required levels of inducer. However, in *gal3* cells grown on noninducing medium the level of the *GAL1* protein is presumably extremely low, as indicated by nondetectability of the *GAL1* mRNA (CITRON and DONELSON 1984; ST. JOHN and DAVIS 1979) in wild-type cells. Thus, initially low basal levels of the *GAL1* protein would seemingly have to be induced to successively higher levels in an autocatalytic manner (BROACH 1979) in order to accumulate sufficient levels of inducer to trigger *GAL4* protein activity . We propose that the specific signal of energy status required for induction in *gal3* cells is required for this autocatalytic increase.

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