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Function of Positive Regulatory Gene *gal4* in the Synthesis of Galactose Pathway Enzymes in *Saccharomyces cerevisiae*: Evidence that the *GAL81* Region Codes for Part of the *gal4* Protein

KUNIHIRO MATSUMOTO,[1] YASUHIKO ADACHI,[2] AKIO TOH-E,[2] AND YASUJI OSHIMA[2]*

Department of Industrial Chemistry, Tottori University, Koyama-cho, Tottori-shi, Tottori 680, Japan,[1] and Department of Fermentation Technology, Osaka University, Yamadakami, Suita-shi, Osaka 565, Japan[2]

A meiotic fine structure map of the *gal4* locus was constructed, which extended over 0.44 units on the chromosome (units in percent frequency of supposed recombination). Several nonsense *gal4* mutations (four UAA and two supposed UGA [*gal4-62* and *gal4-69*]) were placed at various sites on the map. In reversion experiments with 20 independently isolated *gal4* mutants, only the *gal4-62* and *gal4-69* alleles, which are located at the same site on the map, could revert to overcome the superrepression of *GAL80*^{s}*-1* spontaneously with a frequency of approximately 4×10^{-7}. Secondary mutations in the revertants occurred in the region of *gal4-62* or were due to unlinked suppressors. A total of 15 *GAL81* mutations in 19 isolates were found to be located in the same region as *gal4-62* by three-point crosses with the aid of *gal4* mutants; the other four could not be analyzed. The reverted *gal4* gene and *GAL81* mutations were semidominant over the wild-type *GAL4*$^+$ allele and fully dominant over a nonsense *gal4* mutation. Four suppressors (one dominant and three recessive) effective against *gal4-62* and *gal4-69* were isolated. The dominant suppressor was also effective against three independent, authentic auxotrophic UGA nonsense mutations, and one of the three recessive suppressors was effective against the authentic auxotrophic UAA and UAG mutations. These results strongly support the idea that the *gal4* locus is expressed constitutively and codes for a regulatory protein. The *GAL81* site mapped inside the locus codes for a part of the *gal4* protein but does not work as an operator.

The first three galactose pathway enzymes, galactokinase (EC 2.7.1.6), α-D-galactose-1-phosphate uridyltransferase (EC 2.7.7.12), and uridine diphosphoglucose 4-epimerase (EC 5.1.3.2), in *Saccharomyces cerevisiae* are coded by the *gal1*, *gal7*, and *gal10* genes, respectively (3). These structural genes are tightly linked to each other, most probably in the order *gal7-gal10-gal1*, on chromosome II (1) and are expressed coordinately (3). The regulatory genes *gal80*, *GAL81*, and *gal4* are known to operate in the system. Either a recessive *gal80* mutation or a dominant *GAL81* mutation gives rise to constitutive expression of the three structural genes, and a recessive *gal4* mutation results in the absence of the three enzymes simultaneously (4); the wild-type strain shows the inducible phenotype for enzyme synthesis. From the facts that the *GAL81* gene is linked closely to *gal4* and that a *GAL81* mutation is effective only in *cis* position to a functional allele of *gal4*, Douglas and Hawthorne proposed a model for the function of regulatory genes in the system (4), which implied that the *gal80* gene produces a repressor that represses the expression of the *gal4* gene by interacting at the *GAL81* site (the operator of *gal4*) in the absence of galactose. In the presence of galactose, the repressor is inactivated, and the *gal4* gene expresses itself to produce a cytoplasmic positive factor indispensable for expression of the structural genes. Several other possibilities implying a post-translational regulatory role for *gal4* (8, 9, 24) were clearly negated by the recent biochemical evidence communicated by Hopper and his co-workers (6, 7, 15) that the *gal4* gene exerts its function at the level of functional mRNA appearance.

We recently reported evidence that the expression of the *gal4* gene is constitutive and not controlled by the *gal80* gene or by galactose; this evidence was from kinetic studies of galactokinase induction with a temperature-sensitive *gal4* mutant which produces a thermolabile positive factor (11). This observation suggested that the *GAL81* site is not an operator for the *gal4* gene but a part of the *gal4* gene and that it codes

for a site of interaction with the repressor.

This communication deals with the confirmation of this argument by three experiments: (i) meiotic fine structure mapping of the *gal4* and *GAL81* mutations, (ii) the characterization of suppressor mutations at the *GAL81* site and (iii) examination of the semidominance of the *GAL81* mutation and the impedimental effect of the *gal4* mutation in the production of galactokinase activity in heterozygous diploid cells in uninduced condition. The results indicated that the *GAL81* site is situated inside the *gal4* locus, which is expressed constitutively, and that it codes for a portion of the *gal4* protein.

MATERIALS AND METHODS

Yeast strains. The yeast strains used in this study are listed in Table 1. Some of the strains were constructed for use in previous studies (11, 14). For random spore analysis, we isolated spontaneous cycloheximide-resistant mutations in two lines of the pedigree, PY1-1A (*cyhA*), PY1-2D (*cyhA*), and PY1-3D (*cyhA*) from P-28-24C and A6-8 (*cyhB*) from F10D. The *cyhA* and *cyhB* mutants are resistant to 1 and 5 μg of cycloheximide per ml of medium, respectively. Both *cyhA* and *cyhB* are recessive to the wild-type allele, CYH^+, and the wild-type cells cannot grow on medium containing 1 μg of cycloheximide per ml. Although several *cyh* loci have been reported (17, 27), it is not known at which loci our *cyh* mutations occurred. They are nonallelic with each other and unlinked with the *gal4* locus, because tetrad analysis showed a parental ditype/nonparental ditype/tetratype tetrad distribution of 9:15:58 for *gal4-cyhA* combinations and 9:6:29 for *gal4-cyhB* combinations. Two strains marked with various UAA, UAG, and UGA nonsense mutant alleles were kindly provided by B. Ono, Okayama University. Although not described in Table 1, eight strains bearing ochre, tyrosine-inserting suppressors (*SUP2* to *SUP8* and *SUP11*) were obtained from the Yeast Genetics Stock Center, University of California, Berkeley. The other strains were mutants from P-28-24C and F10D or descendants of them by hybridization. Numerous other strains indirectly concerned with the study are also omitted from Table 1.

Media. General usage and compositions of nutrient, minimal, galactose minimal, YPGal, YPGly, YPEth, YPGlyGal, YPEthGal, EBGal, and sporulation media were as described previously (11, 14). In the recombination study by random spore analysis, modified galactose (2%) minimal medium was prepared by modifying Burkholder synthetic medium (2) after several trials; 4 g of $(NH_4)_2SO_4$ per liter and, if necessary, appropriate amounts of amino acids were added instead of L-asparagine, and nicotinic acid, thiamine, inositol, pyridoxine, and panthothenate were omitted from the original formula. Since the same minimal medium with added glucose (2%) in place of galactose supported the growth of all test strains, cell growth on the galactose minimal medium indicates that the cells are able to utilize galactose. To eliminate diploid cells in the random spore analysis, 1 μg of cycloheximide per ml of medium was added.

Techniques. For preparation of spore suspensions for random spore analysis, diploid cells cultivated in nutrient medium at 30°C overnight were inoculated onto whole nutrient agar surfaces in petri dishes, and the plates were incubated at 30°C for 2 days. Cells were collected with bent glass rods and spread onto whole sporulation agar surfaces in petri dishes. Plates were incubated for 2 days at 30°C. After confirmation of sporulation, cells were scratched from the plate with a platinum loop with the aid of 3 ml of 6.7 mM Tris-hydrochloride buffer (pH 7.8) containing 6.4 mg of Zymolyase (Kirin Brewery) per ml, and the suspension was poured into a sterilized centrifuge tube. After standing at 30°C overnight, the suspension was sonicated three times (3 min each) at 19.5 kHz and 200 W (ultrasonic apparatus model 4280; Kaijo Denki). Sterilized water (3 ml) was added, and the suspension was centrifuged at 3,000 × *g* for 10 min. The supernatant fluid was decanted, and the precipitate was mixed with 0.5 ml of streptomycin solution (8 mg/ml of distilled water) and 4.5 ml of sterilized water in the tube on a Vortex mixer and then left to stand for 30 to 60 min at room temperature. The upper fraction containing spores was carefully removed from the sediment of cell debris and diploid cells by using a sterilized pipette and mixed with a final concentration of 0.01% (vol/vol) Tween 80 with a Vortex mixer, before it was spread onto test plates after appropriate dilution. Spore suspensions obtained by this method contained less than 2% diploid cells in the cell populations, as estimated by microscopic examination of each colony developed on nutrient plates without addition of cycloheximide. The frequency of recombination was calculated from the number of colonies developed on the test plates and the total number of spores, which were counted by spreading the same sample onto nutrient plates after appropriate dilution.

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

RESULTS

Isolation and allelic mapping of *gal4* mutations. The respiratory-competent *gal7* and *gal10* mutants are able to grow on YPEth medium but not on YPEthGal medium because of their sensitivity to galactose. (Galactose has a fatal effect on the *gal10* mutant but not on the *gal7* mutant.) A mutation which lowers galactokinase activity, as well as a reverse mutation at *gal7* or *gal10*, relieves the galactose sensitivity (3). It is possible to obtain *gal4* and $GAL80^s$ mutants by selecting the galactose-resistant clones from the *gal7* or *gal10* mutant (11). To isolate *gal4* mutants by this protocol, strain G75-3C (**a** *gal7-2*) was cultivated in 2 ml of nutrient medium at 30°C for 24 h. Cells were harvested, washed, suspended in sterilized water, and spread onto YPEthGal plates to give approximately 10^7 cells per plate. Colonies appearing after 3 to 5 days of incubation at 30°C were isolated and purified by repeated spreading on

TABLE 1. *List of yeast strains*[a]

Strain	Genotype	Source
P-28-24C	**a** Wild type	Our stock culture
F10D	α Wild type	**a** to α mutant of P-28-24C
P612B	α *gal4-2*	Segregant from 1912 (α *gal4-2 gal7-1*) × P-28-24C cross (11). The *gal4-2* allele is suppressible by *SUP3* and *SUP11*.
P612C	**a** *gal4-2*	Segregant from 1912 (α *gal4-2 gal7-1*) × P-28-24C cross (11). The *gal4-2* allele is suppressible by *SUP3* and *SUP11*.
N8-2D	**a** *GAL81-1 gal7-2*	Matsumoto et al. (11)
PY1-1A	**a** *cyhA*	Segregant from cycloheximide-resistant (1 μg/ml) mutant of P-28-24C × P612B cross
PY1-2D	**a** *cyhA*	Segregant from cycloheximide-resistant (1 μg/ml) mutant of P-28-24C × P612B cross
PY1-3D	α *cyhA*	Segregant from cycloheximide-resistant (1 μg/ml) mutant of P-28-24C × P612B cross
A6-8	α *cyhB*	Cycloheximide-resistant (5 μg/ml) mutant from F10D
SL119-1C[b]	**a** ψ^- *leu2-1 met8-1 aro7-1*	B. Ono, Okayama University, Okayama, Japan
637[b]	**a** ψ^+ *cyc1-72 leu2-2 lys2-101 his4-166 trp5-48 ura4-1 can1-100 met8-1 aro7-1 ilv1-2*	B. Ono, Okayama University, Okayama, Japan
G10-3D	α Wild type	Segregant from SA42 × P612B cross. SA42 is a histidine auxotrophic mutant (but wild type for *gal*) of P-28-24C.
G10-6D	α *gal4-2*	Segregant from SA42 × P612B cross. SA42 is a histidine auxotrophic mutant (but wild type for *gal*) of P-28-24C.
G75-3C	**a** *gal7-2*	Segregant from 1098 (**a** *gal7-2* [11]) × AX2-7A cross. AX2-7A is a segregant from the cross between a *leu1* mutant of P-28-24C and F16C (α wild type [19]).
G86-2A	**a** *gal4-62*	Segregant from G75-3CR-22 (mutant from G75-3C; **a** *gal4-62 gal7-2*) × G10-3D cross
G86-2B	α *gal4-62*	Segregant from G75-3CR-22 (mutants from G75-3C; **a** *gal4-62 gal7-2*) × G10-3D cross
G99	**a**/α *GAL80*^{s}*-2*/*GAL80*^{s}*-2*	Diploid constructed by the N270-1B (**a** *GAL80*^{s}*-2*) × N270-1D (α *GAL80*^{s}*-2*) cross. Both N270-1B and N270-1D are haploid segregants from the FJ15B (α *GAL80*^{s}*-2* [14]) × P-28-24C cross.
G100-1A	α *GAL80*^{s}*-1*	Haploid clone obtained by repeated backcrosses of N61-4D (**a** *GAL80*^{s}*-1* [14]) with F10D
G100-3C	**a** *GAL80*^{s}*-1*	Haploid clone obtained by repeated backcrosses of N61-4D (**a** *GAL80*^{s}*-1* [14]) with F10D
G103-18A	α *GAL80*^{s}*-1 GAL81-12 gal10-2*	Constructed by repeated crosses and tetrad analyses among P-28-24C, G100-1A, 1078 (**a** *gal10-2* [14]), and one of the Gal$^+$ revertants from G99 (G99R-10-1B; α *GAL80*^{s}*-2 GAL81-12*)
G104	**a**/α *GAL80*^{s}*-1*/*GAL80*^{s}*-1*	Diploid constructed by G100-1A × G100-3C cross
G104R-14-1A	**a** *GAL80*^{s}*-1 GAL81-7*	Haploid segregant of a constitutive Gal$^+$ revertant from G104
G104R-14-2A	α *GAL80*^{s}*-1 GAL81-7*	Haploid segregant of a constitutive Gal$^+$ revertant from G104
G106-1C	**a** *GAL80*^{s}*-1 GAL81-12 gal4-73*	Segregant from G103-18AR-6 (a galactose-resistant mutant from G103-18A) × G104R-14-1A cross
G108-2D	**a** *GAL81-12 gal4-73*	Segregant from G106-1A (a sister clone of G106-1C; α *GAL80*^{s}*-1 GAL81-12 gal4-73*) × P-28-24C cross
G118-2B	α *GAL80*^{s}*-1 gal4-69*	A segregant from the G75-3CR-43 (**a** *gal4-69 gal7-2*; a galactose-resistant mutant from G75-3C) × G10-3D cross was further crossed with G100-1A, and haploid segregants were selected.

TABLE 1—*Continued*

Strain	Genotype	Source
G118-3C	**a** *GAL80ˢ-1 gal4-69*	Segregant from the G75-3CR-43 (**a** *gal4-69 gal7-2*; a galactose-resistant mutant from G75-3C) × G10-3D cross was further crossed with G100-1A, and haploid segregants were selected.
G126-7A	**a** *GAL80ˢ-1 gal4-62*	Segregant from G86-2B × G100-3C cross
G126-7D	α *GAL80ˢ-1 gal4-62*	Segregant from G86-2B × G100-3C cross
G170-5D	α *GAL80ˢ-1 GAL81-1*	Segregant from 19A3 (**a** *GAL81-1* [14]) × N61-1A (α *GAL80ˢ-1* [11]) cross
G176	**a**/α *GAL80ˢ-2/GAL80ˢ-2*	Diploid constructed by G7-2A (α *GAL80ˢ-2*) × FJ1C (**a** *GAL80ˢ-2* [14]) cross. G7-2A is a segregant from the FJ1C × F16C (cf. G75-3C) cross.
G185-7D	**a** *SUP3*	Segregant from the MG2 (a Gal$^+$ revertant from P612B by a dominant suppressor mutation; α *gal4-2 SUP3*) × P-28-24C cross. That the dominant suppressor is an allele of *SUP3* was confirmed by allelism tests with the authentic testers for the eight tyrosine-inserting suppressors (*SUP2* to *SUP8* and *SUP11*). We observed 4+:0− segregation for the heterozygously marked *his5-2*, *lys1-1*, and *trp5-48* alleles in all 14 asci tested when the suppressor strain was crossed with the standard *SUP3* strain, whereas the combination with the other standard *SUP* strains segregated haploid clones lacking the suppressors (data not shown).
G211-2A	**a** *gal7-2*	Segregant from successive crosses and tetrad dissections of crosses of 1098 (**a** *gal7-2*; cf. G75-3C) with F10D and SA42 (cf. G10-3D)
G211-6A	α *gal7-2*	Segregant from successive crosses and tetrad dissections of cross 1098 (**a** *gal7-2*; cf. G75-3C) with F10D and SA42 (cf. G10-3D)
G250-3C	**a** *gal4-2 gal7-2*	Constructed by repeated crosses and tetrad analyses of crosses among 1098 (cf. G75-3C), SA42 (cf. G10-3D), P612B, and F10D
G250-4C	α *gal4-2 gal7-2*	Constructed by repeated crosses and tetrad analyses of crosses among 1098 (cf. G75-3C), SA42 (cf. G10-3D), P612B, and F10D
G251-4B	α *GAL80ˢ-1 gal7-2*	Segregant from G100-1A × G211-2A cross
G251-5B	**a** *GAL80ˢ-1 gal7-2*	Segregant from G100-1A × G211-2A cross
G254-5C	**a** *gal4-62 sug1*	Segregant from G126-7AR-19 (a Gal$^+$ revertant from G126-7A; **a** *GAL80ˢ-1 gal4-62 sug1*) × G86-2B cross
G261-3C	α *GAL4-69.C51 gal7-2*	Constructed from G118-2BR-1 (a Gal$^+$ revertant from G118-2B; *GAL80ˢ-1 GAL4-69.C51*) by repeated crosses and tetrad dissections of crosses with P-28-24C and G250-4C
G275-4A	**a** *sug2*	Segregant from G86-2BR-17 (a Gal$^+$ revertant from G86-2B; α *gal4-62 sug2*) × P-28-24C cross
G275-4B	α *gal4-62 sug2*	Segregant from G86-2BR-17 (a Gal$^+$ revertant from G86-2B; α *gal4-62 sug2*) × P-28-24C cross
G276-2A	α *gal4-62 sug2*	Segregant from G86-2BR-17 (cf. G275-4A) × G126-7A cross
G277-1D	α *GAL4-62.C102 gal7-2*	Segregant from G86-2BR-2 (a Gal$^+$ revertant from G86-2B; α *GAL4-62.C102*) × G250-3C cross
G278-4A	α *GAL4-62.C1 gal7-2*	Constructed from crosses of G126-7AR-1 (a Gal$^+$ revertant from G126-7A; **a** *GAL80ˢ-1 GAL4-62.C1*) with F10D and G250-3C by repeated crosses and tetrad dissections
G307-1B	**a** *GAL80ˢ-1*	Segregant from G100-1A × P-28-24C cross
G318-6A	**a** *sug1*	Segregant from G254-5C × F10D cross

TABLE 1—*Continued*

Strain	Genotype	Source
G332-1A	α *gal4-69*	Segregant from repeated crosses and tetrad dissections of crosses of G75-3CR-43 (a galactose-resistant mutant from G75-3C; **a** *gal4-69 gal7-2*) with G10-3D and F10D
G332-2A	**a** *gal4-69*	Segregant from repeated crosses and tetrad dissections of crosses of G75-3CR-43 (a galactose-resistant mutant from G75-3C; **a** *gal4-69 gal7-2*) with G10-3D and F10D
G371-2B	**a** *gal4-62 sug2*	Constructed by repeated backcrosses of G86-2BR-17 (cf. G275-4A) with G86-2A
G375-1D	α *gal4-69 sug2*	Segregant from G275-4A × G332-1A cross
G377-1A	**a** *gal4-69 sug1*	Segregant from G318-6A × G332-1A cross
G381-1B	**a** *gal7-2 cyhB*	A6-8 was crossed with P-28-24C, and one of the resultant spore clones was crossed with F10D. The resultant spore clone having a *cyhB* genotype was crossed with G211- 6A, and a spore clone was isolated.
PG2-6C	α *gal4-62 cyhA*	Segregant from PY1-3D × G86-2A cross
M-2A	α *GAL80*ˢ*-1 cyhA*	Segregant from PY1-3D × G307-1D (a sister clone of G307-1B; **a** *GAL80*ˢ*-1*) cross
Y14-3C	**a** *GAL80*ˢ*-1 GAL81-12 gal4-73 cyhA*	Segregant from G106-1C × Y11-2B (segregant from G104R-14-1A × M-2A cross) cross
Y18-4B	α Wild type	Segregant from F10D × ELF52 (mutant from P-28-24C; **a** *leu GAL*⁺) cross
Y25-2D	α Wild type	Constructed by repeated crosses and tetrad dissections of crosses among P-28-24C, F10D, and ELF52 (cf. Y18-4B)
Y42-7A	α *GAL81-1 gal4-74*	Segregant from Y18-4B × A7-21 (a galactose-resistant mutant from N8-2D) cross
Y50-1C	α *GAL81-1 gal4-74*	Segregant from Y42-7A × PY1-2D cross
Y50-9A	**a** *GAL81-1 gal4-74 cyhA*	Segregant from Y42-7A × PY1-2D cross
Y55-19A	α *GAL80*ˢ*-1 GAL81-1 gal4-74 cyhA*	Segregant from Y50-9A × M-2A cross
Y61-6B	α *GAL80*ˢ*-1 GAL81-7 gal7-2 cyhB*	A6-8 was crossed with P-28-24C. One of the segregants (**a** *cyhB*) from the cross was subjected to repeated crossings and tetrad dissections of crosses with G100-1A, G104R-14-2A, and G296-1C (a segregant from the G104R-14-1A × G251-4B cross). Y61-6B was selected from the segregants of the final cross.
Y61-6D	**a** *GAL80*ˢ*-1 GAL81-7 gal7-2 cyhB*	A6-8 was crossed with P-28-24C. One of the segregants (**a** *cyhB*) from the cross was subjected to repeated crossings and tetrad dissections of crosses with G100-1A, G104R-14-2A, and G296-1C (a segregant from the G104R-14-1A × G251-4B cross). Y61-6D was selected from the segregants of the final cross.
Y65-12D	α *gal4-62 SUP3*	Segregant from repeated crosses and dissections of crosses among PG2-2B (a segregant from the same family of PG2-6C; **a** *gal4-62 cyhA*), F10D, P-28-24C, Y25-2D, and G184-3B (a segregant from the MG2 [cf. G185-7D] × P612C cross)
OG3-1D	α *gal4-62 leu2-2 his4-166 lys2-101*	Constructed by repeated crosses and tetrad dissections of crosses among strains 637, F10D, and G86-2B
OG3-6D	**a** *gal4-62 leu2-2 his4-166 lys2-101*	Constructed by repeated crosses and tetrad dissections of crosses among strains 637, F10D, and G86-2B
OG7-2C	α *gal4-62 leu2-1 met8-1*	Constructed by repeated crosses and tetrad dissections of crosses among strains SL119-1C, F10D, and G86-2B

TABLE 1—*Continued*

Strain	Genotype	Source
OG7-4A	**a** *gal4-62 leu2-1 met8-1*	Constructed by repeated crosses and tetrad dissections of crosses among strains SL119-1C, F10D, and G86-2B
OG7-7B	**a** *gal4-62 leu2-1 met8-1*	Constructed by repeated crosses and tetrad dissections of crosses among strains SL119-1C, F10D, and G86-2B
OG17-4B	α *SUPU*	Segregant from OG3-1DR-1 (a mutant from OG3-1D; α *gal4-62 leu2-2 his4-166 lys2-101 SUPU*) × P-28-24C cross
OG18-1B	α *gal4-69 SUPU*	Segregant from OG17-4B × G332-2A cross
OG19-1A	**a** *sup47*	Segregant from OG7-2CR-1 (a mutant from OG7-2C; α *gal4-62 leu2-1 met8-1 sup47*) × P-28-24C cross
OG20-31B	**a** *gal4-69 sup47*	Segregant from 0G19-1A × G332-1A cross

[a] The genetic symbols are those proposed by the Nomenclature Committee for Yeast Genetics (16), whereas the symbols for mating types (**a** and α) follow conventional usage. In the constructed strains, genotypes for markers other than the mating types, *gal*, and the authentic nonsense markers are omitted from the table.

[b] Strains SL119-1C and 637 originated from the laboratory of F. Sherman, University of Rochester, Rochester, N. Y. These strains carry various nonsense mutations: two of these mutations (*aro7-1* and *met8-1*), have the UAG codon, another three (*his4-166*, *leu2-2*, and *lys2-101*) have the UGA codon and the remaining six (*leu2-1*,*cyc1-72*, *trp5-48*, *ura4-1*, *can1-100*, and *ilv1-2*) carry the UAA codon (B. Ono, personal communication).

nutrient plates. One mutant was saved from each 2 ml of the original culture to ensure the independence of the mutation. Each mutant was crossed with the wild-type strain, G10-3D (α), and the ochre nonsense *gal4-2* mutant, G10-6D (α). The resultant diploids were tested for ability to grow on EBGal medium. A total of 20 *gal4* mutants were selected for their inability to complement the *gal4-2* mutation and for the apparent recessiveness of the galactose-negative (Gal$^-$) phenotype to the wild-type allele. From each of the 20 diploids obtained by the *gal4* mutant × G10-3D crosses, two haploid Gal$^-$ segregants having **a** and α mating types and the wild-type allele for *gal7* were selected.

To provisionally classify these 20 *gal4* mutations, we constructed several diploids by combining pairs of different *gal4* mutations. These diploids were sporulated, treated with Zymolyase, and sonicated, and the spore suspensions were spread onto modified galactose minimal plates to give 10^4 to 10^5 spores per plate. The plates were incubated at 30°C for 9 days. Some of the combinations were found to produce galactose-positive (Gal$^+$) colonies on the plates with significant frequencies, whereas others produced few. Although cycloheximide was not added to the medium, contamination by diploid cells was less than 2%, as described above. Since all of the diploids showed the Gal$^-$ phenotype, the occurrence of the Gal$^+$ colonies was due to recombination between two mutant alleles or to reversion in either of the *gal4* mutations. According to the above-described experiments, the *gal4* mutants which failed to produce Gal$^+$ colonies and the mutants which produced them with a frequency lower than 0.01% of the total spores tested were grouped into same class. The *gal4-1*, *gal4-2*, and *gal4-4* mutants described previously (11) were also used in the experiments. Since the *gal4-3* mutation (11) was, like *gal4-4*, originally isolated from a *GAL81-1 gal7-2* strain, but was not separated from the *GAL81-1* allele, and since it is a also temperature-sensitive mutant, it was excluded from the present study. According to this classification, the 23 *gal4* mutations were divided into eight classes. Class 1 consisted of *gal4-1*, *gal4-2*, and *gal4-4* alleles; class 2 consisted of *gal4-52*; class 3 consisted of *gal4-53*, *gal4-55*, *gal4-60*, *gal4-68*, and *gal4-71*; class 4 consisted of *gal4-54*; class 5 consisted of *gal4-56* and *gal4-67*; class 6 consisted of *gal4-57*, *gal4-58*, *gal4-59*, *gal4-61*, *gal4-63*, *gal4-65*, *gal4-66*, and *gal4-70*; class 7 consisted of *gal4-62* and *gal4-69*; and class 8 consisted of *gal4-64*. All of these mutations except *gal4-2* are unsuppressible by the dominant, ochre suppressor *SUP3* or *SUP11*.

Then one or two *gal4* mutant alleles from each class were selected and subjected to meiotic recombination study with the aid of the *cyhA* mutation. These were *gal4-2*, *gal4-52*, *gal4-71*, *gal4-54*, *gal4-56*, *gal4-67*, *gal4-70*, *gal4-62*, *gal4-69*, and *gal4-64*. Some of the selected mutants were crossed with the *cyhA* mutants PY1-1A and PY1-3D, and two *gal4 cyhA* double mutants having **a** and α mating types were obtained by tetrad analysis of the diploids. Then we constructed various diploids heterozygous for two different *gal4* mutations and having the *cyhA*/+ genotype. The diploids were grown on nutrient medium, sporulated, and subjected to random

spore analysis by spreading on modified galactose minimal medium containing the appropriate amino acid(s) and cycloheximide (Table 2). Gal$^+$ colonies appeared most infrequently in the

TABLE 2. *Meiotic recombination frequencies in various pairwise combinations of mutant gal4 alleles*[a]

Combination of *gal4* alleles	No. of spores tested ($\times 10^6$)	No. of Gal$^+$ colonies	Frequency of Gal$^+$ (%)	Range of values[b]
2 × 54	2.58	1,027	0.400	0.375-0.424
2 × 62	2.55	293	0.115	0.102-0.129
2 × 70	2.09	66	0.032	0.025-0.040
52 × 54	8.88	617	0.069	0.064-0.075
52 × 64	0.22	17	0.077	0.048-0.123
54 × 62	9.78	163	0.166	0.143-0.194
54 × 64	1.43	148	0.104	0.088-0.122
54 × 70	6.96	2,158	0.310	0.297-0.323
56 × 62	2.85	264	0.093	0.082-0.105
56 × 67	25.60	102	0.004	0.003-0.005
62 × 62	5.92	1	0	
62 × 67	1.00	91	0.092	0.075-0.113
62 × 69	6.40	1	0	
62 × 70	0.92	93	0.101	0.083-0.124
62 × 71	8.92	103	0.012	0.010-0.014
64 × 67	2.78	56	0.020	0.016-0.026
69 × 70	8.78	1,141	0.130	0.123-0.138
69 × 71	13.20	264	0.020	0.018-0.023
70 × 70	3.98	1	0	
70 × 71	12.70	457	0.036	0.033-0.039
76 × 2	5.62	334	0.059	0.053-0.066
76 × 62	19.81	1,063	0.054	0.051-0.057
76 × 67	7.62	768	0.101	0.094-0.108
76 × 70	17.14	342	0.020	0.018-0.022
76 × 71	4.73	79	0.017	0.013-0.021
77 × 52	15.17	753	0.050	0.046-0.053
77 × 54	41.63	4,284	0.103	0.100-0.106
77 × 62	16.87	1,734	0.103	0.098-0.108
77 × 67	80.08	1,008	0.013	0.012-0.013
78 × 2	66.16	2,521	0.038	0.037-0.040
78 × 70	22.53	1,127	0.050	0.047-0.053
78 × 71	10.08	726	0.072	0.067-0.077

[a] A spore suspension of each diploid was spread onto modified galactose minimal plates containing 1 μg of cycloheximide per ml after appropriate dilution. The plates were incubated for 9 days at 30°C, and the number of colonies appearing was counted. The total number of spores tested was scored on nutrient plates containing 1 μg of cycloheximide per ml.

[b] The range of values was calculated to give 5% probability based on chi-square statistics with the following equation:

$$\text{Range of value} = \frac{(3.841 + 2R) \pm \sqrt{(3.841 + 2R)^2 - 4(3.841 + N)R^2/N}}{2(3.841 + N)} \times 100$$

where N is the total number of spores tested and R is the observed number of Gal$^+$ colonies.

gal4-62 × *gal4-69* combination (1 in 6.40 × 10^6 spores) and most frequently in the *gal4-2* × *gal4-54* combination (0.40% of Gal$^+$ appearance). These data can be summarized in a map, along with data for a few other mutants described below (Fig. 1). Each of the 13 other *gal4* mutants produced Gal$^+$ colonies in very low frequency (less than 0.01%), if at all, in combination with one of the selected *gal4* mutations, and each mutation was therefore mapped at the same site as that mutation.

In another batch of experiments, 72 *gal4* mutants were isolated by the same procedure from G211-2A (a *gal7-2*) and further tested for susceptibility to *SUP3* by testing the growth of diploids prepared by crosses between the galactose-resistant isolates and Y65-12D (α *gal4-62 SUP3*) on EBGal medium. By this procedure, we obtained three nonsense *gal4* mutants, *gal4-76, gal4-77,* and *gal4-78.* Their susceptibilities to the *SUP3* suppressor were further confirmed by tetrad analysis of diploids prepared by crosses between G185-7D (a *SUP3*) and some of the Gal$^-$ (on EBGal medium) clones showing galactose resistance on YPEthGal medium, which were selected from the tetrad clones of diploids prepared by crosses between the three original galactose-resistant isolates and Y25-2D (α wild type). These ochre nonsense alleles were also placed in the fine structure map of *gal4* by the recombination experiments (Table 2 and Fig. 1). The results indicated that apart from some minor inconsistencies, the individual map distances are, in general, consistent with each other.

Constitutive Gal$^+$ revertants from the *gal4* mutants. If the *GAL81* site functions as

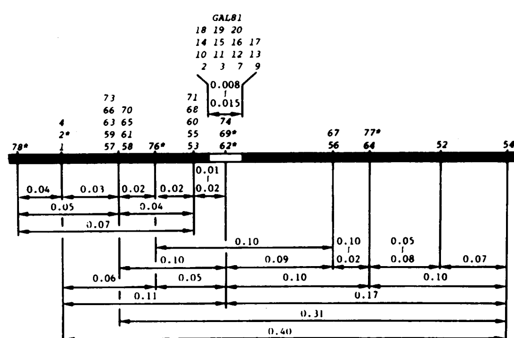

FIG. 1. *Meiotic fine structure map of the gal4 locus. The map was constructed with the data listed in Tables 2 and 9. The width of the GAL81 region (0.008 to 0.015) was deduced from the data in Table 8. The mutant alleles marked with an asterisk are susceptible to nonsense suppressors. The recombination frequencies are expressed as percent appearance of Gal$^+$ or galactose-resistant spores.*

the operator of *gal4*, as suggested by Douglas and Hawthorne (4), it would be extremely difficult to obtain a Gal+ revertant from a *GAL80^s* *gal4* double mutant, since two mutational events would be required in a cell, as a Gal+ reversion due to a mutation at a presumptive controlling site of the structural genes has never been observed (14). These events would be a reverse mutation from *gal4* to *GAL4*+ occurring simultaneously either with a mutation of *gal81*+ (wild type) to *GAL81* (insensitive to superrepression by *GAL80^s*) or with a mutation of the *GAL80^s* allele (the superrepressible allele) to *gal80* (an allele unable to repress the *gal81*+ allele) or with a restoration of the wild-type *GAL80*+ allele. If the *gal4* gene expresses itself constitutively and the *GAL81* site plays a different role, it would be possible to obtain Gal+ revertants from a certain *GAL80^s gal4* mutant by a single secondary mutation. To test the above possibilities, double mutants were constructed which had the *GAL80^s-1* allele and 1 of the 20 *gal4* mutant alleles isolated. These were cultivated in 2 ml of nutrient medium at 30°C for 24 h, harvested, washed, suspended in a few drops of sterile water, and spread onto galactose minimal medium to give 10^7 cells per plate. The plates were incubated at 30°C for 9 to 14 days. Only two of the *GAL80^s-1 gal4* genotypes, *GAL80^s-1 gal4-62* (G126-7A; a) and *GAL80^s-1 gal4-69* (G118-2B; α), gave Gal+ colonies. We observed 18 spontaneous Gal+ colonies in 4.67×10^7 colonies of G126-7A and 22 such colonies in 5.18×10^7 colonies of G118-2B. These frequencies (approximately 4×10^{-7}) strongly suggest that each of the Gal+ colonies resulted from a single mutation. It is noteworthy that both the *gal4-62* and *gal4-69* alleles were mapped at the same position on the *gal4* locus (Table 2 and Fig. 1). No Gal+ colonies developed on the plates spread with the 18 other newly isolated *gal4* mutants.

To characterize the secondary mutations in the revertants from the *GAL80^s-1 gal4-62* and *GAL80^s-1 gal4-69* strains, 17 Gal+ colonies from strain G126-7A and 3 colonies from G118-2B were isolated and subjected to further analysis. Table 3 shows the production of galactokinase activity in the Gal+ clones cultivated under induced and uninduced conditions. All revertants from strain G126-7A showed constitutive production of the enzyme activity, but at lower levels (5 to 25%) than the induced level of the wild-type strains, F10D and P-28-24C. It is noteworthy that the constitutive revertants from G126-7A showed no significant increases in activity; rather, the activity levels decreased when the revertants were cultivated under induced conditions. Similar behavior was observed with most of the constitutive mutants, irrespective of whether they were due to a suppressor or a secondary mutation at or close to the *gal4* locus (Tables 4 through 6). The reasons for the decrease in enzyme activity are obscure. On the other hand, all three revertants from strain G118-2B showed the inducible phenotype, although the activity levels were as low as 5% of the fully induced level of the wild-type strains.

To examine the linkage between the reversion site and the *gal4* locus, each revertant was crossed with the *GAL80^s-1 GAL81-1* (inducible [14]) strain G170-5D or with a *GAL80^s-1 GAL81-7* (semiconstitutive [data not shown]) strain (G104R-14-1A or G104R-14-2A). If the reversion occurs at or close to the *gal4* locus, Gal− segregants arising from spores having the

TABLE 3. *Characterization of the Gal+ revertants from the GAL80^s-1 gal4-62 and GAL80^s-1 gal4-69 strains*

Strain	Galactokinase activity[a]		Tetrad segregation on EBGal[b]		
	Uninduced	Induced	4+:0−	3+:1−	2+:2−
GAL80^s-1 gal4-62					
G126-7A (original)	<0.01	<0.01	0	0	8
G126-7AR-1	1.37	1.13	30	0	0
G126-7AR-6	0.51	0.49	7	0	0
G126-7AR-7	0.30	0.29	11	0	0
G126-7AR-8	1.14	0.64	11	0	0
G126-7AR-9	0.62	0.48	14	2	0
G126-7AR-11	0.80	0.69	12	0	0
G126-7AR-12	0.73	0.70	16	0	0
G126-7AR-13	0.94	0.70	11	0	0
G126-7AR-14	0.62	0.48	3	4	0
G126-7AR-15	0.54	0.53	11	0	0
G126-7AR-16	0.99	0.91	19	1	0
G126-7AR-17	1.37	1.21	18	0	0
G126-7AR-18	1.28	0.91	8	0	0
G126-7AR-19	0.62	0.25	3	6	6
G126-7AR-20	0.33	0.27	9	0	0
G126-7AR-21	0.68	0.69	10	0	0
G126-7AR-22	0.80	0.56	21	5	0
GAL80^s-1 gal4-69					
G118-2B (original)	<0.01	<0.01	0	0	7
G118-2BR-1	<0.01	0.21	13	0	0
G118-2BR-2	<0.01	0.31	9	0	0
G118-2BR-3	0.02	0.24	14	0	0
Wild type					
F10D	0.02	5.60			
P-28-24C	<0.01	5.49			

[a] Cells of each revertant were grown to logarithmic phase in YPGly (uninduced) or YPGlyGal (induced) medium at 30°C with shaking. Galactokinase activity was determined by using permeabilized cells as an enzyme source. Specific activity is expressed as units per milliliter per optical density unit of the culture at 660 nm.

[b] The Gal+ revertants and the original strains (G126-7A and G118-2B) were crossed with the *GAL80^s-1 GAL81-1* or *GAL80^s-1 GAL81-7* strain, and the diploids were subjected to tetrad analysis.

TABLE 4. *Characterization of the Gal$^+$ revertants from the gal4-62 strain G86-2B*

Strain	Galactokinase activity[a]		Tetrad segregation on EBGal[b]		
	Unin-duced	In-duced	4+:0−	3+:1−	2+:2−
G86-2B (original)	0.01	0.01	0	0	8
G86-2BR-3	0.06	6.67	10	0	0
G86-2BR-4	0.03	6.88	17	0	0
G86-2BR-5	0.02	6.04	15	0	0
G86-2BR-6	0.01	4.61	9	0	0
G86-2BR-7	0.09	1.05	11	0	0
G86-2BR-10	0.03	5.83	13	0	0
G86-2BR-11	0.02	4.93	13	0	0
G86-2BR-13	0.04	4.98	10	0	0
G86-2BR-14	0.02	6.38	15	0	0
G86-2BR-15	0.02	6.29	9	0	0
G86-2BR-1	0.40	0.30	9	0	0
G86-2BR-2	1.78	1.16	13	0	0
G86-2BR-8	0.85	0.57	13	0	0
G86-2BR-9	0.43	0.20	13	0	0
G86-2BR-12	0.67	0.73	15	0	0
G86-2BR-16	1.01	0.75	14	0	0
G86-2BR-17	1.37	0.70	1	11	7
G86-2BR-18	0.88	0.50	9	0	0
G86-2BR-19	0.43	0.18	13	0	0

[a] Cells of each revertant were grown to logarithmic phase in YPGly (uninduced) or YPGlyGal (induced) medium at 30°C with shaking. Galactokinase activity was determined by using permeabilized cells as an enzyme source. Specific activity is expressed as units per milliliter per optical density unit of the culture at 660 nm.

[b] Each Gal$^+$ revertant was crossed with the wild-type strain P-28-24C, and the diploids were subjected to tetrad analysis.

original genotype (i.e., *$GAL80^{s}$-1 gal4-62*) formed by recombination should be rare in tetrad analyses of the above crosses. Most of the diploids showed a 4+:0− segregation for growth on EBGal medium, but diploids from five revertants (G126-7AR-9, G126-7AR-14, G126-7AR-16, G126-7AR-19, and G126-7AR-22) showed 3+:1− and 2+:2− segregations in addition to 4+:0− (Table 3). These results indicated that the secondary mutation in all except these five revertants occurred at or near the *gal4* locus. Since growth characteristics of some of the segregants from the four diploids prepared with G126-7AR-9, G126-7AR-14, G126-7AR-16, and G126-7AR-22 were ambiguous on EBGal plates, we did not study them further. The secondary mutation in G126-7AR-19 is apparently unlinked and might be due to an extragenic suppressor of the *gal4-62* allele. This is discussed below.

One trivial explanation of the above results is that the *gal4-62* strain G126-7A carries an adventitious *GAL81* mutation. To test this possibility, approximately 10^7 cells of a *gal4-62* single mutant, G86-2B (α), were spread onto galactose minimal medium, and the plates were incubated for 3 to 13 days at 30°C. Gal$^+$ colonies appeared with a frequency of 5.0×10^{-7} (23 Gal$^+$ from 4.61 $\times 10^7$ colonies). A total of 19 clones were isolated at random, and the galactokinase activity of each isolate grown in YPGly or YPGlyGal medium was determined (Table 4); 9 mutants were found to produce the enzyme constitutively, and 10 produced it inducibly. Most of the inducible revertants showed the same phenotype as the wild-type cells. The nine constitutive revertants, in general, had basal activity of the enzyme which was approximately 3 to 20% of the induced level of the wild-type cells and, like the revertants from strain G126-7A, showed no significant increase in activity when galactose was added. The results clearly negate the possibility that the original *gal4-62* mutation contained an adventitious *GAL81* mutation, since in that case all revertants would be constitutive.

Site of Gal$^+$ reversions is at or near the *gal4-62* or *gal4-69* allele. For further characterization of the revertants from the *$GAL80^{s}$-1 gal4-62* and *$GAL80^{s}$-1 gal4-69* strains, two re-

TABLE 5. *Effects of various suppressors of the gal4-62 and gal4-69 alleles on enzyme synthesis*[a]

Strain or combination	Genotype	Galactokinase activity	
		Unin-duced	Induced
OG3-1D	*gal4-62*	0.02	0.02
OG7-2C	*gal4-62*	0.01	<0.01
G332-1A	*gal4-69*	0.02	0.04
G254-5C	*gal4-62 sug1*	0.62	0.26
G276-2A	*gal4-62 sug2*	1.48	0.91
G86-2BR-17[b]	*gal4-62 sug2*	1.37	0.70
G377-1A	*gal4-69 sug1*	0.59	0.39
G375-1D	*gal4-69 sug2*	0.83	0.58
OG3-1DR-1	*gal4-62 SUPU*	0.02	1.98
OG18-1B	*gal4-69 SUPU*	0.03	1.92
OG7-2CR-1	*gal4-62 sup47*	0.01	1.02
OG20-31B	*gal4-69 sup47*	0.03	0.50
G254-5C / G86-2B	*gal4-62 sug1* / *gal4-62 +*	0.02	<0.01
G86-2BR-17 / G86-2A	*gal4-62 sug2* / *gal4-62 +*	0.03	0.02
OG3-1DR-1 / OG3-6D	*gal4-62 SUPU* / *gal4-62 +*	0.04	1.85
OG7-2CR-1 / OG7-4A	*gal4-62 sup47* / *gal4-62 +*	<0.01	<0.01
G318-6A	*$GAL4^+$ sug1*	0.03	4.64
G275-4A	*$GAL4^+$ sug2*	<0.01	5.99
OG17-4B	*$GAL4^+$ SUPU*	<0.01	4.28
OG19-1A	*$GAL4^+$ sup47*	<0.01	5.41

[a] Cells were cultivated in YPGly (uninduced) or YPGlyGal (induced) medium at 30°C by shaking and were harvested at logarithmic phase. Galactokinase activity was determined by using permeabilized cells as an enzyme source. Specific activity is expressed as units per milliliter per optical density unit of the culture at 660 nm.

[b] See Table 4.

TABLE 6. *Semidominance of the GAL81, GAL4-62.C, and GAL4-69.C mutants over the various gal4 alleles*

Genotype[a]	Galactokinase activity[b]	
	Uninduced	Induced
GAL81-1/GAL81-1	4.16	5.07
GAL81-1/gal81$^+$	2.86	4.63
GAL81-1/gal4-2	4.59	6.28
GAL81-7/GAL81-7	6.13	6.17
GAL81-7/gal81$^+$	3.70	5.50
GAL81-7/gal4-2	5.33	5.99
GAL81-20/GAL81-20	5.14	5.32
GAL81-20/gal81$^+$	2.48	5.68
GAL81-20/gal4-2	5.91	5.68
GAL4-62.C1/GAL4-62.C1	1.91	1.43
GAL4-62.C1/GAL4$^+$	0.24	4.75
GAL4-62.C1/gal4-2	1.45	0.73
GAL4-62.C1/gal4-5[c]	1.17	0.59
GAL4-62.C1/gal4-20[c]	0.76	0.64
GAL4-62.C1/gal4-22[c]	1.16	0.57
GAL4-62.C1/gal4-52	1.31	0.52
GAL4-62.C1/gal4-71	0.63	0.34
GAL4-62.C18/GAL4-62.C18	1.89	1.22
GAL4-62.C18/GAL4$^+$	0.08	4.88
GAL4-62.C18/gal4-2	1.21	0.70
GAL4-69.C51/GAL4-69.C51	0.67	4.21
GAL4-69.C51/GAL4$^+$	0.03	5.26
GAL4-69.C51/gal4-2	0.70	3.51
GAL4-62.C102/GAL4-62.C102	1.73	1.35
GAL4-62.C102/GAL4$^+$	0.07	4.80
GAL4-62.C102/gal4-2	1.32	0.98
Wild type (P-28-24C × F10D)	<0.01	5.27

[a] Detailed descriptions of the strain combinations are omitted.

[b] Cells were cultivated in YPGly (uninduced) or YPGlyGal (induced) medium at 30°C with shaking, and they were harvested at logarithmic growth phase. Galactokinase activity was determined by using permeabilized cells as an enzyme source. Specific activity was determined at least three times and is expressed as mean values in units per milliliter per optical density unit of the culture at 660 nm.

[c] The *gal4-5*, *gal4-20*, and *gal4-22* mutations were isolated by the procedure used to isolate mutations from G75-3C and G211-2A.

vertants (G126-7AR-1 and G118-2BR-1) were selected. They were back-crossed with the α *GAL80*^{s}*-1 gal4-62* (G126-7D) and **a** *GAL80*^{s}*-1 gal4-69* (G118-3C) strains, and both of the diploids could grow on EBGal medium. This fact indicates that each secondary mutation is dominant over its wild-type counterpart. Tetrad analysis showed a 2+:2− segregation for growth on EBGal medium for all 25 asci from the G126-7AR-1 × G126-7D cross and for 11 asci from the G811-2BR-1 × G118-3C cross. This fact indicates that each reversion event is a single mutation which gives rise to the synthesis of the galactose pathway enzymes. Other experiments (data not shown) indicated that in the presence of the wild-type allele *GAL80*$^+$ in place of *GAL80*^{s}*-1*, all of the reversions led to constitutive synthesis of the enzymes. Thus, the secondary mutations at or close to the *gal4* locus were designated *C1* for G126-7AR-1 and *C51* for G118-2BR-1 (according to their isolation numbers), and the whole *gal4* loci were designated, respectively, *GAL4-62.C1* and *GAL4-69.C51* (*C* for constitutive, irrespective of the inducible or constitutive phenotype in combination with *GAL80*^{s}*-1*). (For the revertants from G118-2B, the allele number is expressed by the isolation number of the clone plus 50.) Nomenclature for mutant G126-7AR-19 should not follow this guideline, because the Gal$^+$ reversion of this mutant is due to a suppressor mutation outside *gal4*. This is described below.

The gene symbols for the Gal$^+$ revertants from G86-2B listed in Table 4 were assigned by the same method. When the revertants from G86-2B were crossed with wild-type strain P-28-24C (**a**), the diploids originating from the 10 inducible revertants showed the same phenotype as the wild-type strain (data not shown). Eight of the constitutive revertants produced the enzyme activity in the uninduced condition at severely reduced levels in comparison with the respective haploid mutants (e.g., G86-2BR-2 in Tables 4 and 6) and at higher levels in the inducible condition, whereas one of the diploids, which was prepared by the G86-2BR-17 × P-28-24C (=G275) cross, showed the same phenotype as the wild-type strain (data not shown). All of the diploids except G275 gave 4+:0− segregation on EBGal medium on tetrad analysis (Table 4). Hence, 18 of the 19 Gal$^+$ revertants were due to a secondary mutation at or close to the *gal4* locus; the constitutive revertants were semidominant or recessive with respect to the *GAL4*$^+$ allele, whereas the inducible revertants belonged to the same category as the wild-type strain, and the remaining constitutive revertant (G86-2BR-17) was recessive. Thus, the *gal4* loci of the Gal$^+$ revertants from G86-2B were designated, for example, *GAL4-62.I3* (*I* for inducible) for the inducible revertant G86-2BR-3 and *GAL4-62.C101* for the constitutive revertant G86-2BR-1. (For the semidominant constitutive revertants from G86-2B, the *gal4* locus was denoted by the isolation number of the clone plus 100.) Since the secondary mutation in G86-2BR-17 seems to be due to an extragenic suppressor for the *gal4-62* mutation, the revertant should not be designated by the above nomenclature.

To estimate the distance between the primary and secondary mutations in the revertants, strains G126-7AR-1 (**a** *GAL80*^{s}*-1 GAL4-62.C1*), G118-2BR-1 (α *GAL80*^{s}*-1 GAL4-69.C51*), and

G86-2BR-2 (α *GAL80*$^{+}$ *GAL4-62.C102*) were subjected to further analysis. To eliminate the *GAL80*^{s}*-1* allele in the first two strains, they were crossed with a wild-type strain [F10D (α) or P-28-24C (**a**)], and the resultant diploids were subjected to tetrad analysis. We selected some of the Gal^{+} segregants from the asci showing 2+:2− segregation, although some of the asci showed 4+:0− or 3+:1− segregation. These segregants must have a genotype of *GAL80*$^{+}$ (the wild-type allele of *gal80*) and *GAL4-62.C1* or *GAL4-69.C51*. The segregants and strain G86-2BR-2 were further crossed with a *gal4-2 gal7-2* strain, [G250-3C (**a**) or G250-4C (α)], and the diploids were subjected to tetrad analysis. Tetrad segregants from those diploids were tested on YPEthGal medium for susceptibility to galactose, and we selected some of the segregants which failed to grow on this medium. These segregants (G278-4A, G277-1D, and G261-3C) should have *GAL4-62.C1 gal7-2*, *GAL4-62.C102 gal7-2*, and *GAL4-69.C51 gal7-2* genotypes, respectively. The segregants were crossed with the *GAL4*$^{+}$ *gal7-2 cyhB* strain G381-1B (**a**). Both of the haploid strains in each cross were galactose sensitive due to the *gal7-2* mutation. The resultant diploids were also galactose sensitive and could not grow on YPEthGal plates. The diploids were sporulated, treated with Zymolyase, and sonicated. If recombinations between the primary and secondary mutation sites occurred in the diploid cells during meiosis, spores having the *gal4-62* (or *gal4-69*) *gal7-2* genotype would appear. These spores would be resistant to galactose and would grow on YPEthGal medium. We tested 10^5 to 10^6 spores on YPEthGal plates supplemented with 1 μg of cycloheximide per ml and observed very low frequencies (less than 0.0023%) of spontaneous galactose-resistant colonies from these crosses (Table 7). Although these frequencies are greater than the frequency observed in the spores derived from a *GAL4*$^{+}$/*GAL4*$^{+}$ *gal7-2*/*gal7-2 cyhB*/*CYHB*$^{+}$ diploid (0.0006%), they are low enough to conclude that the secondary mutations occurred at or close to the *gal4-62* or *gal4-69* allele.

Meiotic fine linkage analysis of the *GAL81* mutants. To study the distribution of the *GAL81* sites, we isolated 18 new *GAL81* mutants of independent origin in addition to the *GAL81-1* mutant which was isolated previously (14). To facilitate the isolation, we used the *GAL80*^{s}*-1*/*GAL80*^{s}*-1* diploid strain G104 and collected nine *GAL81* mutants (*GAL81-2*, *GAL81-3*, and *GAL81-5* to *GAL81-11*). The remaining nine *GAL81* mutants (*GAL81-12* to *GAL81-20*) were isolated from the *GAL80*^{s}*-2*/*GAL80*^{s}*-2* diploids G99 and G176. It was confirmed that all of the newly isolated *GAL81* mutations (in combination with the *GAL80*$^{+}$ genotype) showed the constitutive phenotype for enzyme production and that the constitutive mutations were linked closely to the *gal4* locus and were dominant over their wild-type counterparts.

We constructed *GAL80*^{s}*-1 GAL81-x gal7-2* triple mutants (where *GAL81-x* indicates various alleles of *GAL81* mutations) by crossing the *GAL80*^{s}*-1 gal7-2* strain G251-4B (α) or G251-5B (**a**) and *GAL80*^{s}*-1* (or *GAL80*^{s}*-2*) *GAL81-x* strains. Since the *GAL80*^{s}*-2* allele showed more severe repression than the *GAL80*^{s}*-1* allele (14), it is possible to infer that some of the *GAL81-x* alleles which were isolated from the *GAL80*^{s}*-1*/*GAL80*^{s}*-1* diploid G104 were uninducible in combination with *GAL80*^{s}*-2*, as observed with the *GAL80*^{s}*-2 GAL81-1* strain (14), and gave the galactose resistance phenotype in the *GAL80*^{s}*-2 GAL81-1 gal7-2* triple mutant. To eliminate this

TABLE 7. *Meiotic recombination frequencies between the gal4-62 or gal4-69 allele and the secondary constitutive mutation sites*[a]

Combination: Strain	Combination: Genotype	No. of spores tested (×10^5)	No. of colonies appearing on YPEthGal	Frequency (%)
G381-1B / G278-4A	**a** + *gal7-2 cyhB* / α *GAL4-62.C1 gal7-2* +	9.33	17	0.0018
G381-1B / G277-1D	**a** + *gal7-2 cyhB* / α *GAL4-62.C102 gal7-2* +	1.76	4	0.0023
G381-1B / G261-3C	**a** + *gal7-2 cyhB* / α *GAL4-69.C51 gal7-2* +	13.62	25	0.0018
G381-1B / G211-6A	**a** *gal7-2 cyhB* / α *gal7-2* +	1.66	1	0.0006

[a] Spore suspensions of the diploids were spread onto YPEthGal medium and onto nutrient plates containing 1 μg of cycloheximide per ml after appropriate dilution. The YPEthGal plates were incubated for 7 days, and the nutrient plates were incubated for 2 days at 30°C. Colonies appearing on the plates were scored, and the frequencies of appearance of galactose-resistant colonies were calculated.

difficulty, we used the *GAL80*ˢ*-1* allele in the recombination analysis. The *GAL81-x* mutants isolated from G99 and G176 (*GAL80*ˢ*-2/GAL80*ˢ*-2*) were crossed once with a wild-type strain (P-28-24C or F10D), and *GAL81-x* single mutants (without *GAL80*ˢ*-2*) were selected from the tetrad segregants of the diploids. These single *GAL81-x* mutants were crossed with *GAL80*ˢ*-1* strain G100-1A (α) or G307-1B (**a**), and the diploids were subjected to tetrad analysis. From the meiotic segregants, *GAL80*ˢ*-1 GAL81-x* recombinants were selected and crossed with *GAL80*ˢ*-1 gal7-2* strain G251-4B (α) or G251-5B (**a**). The diploids were sporulated, and four-spore asci were dissected. The asci showed 4+:0−, 3+:1−, and 2+:2− segregations on YPEthGal medium. From asci showing 2+:2− segregation, we selected spore clones unable to grow on YPEthGal medium. These strains should have the *GAL80*ˢ*-1 GAL81-x gal7-2* genotype, in which *GAL81-x* is epistatic to *GAL80*ˢ*-1*, as the selected clones were galactose sensitive.

All of the triple mutants thus obtained were crossed with *GAL80*ˢ*-1 GAL81-7 gal7-2 cyhB* strain Y61-6B (α) or Y61-6D (**a**). The diploids were sporulated and subjected to random spore analysis on nutrient and YPEthGal plates containing 1 μg of cycloheximide per ml. If a recombination occurred between two different *GAL81* alleles, it could be scored by counting the galactose-resistant colonies on the YPEthGal plate, as the $gal81^+$ wild-type allele is repressed by the *GAL80*ˢ*-1* allele and the galactose sensitivity due to the *gal7-2* mutation is relieved by the repression of galactokinase synthesis. Results showed that the 15 *GAL81* mutations occurred at the same site or at sites tightly linked to the *GAL81-7* mutation, as the frequencies of occurrence of galactose-resistant colonies were less than 0.0077% in at least 0.48×10^5 spores tested (Table 8). However, the diploids constructed with the triple mutants having the remaining four *GAL81* alleles (*GAL81-1, GAL81-5, GAL81-6,* and *GAL81-8*) showed significant growth on YPEthGal medium at 30°C after a few days. This fact makes it impossible to perform the recombination analysis of these four alleles by this method. The *GAL81-1* allele gives rise to the inducible phenotype in combination with *GAL80*ˢ*-1* (14), and the other three showed the same inducible phenotype with *GAL80*ˢ*-1* (data not shown). These phenotypes might partially relieve the sensitivity of the *gal7* mutation to galactose, but the exact cause of the ambiguous results is not known. For the same reason, we could not analyze the *GAL4-62.C* and *GAL4-69.C* mutations in combination with *gal7-2* and *GAL80*ˢ*-1* mutations.

TABLE 8. *Meiotic recombination data for pairwise combinations of GAL81 alleles with the GAL81-7 allele as the standard*

GAL81 allele[a]	No. of spores tested (×10⁵)	No. of colonies appearing on YPEthGal[b]	Frequency (%)
GAL81-2	4.07	9	0.0022
GAL81-3	2.17	5	0.0023
GAL81-9	8.29	15	0.0018
GAL81-10	0.65	0	0.0016
GAL81-11	3.49	14	0.0040
GAL81-12	2.86	11	0.0038
GAL81-13	2.12	6	0.0028
GAL81-14	0.65	5	0.0077
GAL81-15	9.17	28	0.0031
GAL81-16	1.93	14	0.0073
GAL81-17	1.77	8	0.0045
GAL81-18	13.70	4	0.0003
GAL81-19	0.48	3	0.0062
GAL81-20	2.30	11	0.0048
GAL81-7[c]	1.50	3	0.0020

[a] Although detailed descriptions of the strain combinations are omitted, diploids were constructed by crossing *GAL80*ˢ*-1 gal7-2 GAL81-7 cyhB* strains Y61-6B (α) and Y61-6D (**a**) with *GAL80*ˢ*-1 gal7-2 GAL81-x*, where *GAL81-x* indicates the various alleles of the *GAL81* mutation described in the table. The diploids were subjected to random spore analysis on YPEthGal plates.

[b] The appearance of galactose-resistant colonies was scored after a 7-day incubation of the plates at 30°C.

[c] The homoallelic combination for *GAL81-7*.

GAL81 region is mapped at or close to the *gal4-62* site. Although the map positions of the four ambiguous *GAL81* mutations were unknown, we determined the positions of the *GAL4-62.C* and *GAL4-69.C* mutations relative to the other 15 *GAL81* alleles by linkage analysis. Since the recombination frequencies for all combinations of the 15 *GAL81* alleles so far determined were very low (less than 0.0077%) (Table 8) and the secondary constitutive mutations in *GAL4-62.C* and *GAL4-69.C* were suggested to occur at or close to the *gal4-62* and *gal4-69* alleles (Table 7), which were mapped close to each other (recombination frequency, less than 0.0002%) (Table 2 and Fig. 1), the relative positions of the two regions could be determined by linkage analysis of a certain *GAL81* allele and the *gal4-62* or *gal4-69* allele.

To determine the relative positions of the *GAL81* and *gal4-62* or *gal4-69* alleles, we decided to use three-point crosses. Therefore, we isolated helper *gal4* mutations from the *GAL81-12* strain G103-18A (α *GAL80*ˢ*-1 GAL81-12 gal10-2*) and from the *GAL81-1* strain N8-2D (**a** *GAL81-1 gal7-2*) with the aid of the galactose sensitivity of the *gal10-2* and *gal7-2* alleles. After several trials we isolated one galactose-resistant

colony on an YPEthGal plate from each of the above two strains. These isolates could not grow on EBGal medium. By repeated crosses and tetrad analyses of the isolates from crosses of G103-18A with the *GAL80*^{s}*-1 GAL81-7* strain G104R-14-1A, the wild-type strain P-28-24C, and the *GAL80*^{s}*-1 cyhA* strain M-2A, we obtained strains G106-1C (**a** *GAL80*^{s}*-1 GAL81-12 gal4-73*), G108-2D (**a** *GAL81-12 gal4-73*), and Y14-3C (**a** *GAL80*^{s}*-1 GAL81-12 gal4-73 cyhA*). The *gal4-73* mutant allele might be connected with the *GAL81-12* allele, as it was marked in the original strain. A similar *gal4* mutation, *gal4-74*, was obtained by selection of a galactose-resistant colony on YPEthGal medium spread with N8-2D. It was crossed with Y18-4B (α wild type), PY1-2D (**a** *cyhA*), and M-2A (α *GAL80*^{s}*-1cyhA*), and strains Y42-7A (α *GAL81-1 gal4-74*), Y50-9A (**a** *GAL81-1 gal4-74 cyhA*), and Y55-19A (α *GAL80*^{s}*-1 GAL81-1 gal4-74 cyhA*) were selected from the tetrad segregants, as listed in Table 1. The assignments of genotype were further confirmed by the failure of diploids prepared by crossing G108-2D or Y42-7A with *gal4-2* strain P612B (α) or P612C (**a**) to grow on EBGal medium.

By using the *GAL81-12 gal4-73* and *GAL81-1 gal4-74* strains with or without the *GAL80*^{s}*-1* marker and the strains marked with the *GAL80*^{s}*-1* and/or *gal4-62* allele, we conducted a linkage analysis of the region between the *GAL81* and *gal4-62* sites. Three different types of crosses were made: (i) *GAL80*^{s}*-1 GAL81-12 gal4-73/GAL80*^{s}*-1 gal81*$^{+}$ *GAL4*$^{+}$ (cross A), (ii) *GAL81-12 gal4-73/gal81*$^{+}$ *gal4-62* (cross B), and (iii) *GAL80*^{s}*-1 GAL81-12 gal4-73/GAL80*^{s}*-1 gal81*$^{+}$ *gal4-62* (cross C) (Fig. 2), and similar diploids having the *GAL81-1* and *gal4-74* alleles were constructed. If both the *GAL81-12* and *gal4-62* mutations occurred at the same site in the *gal4* locus, the same frequency of Gal^{+} recombinant spores would be expected in all three crosses by random spore analysis on modified galactose minimal medium. If the *gal4-62* site is situated between *GAL81-12* and *gal4-73*, Gal^{+} colonies would occur most frequently in cross A and with almost the same frequency in crosses B and C. If the gene order is *gal4-73–GAL81-12–gal4-62*, Gal^{+} colonies would be expected most frequently in cross B and with almost the same frequency in crosses A and C. If the gene order is *GAL81-12–gal4-73–gal4-62*, Gal^{+} would appear very rarely in cross C. These predictions were tested with the strains constructed with the *gal4-73* mutant isolated from G103-18A, as described above. The results (Table 9) showed no essential differences in the frequencies of Gal^{+} colonies for the three types of crosses. This

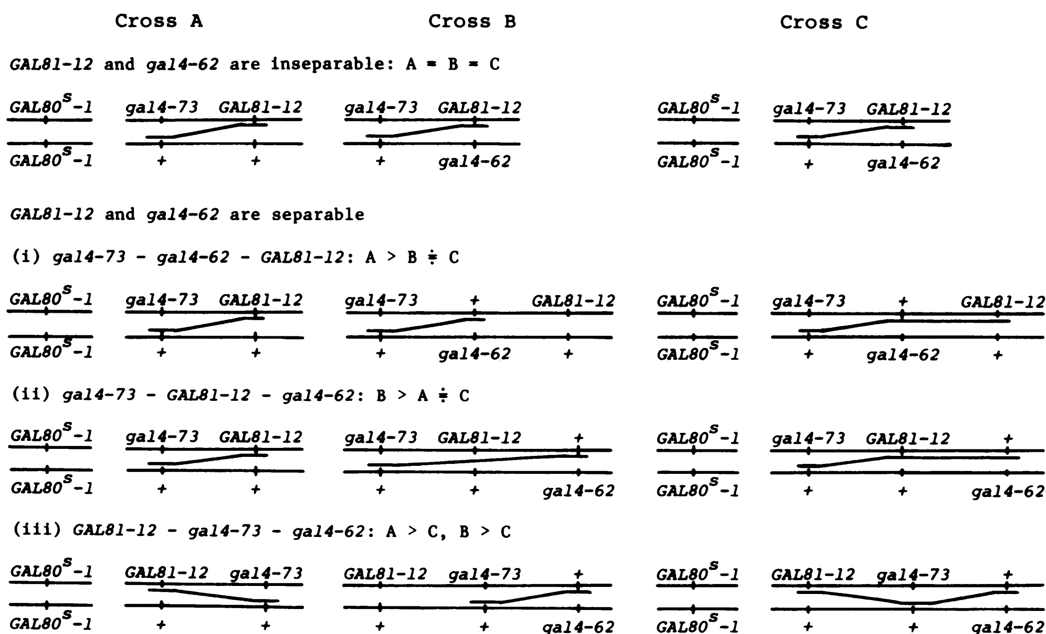

FIG. 2. *Three types of crosses for linkage analysis between the gal4-62 and GAL81-12 alleles. On the assumption that the gal4-62 and GAL81-12 alleles are separable, three different patterns of segregation with respect to the allelic order can be expected, and these patterns are different from the pattern expected on the assumption that gal4-62 is inseparable from GAL81-12, as illustrated in the figure.*

TABLE 9. *Meiotic recombination data for gal4-62 and the GAL81 mutation sites*[a]

Cross			No. of spores tested (×10^5)		No. of Gal$^+$ colonies		Frequency (%)	
Type[b]	Strain	Genotype	Expt 1	Expt 2	Expt 1	Expt 2	Expt 1	Expt 2
With *GAL18-12 gal4-73* strains								
A	G106-1C / M-2A	*GAL80^s-1 gal4-73 GAL81-12* + / *GAL80^s-1* + + *cyhA*	5.05	0.58	664	63	0.13	0.11
B	G108-2D / PG2-6C	+ *gal4-73 GAL81-12* + / + *gal4-62* + *cyhA*	3.00		287		0.096	
C	Y14-3C / G126-7D	*GAL80^s-1 gal4-73 GAL81-12 cyhA* / *GAL80^s-1 gal4-62* + +	0.51	3.09	59	366	0.11	0.12
With *GAL81-1 gal4-74* strains								
A	Y55-19A / G307-1B	*GAL80^s-1 gal4-74 GAL81-1 cyhA* / *GAL80^s-1* + + +	0.74	1.51	1	5	0.0014	0.0033
B	Y50-1C / PG2-6C	+ *gal4-74 GAL81-1* + / + *gal4-62* + *cyhA*	1.60	2.30	1	2	0.0006	0.0009
C	Y55-19A / G126-7A	*GAL80^s-1 gal4-74 GAL81-1 cyhA* / *GAL80^s-1 gal4-62* + +	0.83	0.38	1	0	0.0012	<0.0003
Control	G100-3C / M-2A	*GAL80^s-1* + + + / *GAL80^s-1* + + *cyhA*	2.78		0		0	

[a] Spore suspensions of the diploids were spread onto modified galactose minimal plates containing 1 μg of cycloheximide per ml after appropriate dilution. The plates were incubated for 9 days at 30°C, and the colonies appearing were scored. The total number of spores tested was scored on nutrient plates containing 1 μg of cycloheximide per ml.

[b] Allelic configurations in three different crosses (A, B, and C) are illustrated in Fig. 2.

fact supports the first possibility, that the *GAL81-12* and *gal4-62* mutations occurred at almost the same position in the *gal4* locus. The same conclusion was suggested by similar experiments with mutants having the *GAL81-1 gal4-74* genotype (Table 9); i.e., the *gal4-62* and *gal4-74* mutations occurred at almost the same site close to the *GAL81-1* mutation. In other words, the *GAL81* region adjoins or includes the *gal4-62, gal4-69,* and *gal4-74* sites and is located inside the *gal4* locus (Fig. 1).

With the recombination data described above and the data for the *gal4-73* × *gal4-70, gal4-73* × *gal4-71, gal4-73* × *gal4-64,* and *gal4-73* × *gal4-52* combinations, in which the frequencies of Gal$^+$ appearance were calculated to be 0.012% (30 Gal$^+$ from 2.52 × 10^5 spores tested), 0.088% (361 Gal$^+$ from 4.11 × 10^5 spores), 0.119% (749 Gal$^+$ from 6.29 × 10^5 spores), and 0.175% (496 Gal$^+$ from 2.83 × 10^5 spores), respectively, we tentatively mapped the *gal4-73* allele at the same point as *gal4-70* (Fig. 1). Since Gal$^+$ appeared very rarely in the *gal4-74* × *gal4-62* combination (cross B experiment of *gal4-74*; Table 9), *gal4-74* was mapped at the same site as *gal4-62* and *gal4-69* (Fig. 1).

Suppressors for the *gal4-62* and *gal4-69* alleles. All of data described above strongly suggest that the *GAL81* mutation occurs at or close to the *gal4-62* and *gal4-69* mutations. All of the *GAL81* mutations were placed in a narrow region with a limited recombination frequency of at most 0.015%, because the two highest frequencies observed were 0.0077% (between *GAL81-7* and *GAL81-14*) and 0.0073% (between *GAL81-7* and *GAL81-16*) (Table 8). Thus, the *GAL81* region is located inside the *gal4* locus and occupies a region which is not more than 4% of the *gal4* locus, as the whole *gal4* locus was shown to extend 0.44% in recombination frequency (Fig. 1). These arguments imply that the *GAL81* region might code for protein as a part of the *gal4* locus.

To strengthen this view, we characterized the suppressor mutations found in the Gal$^+$ revertants from G126-7A (a *GAL80^s-1 gal4-62*) (Table 3) and G86-2B (α *gal4-62*) (Table 4). The revertants G126-7AR-19 and G86-2BR-17 were crossed with strains G126-7D (α *GAL80^s-1 gal4-62*) and G86-2A (a *gal4-62*), which have the same *gal* genotypes as their respective original strains but complementary mating types. The diploids could not grow on EBGal medium or produce galactokinase activity. The diploids were sporulated and dissected, and the segregants were tested on EBGal plates. All of the tetrads tested (11 tetrads from the G126-7AR-19 × G126-7D cross and 11 from the G86-2BR-17 × G86-2A cross) showed a 2Gal$^+$:2Gal$^-$ segregation, and all of the Gal$^+$ clones produced galac-

tokinase activity constitutively, as did the original isolates. These observations indicate that both secondary mutations are recessive single mutations and act as suppressors for the *gal4-62* allele. Tetrad data listed in Table 3 and 4 show that the suppressors are unlinked to *gal4*. The secondary mutation in G126-7AR-19 was designated *sug1* (suppressor for *gal*) and that in G86-2BR-17 was designated *sug2*. Complementation and allelism tests were done with the diploid obtained by the G254-5C (**a** *gal4-62 sug1*) × G275-4B (α *gal4-62 sug2*) cross. This diploid failed to grow on EBGal medium and showed a trace level of galactokinase activity (0.04 U/ml per optical density unit of the culture at 660 nm in induced cells and 0.16 U in uninduced cells). A total of 12 asci from the diploid were dissected; from these only one complete tetrad was recovered, this showing a 4Gal$^+$:0Gal$^-$ segregation on EBGal medium. Three spores were recovered from eight asci, all of which showed a 2Gal$^+$:1Gal$^-$ segregation, and two spores were recovered from the three remaining asci; of these one showed a 0Gal$^+$:2Gal$^-$ segregation, and the other two showed a 2Gal$^+$:0Gal$^-$ segregation. These segregation data strongly suggest that spores having the *gal4-62 sug1 sug2* genotype could not germinate. This fact was confirmed by complementation tests for the segregants, in which no *sug1 sug2* double mutant was recovered. This segregation pattern also indicates that the *sug1* and *sug2* suppressors are not linked to each other.

Suppressors *sug1* and *sug2* are not linked to *gal80* and *gal7*. Linkage of suppressors *sug1* and *sug2* to the *gal80* and *gal7* loci was examined. If *sug1* (or *sug2*) is closely linked to *gal80*, then most Gal$^-$ segregants from the G126-7AR-19 (**a** *GAL80^s-1 gal4-62 sug1*) × G86-2B (α *gal4-62*) cross (G254) should carry the *GAL80$^+$* allele, and those from the G86-2BR-17 (α *gal4-62 sug2*) × G126-7A (**a** *GAL80^s-1 gal4-62*) cross (G276) should carry the *GAL80^s-1* allele. (In the former case, for example, the *GAL80^s-1* allele was mutated to *sug1* in the original revertant.) When the diploids, G254 and G276 were dissected, phenotypes on EBGal medium segregated 2+:2− in all of 7 (G254) and 11 (G276) asci. Then each clone was crossed with the wild-type strains to discover whether it contained the *GAL80^s-1* mutation. The suppressors were found to segregate independently from *gal80*, because *GAL80$^+$ gal4-62 sug1* segregants were obtained from G254 and *GAL80^s-1 gal4-62 sug2* segregants were obtained from G276 (data not shown). It was confirmed that the *gal4-62 sug1* genotype also gives rise to the constitutive but low activity of galactokinase, as in the *gal4-62 sug2* strain (Table 5).

If *sug1* (or *sug2*) were the promoter mutation for the structural genes *gal17, gal10,* and *gal1*, it must be linked to *gal7*, in which case almost all tetrads from a *gal4-62 sug1* (or *gal4-62 sug2*) × *gal7* cross would show a 2+:2− segregation on EBGal medium. We tested this possibility by tetrad analysis of the G254-5C (**a** *gal4-62 sug1*) × G211-6A (α *gal7-2*) and G86-2BR-17 (α *gal4-62 sug2*) × G211-2A (**a** *gal7-2*) crosses. Five of 10 asci from the former cross and 4 of 15 asci from the latter cross showed a 1Gal$^+$:3Gal$^-$ segregation, and 1 of the asci in the latter cross showed a 0Gal$^+$:4Gal$^-$ segregation; all of the remaining asci showed a 2Gal$^+$:2Gal$^-$ segregation. These segregation data clearly negated the linkage between *gal7* and the suppressors.

***sug1* and *sug2* are specific for *gal4-62* and *gal4-69*.** To investigate the effect of each suppressor on the wild-type allele of *gal4*, we selected the Gal$^+$ spore culture G318-6A from a 3Gal$^+$:1Gal$^-$ tetrad from the G254-5C × F10D cross and a similar spore culture, G275-4A, from the G86-2BR-17 × P-28-24C cross. When strains G318-6A and G275-4A were crossed with the *gal4-62* strain G86-2B, the diploids showed a segregation pattern for galactose utilization which suggested that G318-6A and G275-4A carried the *GAL4$^+$ sug1* (or *sug2*) genotype (data not shown). Since the two strains showed the inducible phenotype (Table 5), we concluded that the suppressors were ineffective against *GAL4$^+$* and that the constitutive synthesis of galactokinase activity is caused by the combination of the suppressors with the *gal4-62* allele.

The specificity of suppression by *sug1* and *sug2* toward the other *gal4* mutants was examined by crossing the *gal4-62 sug1* and *gal4-62 sug2* strains with six other *gal4* mutants (*gal4-2, gal4-7, gal4-53, gal4-60, gal-64,* and *gal4-69*). Tetrad analysis of the diploids revealed that all except one of the crosses produced more than two Gal$^-$ clones in each ascus, the exception being the diploid prepared with the strain having the *gal4-69* genotype, which showed 2Gal$^+$:2Gal$^-$ segregation in all 19 and 11 asci tested for *sug1* and *sug2*, respectively. These segregation patterns indicate that both the *sug1* and *sug2* suppressors are specific for the *gal4-69* allele but not for the other *gal4* mutations tested. This fact suggests the possibility that the *gal4-62* and *gal4-69* mutations are suppressible by a certain nonsense suppressor. This possibility was tested by crossing G86-2A (**a** *gal4-62*) and G332-2A (**a** *gal4-69*) with strains having the *SUP2* to *SUP8* and *SUP11* suppressors, which were obtained from Berkeley stock center, or with testers constructed in our laboratory. However, none of these strains could suppress the *gal4-62* or *gal4-69* mutation (data not shown). During these

experiments, we found that *sug1* and *sug2* did not suppress the ochre (UAA) nonsense mutations *ade2-1, his5-2, ilv1-2, leu2-1, lys1-1, trp5-48,* and *ura4-1,* the amber (UAG) nonsense mutations *aro7-1, met8-1,* and *trp1-1,* and the UGA nonsense mutations *his4-166, leu2-2,* and *lys2-101.* (We did not test the other two ochre mutations, *cyc1-72* and *can1-100,* and an amber mutation, *cyc1-76,* which were marked in the testers.)

UGA nonsense suppressor and an omnipotent suppressor can suppress *gal4-62* and *gal4-69*. The above observations, however, do not mean that *sug1* and *sug2* are not nonsense suppressors. To further characterize the *gal4-62* and *gal4-69* mutations, we conducted reversion experiments with strains OG3-1D (α *gal4-62 his4-166 leu2-2 lys2-101*) and OG7-2C (α *gal4-62 leu2-1 met8-1*). All three auxotrophic markers in OG3-1D are UGA nonsense mutations, whereas those in OG7-2C are UAA (*leu2-1*) and UAG (*met8-1*) nonsense mutations. Strains OG3-1D and OG7-2C were cultivated on nutrient medium. Cells of OG3-1D were harvested, washed, and spread onto galactose minimal medium supplemented with appropriate amounts of histidine and lysine (medium A), histidine and leucine (medium B), or lysine and leucine (medium C). Similarly, cells of OG7-2C were plated onto galactose minimal medium supplemented with leucine (medium D) or methionine (medium E). These plates were incubated at 30°C. In strain OG3-1D, 38 colonies (7 from medium A, 15 from medium B, and 16 from medium C) were collected after 5 to 12 days of incubation. It was confirmed that all of the isolated clones could grow on either medium A, B, or C and also on EBGal medium. The mutation was further characterized in one of the isolates, OG3-1DR-1. It was crossed with P-28-24C (**a** wild type), the diploid was sporulated, and four-spore asci were dissected. Two or more spore clones showed the positive phenotype for all of the markers in all of the asci, and always two spore clones showed the positive phenotype for all of the traits in each ascus. These facts strongly suggest that the reversion was caused by a suppressor mutation. When OG3-1DR-1 was crossed with OG3-6D, which has the same genotype for *gal* and the auxotrophic markers but has a mating type complementary to that of OG3-1D, the diploid segregated two spore clones showing the positive phenotype, whereas the other two spore clones showed the negative phenotype for all of the Gal, Leu, His, and Lys traits in all 10 asci tested. This fact clearly indicates that the suppressor is effective against all of the markers, including *gal4-62*. Since the diploid is heterozygous for the suppressor and showed the same level of suppression as haploid *gal4-62* cells carrying the suppressor (Table 5), the suppressor is dominant over the wild-type counterpart. The suppressor was tested for its effect on the UAA (*leu2-1*) and UAG (*met8-1*) nonsense mutations by tetrad analysis of the diploid prepared by the OG3-1DR-1 (α *gal4-62 his4-166 leu2-2 lys2-101 SUP*) × OG7-7B (**a** *gal4-62 leu2-1 met8-1*) cross. Results clearly indicated that the suppressor is ineffective against the UAA and UAG mutations, as the Leu trait ($leu2\text{-}1^{UAA}/leu2\text{-}2^{UGA}$) showed 2+:2−, 1+:3−, and 0+:4− segregations, the Met trait ($met8\text{-}1^{UAG}/+$) showed a 2+:2− segregation, the Gal phenotype showed a 2+:2− segregation, and the His and Lys phenotypes showed 4+:0−, 3+:1−, and 2+:2− segregations in all seven asci tested. The dominant suppressor is not allelic with the *sug1* and *sug2* genes, since many Gal$^-$ spore clones were found in tetrad analyses of diploids prepared by the OG3-1DR-1 × G254-5C (**a** *gal4-62 sug1*) and OG3-1DR-1 × 371-2B (**a** *gal4-62 sug2*) crosses in five and eight asci dissected, respectively (data not shown). Hence, the dominant suppressor in OG3-1DR-1 is different from *sug1, sug2, SUP2* to *SUP8,* and *SUP11* and was thus tentatively designated *SUPU* (dominant suppressor for *um*ber nonsense codon). Since the *SUPU* suppressor is effective against the UGA nonsense mutations *his4-166, leu2-2,* and *lys2-101,* the *gal4-62* allele might be also a UGA nonsense mutation.

Isolation of similar suppressor mutations was also tried with OG7-2C. On inspection after 5 to 7 days of incubation at 30°C, galactose minimal plates supplemented with an appropriate amount of leucine (medium D) or methionine (medium E) showed no colonies. However, after 14 days of incubation tiny, slow-growing colonies appeared with very low frequencies as four and one colonies per 10^8 cells on the plates containing media D and E, respectively. We isolated one of them, OG7-2CR-1, for further analysis. When OG7-2CR-1 was crossed with OG7-4A (**a** *gal4-62 leu2-1 met8-1*), a sister clone of OG7-2C, the diploid showed the Gal$^-$ phenotype, the tetrad segregants from the diploid showed a 2+:2− segregation for the Gal, Leu, and Met traits, and all of the positive clones for one phenotype showed the positive phenotype for the other markers in 11 asci tested. Thus, the suppressor mutation in OG7-2CR-1 is a single recessive mutation effective against UAA, UAG, and probably UGA nonsense mutations. Since the diploid cells constructed by crosses between OG7-2CR-1 and G254-5C (**a** *gal4-62 sug1*) or G371-2B (**a** *gal4-62 sug2*) showed the Gal$^-$ phenotype, the recessive suppressor is different from *sug1* and *sug2*. During the course of the study,

we observed that the recessive suppressor is probably linked to *sup45*, as it showed parental ditype/nonparental ditype/tetratype tetrad ratios of 5:0:4 with *lys2* and 9:0:12 with *met8* on chromosome II. We therefore designated the suppressor *sup47*, as a member of the same family as the *sup45* suppressor (5; B. Ono, personal communication). The effects of the *sug1*, *sug2*, *sup47*, and *SUPU* suppressors on the *gal4-62* and *gal4-69* alleles for galactokinase synthesis are shown in Table 5. It is evident that *sug1* and *sug2* give rise to constitutive galactokinase synthesis in combination with the *gal4-62* and *gal4-69* alleles, whereas the *sup47* and *SUPU* suppressors give rise to an inducible phenotype with the *gal4-62* and *gal4-69* alleles. These facts strongly suggest that the amino acid inserted by *sug1* or *sug2* is different from that inserted by *sup47* or *SUPU*, if the suppressors are informational suppressors. The enzyme levels produced with the *gal4* mutant alleles acted on by the suppressors were lower than the fully induced levels of wild-type cells, as observed in the other systems, and the suppressors had no effect on the wild-type allele of the *gal4* gene (Table 5).

Semidominance of the *GAL81*, *GAL4-62.C*, and *GAL4-69.C* mutations over the various *gal4* alleles. In a previous communication (11), we presented evidence that the expression of the *gal4* gene is constitutive and not under the control of *gal80*; this evidence was from a kinetic study of the thermolabile *gal4* mutation. If this is true and if the active final products of *gal4* are built from a few identical subunits, the *GAL81*, *GAL4-62.C*, and *GAL4-69.C* mutants should be semidominant over the wild-type or mutant alleles of *gal4* in the constitutive expression of the structural gene(s). To test this possibility, we constructed diploids having a *GAL81* (or *GAL4-62.C* or *GAL4-69.C*)/*GAL4*$^+$ (or *gal4* mutation) genotype. The results (Table 6) clearly showed that the activity levels in the uninduced condition were always markedly lower in diploids bearing the wild-type allele of *gal4* than in the homozygous diploid, but that in the inducible condition they reached almost the same level as in the wild-type strain. When the constitutive alleles were combined with the *gal4-2* allele, which is suppressible by the UAA suppressors *SUP3* and *SUP11*, the diploids always showed a similar or higher level compared with the corresponding homozygous diploid in both uninduced and induced conditions. Several other *gal4* mutant alleles were studied in combination with the *GAL4-62.C1* allele. These diploids always showed significantly lower activity in both the uninduced and induced conditions than the homozygous diploid, although the activity levels varied depending on the *gal4* mutant allele. These observations strongly support the ideas that the *gal4* gene is expressed in the uninducible condition as well as in the inducible condition and that the primary products produced by the *gal4* gene aggregate to form an active cytoplasmic regulatory factor.

DISCUSSION

A fine structure map of the *gal4* locus was constructed by random spore analysis on the assumption that the appearance of Gal$^+$ colonies depends on meiotic recombination between two mutant *gal4* alleles. Although the method of map construction adopted here was not strictly the same as the mitotic and meiotic mapping methods described by Moore and Sherman (13), those authors claimed that map distances and sometimes allele orders are not necessarily proportional or otherwise simply related to physical distances measurable by the number of base pairs. With this claim in mind, we performed the *gal4* mapping at least three times independently and observed the same gene orders in all cases, with minor variations in the recombination frequencies. The data derived from one of the three independent experiments, along with some supplementary data (Tables 2 and 9), allowed us to locate *gal4* mutations in a single linear map (Fig. 1), which covers a region expressible by a recombination frequency of 0.44%. Four of the mutations (*gal4-2*, *gal4-76*, *gal4-77*, and *gal4-78*) were found to be suppressed by a UAA suppressor (*SUP3*), and two closely linked mutations (*gal4-62* and *gal4-69*) were suppressed by the newly isolated, dominant, presumed UGA suppressor *SUPU*, a recessive omnipotent suppressor (*sup47*), and two uncharacterized suppressors (*sug1* and *sug2*). Since these nonsense mutations were located at various sites on the map, it is possible to conclude that *gal4* codes for a protein molecule.

The *gal4-62* and *gal4-69* mutant alleles, which might occupy the same site on the map, are particularly interesting *gal4* mutations. They could revert to give the constitutive Gal$^+$ phenotype, presumably by a single secondary mutation. On the other hand, all of the *GAL81* mutations analyzed were located in a narrow region having a recombination frequency of at most 0.015% (Table 8). The *gal4-62* and *gal4-69* sites also appear to lie in the *GAL81* region, as *gal4-62* was mapped at the same site as *GAL81-12* and *GAL81-1* (Table 9). Thus, we may conclude that the *GAL81* region is located at the *gal4-62* and *gal4-69* sites and occupies not more than 4% of the total length of the *gal4* locus

(Fig. 1). The facts that the *GAL81* region lies inside the *gal4* locus and that the *gal4-62* and *gal4-69* alleles are probably UGA nonsense mutations support the view that the *GAL81* region is not an operator but codes for a portion of the *gal4* protein. This argument requires that the *gal4* gene is expressed constitutively rather than inducibly, as described in a previous communication (11). The fact that the constitutivities of the *GAL81* and *GAL4-62.C* (or *GAL4-69.C*) alleles were semidominant over the wild-type and *gal4* mutant alleles (Table 6) also supports the idea that *gal4* is expressed constitutively, if it is assumed that the *gal4* product has a subunit structure.

The above arguments indicate that a revision of the Douglas-Hawthorne model (4) is necessary. In the revised model, the expression of the *gal4* gene would be constitutive but not under the control of *gal80* and the inducer (galactose). The most simple mechanism to explain the observations is that the *GAL81* site codes for an affinity site in the *gal4* protein, the positive factor, for its interaction in the cytoplasm (or in the nucleoplasm) with the *gal80* product, the negative factor. The presence of inducer inhibits the negative factor and makes the positive factor effective for expression of the structural genes. Present data, however, do not tell us whether the negative and positive factors aggregate irrespective of the presence of inducer or are separated by the presence of inducer. The same conclusion was drawn from mating experiment data by Perlman and Hopper (15). Since preexisting *gal4* activity is directly blocked by the introduction of the *GAL80*^{s} product during zygote formation, these authors further suggested that the negative factor may be dissociated from the positive factor by the presence of inducer. Alternatively, it is possible that the presumptive promoter contiguous to the structural genes *gal7, gal10,* and *gal1* has two controlling sites, one of which has affinity for repressor (the *gal80* product) and the other of which has affinity for the positive factor produced by the *gal4* gene. Although we made several attempts to isolate mutations at the promoter, no positive results were obtained (14; unpublished data). This fact suggests a novel feature of the promoter, and the latter mechanism cannot be neglected with the present data, although Littlewood et al. (10) claimed that this mechanism is implausible from a similar observation in a regulatory system for phosphatase synthesis in *Neurospora crassa*. Hopper and Rowe (7), however, have shown that the mRNA's for the *gal1* and *gal7* genes, the two outside genes of the structural gene cluster, are distinctly different in migration behavior on polyacrylamide gel electrophoresis. If this difference were due to two distinct promoters in the cluster rather than to post-transcriptional processing, this would account for the observation that we could not isolate promoter mutations. In either mechanism, the *gal4* product should interact directly with the *gal80* product, because a specific, allelic relationship was observed between a dominant *GAL81* constitutive allele and a certain dominant superrepressible *GAL80*^{s} allele (14).

The essential difference between the Douglas-Hawthorne model and the revised one is that the former mechanism implies a protein-to-DNA interaction between *gal80* and *gal4* and the revised model implies a protein-to-protein interaction. The hierarchical relationship of the *gal80* and *gal4* genes deduced from the epistasis and hypostasis relationships seen in the genetic data is explained by a stepwise expression of the *gal4* and structural genes under the control of *gal80* and inducer in the Douglas-Hawthorne model and by the special spatial and steric relationships of the factors in the cytoplasmic aggregate or at the promoter(s) of the structural genes in the revised model.

The other system for regulation of enzyme synthesis under investigation in our laboratory is the system for repressible acid and alkaline phosphatases in *S. cerevisiae*. Derepression of both of the repressible enzymes occurs on depletion of inorganic phosphate in the medium. The syntheses of the two enzymes are regulated by a single, common genetic system (20, 23). Also involved are positive (the *phoB* and *phoD* products and possibly the *phoS* product) and negative (the *phoR* and *phoU* products) factors produced by regulatory genes (21–23, 25). Epistasis and hypostasis tests of these genes clearly indicated a cascade of molecular events in the transmission of signals caused by the presence or absence of inorganic phosphate in the sequence *phoS* → *phoR* and *phoU* → *phoD* and/or *phoB* and to the structural genes (*phoE* for repressible acid phosphatase [18, 22] and *phoH* for repressible alkaline phosphatase [unpublished data]). Our recent observations, however, suggest that the cascade of events is not due to the stepwise synthesis of the regulatory factors, but is probably due to the steric relationship of the factors (19; unpublished data).

In *N. crassa*, Metzenberg and his co-workers elucidated a similar genetic regulatory system for acid and alkaline phosphatases and the other activities related to phosphorus metabolism. These enzymes are derepressed by a reduction in the levels of inorganic phosphate in the medium and are repressed in high-phosphate me-

dium, as in the same enzyme systems in *S. cerevisiae*. Regulation of the syntheses of these enzymes is attributed to a genetic regulatory system composed of three genes, *nuc-2, preg,* and *nuc-1* (10). The functions of these genes are exerted in a definite sequence, in which the *nuc-1* product is the final and indispensable step in turning on the structural genes. The constitutive mutation at *preg* is epistatic to *nuc-2*, and the *nuc-2* mutation can block enzyme synthesis if the wild-type allele of *preg* is present. This description indicates that surprisingly similar mechanisms may function in the regulation of acid and alkaline phosphatase syntheses in *S. cerevisiae* and *N. crassa* and in the regulation of the galactose pathway enzymes, although no gene corresponding to *phoS* or *nuc-2* in the repressible phosphatase systems was identified in the *gal* system. According to the model proposed by Metzenberg and Nelson (12), the function of the regulatory genes for phosphatases in *N. crassa* does not involve stepwise synthesis of the regulatory factors, but it does involve cytoplasmic interaction of the factors; the normal product of the *nuc-1* gene (which seems to correspond to the product of the *phoD* and/or *phoB* gene or *gal4* in *S. cerevisiae*) is required to turn on the expression (transcription) of the structural genes. The normal product of the *preg* gene (corresponding to *phoR* and *phoU* or *gal80* in *S. cerevisiae*) is required to nullify the activity of *nuc-1* product. The normal product of the *nuc-2* gene (corresponding to *phoS* in *S. cerevisiae*) is required to nullify the activity of the *preg* product or prevent its synthesis in the absence of inorganic phosphate. Phosphate or a derivative of it nullifies the activities of the *nuc-2* product or prevents its synthesis. In this model, the constitutive synthesis of the enzymes is due to the quantitative, but not qualitative, modification of the regulatory factors. For example, the constitutive $nuc\text{-}1^c$ mutation was explained by an elevated amount of the normal *nuc-1* product that exceeds the stoichiometric amount of the $preg^+$ product.

Since all of the *gal4* mutants described in this communication occurred in the region coding for the structure of the *gal4* protein, as evidenced by the fact that the nonsense mutations were scattered over the map region, the explanation offered by Metzenberg and Nelson (12) might not hold in the present case. They observed different levels of enzyme activities in response to different gene dosages at the *nuc-2* and *preg* loci with the mutant alleles. This observation is analogous to the present observation that the *GAL81, GAL4-62.C,* and *GAL4-69.C* alleles were semidominant over the wild-type and *gal4* mutant alleles (Table 6), which we interpreted by supposing a subunit structure for the *gal4* factor, because the nonsense *gal4* allele *gal4-2* did not show the deleterious effect on the enzyme level shown by the constitutive mutant alleles. To explain a similar phenomenon involving the positive regulatory gene of quinic acid catabolism in *N. crassa* (*qa-1*), Valone et al. (26) suggested that the regulatory product of the gene is a multimeric protein capable of producing hybrids with homologous mutant proteins. Confirmation of these arguments remains for future study.

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