Mol Gen Genet (1983) 191:31–38

MGG

Regulation of Expression of the Galactose Gene Cluster in *Saccharomyces cerevisiae*

Isolation and Characterization of the Regulatory Gene *GAL4*

Hideaki Hashimoto*, Yoshiko Kikuchi, Yasuhisa Nogi, and Toshio Fukasawa
Laboratory of Molecular Genetics, Keio University School of Medicine, Shinjuku, Tokyo 160, Japan

Summary. The *GAL4* gene positively regulating the expression of the gene cluster *GAL7-GAL10-GAL1* in the yeast *Saccharomyces cerevisiae* was isolated for its ability to suppress a recessive mutation in that gene. When the isolated gene was incorporated into a multi-copy plasmid, the *GAL* cluster genes in the host chromosome partially escaped the normal control; a yeast that harbors the plasmid bearing the *GAL4* gene synthesized the galactose-metabolizing enzymes encoded by the *GAL* cluster genes at a low but significant level in the absence of galactose. If the *GAL7* gene was amplified along with *GAL4* on the multi-copy plasmid, the constitutive synthesis of Gal-1-P uridylyl transferase encoded by *GAL7* was further pronounced and the enzyme activity reached the level of the fully induced wild-type yeast. Such an escape synthesis of the *GAL* enzymes was not detected if *GAL4* or both *GAL4* and *GAL7* were carried by a single-copy plasmid. The results suggest that the escape synthesis of *GAL* enzymes observed in the *GAL4*-amplified yeast was a consequence of overproduction of the *GAL4* protein. The *GAL80* gene negatively regulating the *GAL* cluster genes was also isolated, and when amplified together with *GAL4*, no escape synthesis of the *GAL* enzymes was observed, suggesting that the balanced synthesis of two regulatory proteins was essential to maintain the repressed state of the *GAL* cluster genes.

Introduction

The genes *GAL1*, *GAL7*, and *GAL10* respectively coding for galactokinase, Gal-1-P uridylyl transferase, and UDPG-4-epimerase in the galactose utilization pathway of the eucaryotic microorganism *Saccharomyces cerevisiae* are localized within a narrow region on chromosome II to form a gene cluster (*GAL* cluster; Douglas and Hawthorne 1964; Bassel and Mortimer 1971; St. John and Davis 1981). Those enzymes are typically inducible and the induced syntheses are coordinative (Douglas and Hawthorne 1964; Broach 1979). Yet, recent experiments (St. John et al. 1981; Segawa and Fukasawa 1980) strongly suggest that each gene of the *GAL* cluster constitutes a separate transcriptional unit, unlike the genes of the gal operon in *Escherichia coli* which are transcribed as a unit to yield the polycistronic mRNA (Buttin 1963). Expression of the yeast *GAL* cluster is known to be regulated by a complex regulatory mechanism involving three unlinked genes, namely *GAL4*, *GAL80* (Douglas and Hawthorne 1966) and *GAL11* (Nogi and Fukasawa 1980), of which the first two are believed to play the primary role. The *GAL4* gene codes for a positive regulatory factor which is required to promote the transcription of the genes in the *GAL* cluster (St. John and Davis 1979, 1981). The *GAL80* gene (originally called *GALi*) codes for a negative regulatory factor which is necessary to maintain the repressed state of the *GAL* cluster in the absence of galactose. These regulatory factors are presumed to be proteins because temperature-sensitive mutations and suppressible nonsense mutations are known to occur in both genes (Douglas and Hawthorne 1972; Klar and Halvorson 1974; Matsumoto et al. 1978). As the first step to elucidate the molecular nature of the regulatory machinery, we have isolated those two regulatory genes by complementation of recessive mutations occurring in the respective genes. In this paper, we present experiments concerning the dosage effect of the *GAL4* gene on the expression of the *GAL* cluster by making use of the multi-copy vector or the single-copy vector. The results indicate that the *GAL* cluster genes partially escape the normal control if the gene copy of *GAL4* is amplified in a cell. Such an escape synthesis was remarkably enhanced, if a gene of *GAL* cluster, i.e. *GAL7*, was amplified along with *GAL4*: Gal-1-P uridylyl transferase, the enzyme encoded by *GAL7* was constitutively synthesized in a yeast bearing a multi-copy plasmid with both genes to the level of a fully induced, wild-type yeast. We further suggest that the escape synthesis caused by the amplified *GAL4* is a consequence of the imbalance of the copy number of the regulatory genes *GAL4* and *GAL80* because the effect of amplification of the *GAL4* gene was completely suppressed by the concomitant amplification of the isolated *GAL80* (Nogi et al., in preparation). In the light of these results, possible models for the regulatory mechanism are discussed. During the course of the preparation of this manuscript, two independent groups have reported the successful isolation and the characterization of *GAL4* (Johnston and Hopper 1982; Laughon and Gesteland 1982).

Materials and Methods

Strains, Recombinant Plasmids and Gene Library. The *E. coli* K12 strains JA221 (*recA hsdM hsdR leuB trpΔE*

* On a leave absence from Nikka Whisky Co.

Offprint requests to: T. Fukasawa

Table 1. Yeast strains and recombinant plasmids used

1. Yeast strains

Designation	Genotype	Source/Reference
YK3	*a trp1 his3*-532	A Gal$^+$ revertant from D13-1A
YK4-4	*α gal7 trp1*	N492-2A × YK3
C5014-2B	*a ura3 arg4 his1 hom3*	T. Takahashi
YK5-6B	*α gal4 trp1 his3 leu2 ade*	NM1-6C × YK3
YH2-9D	*α gal7 trp1 his3*	YK3 × YK4-4
YH5-2A	*α gal4 trp1 ura3 arg4 leu2 ade his*	YK5-6B × C5014-2B
D13-1A	*a his3*-532 *trp1 gal2 SUC2 CUP1 mal*	Struhl et al. (1979)
N492-2A	*α gal7 trp1 ura*	Our stock
MC16	*α ade2 leu2 his4 SUF2*	Beggs (1978)
278-1B	*a gal4 his1 ura1 ade6 trp1*	Douglas and Hawthorne (1964)
8MC16-1D	*a gal4 leu2-3 his*	MC16 × 278-1B
NM1-6C	*α gal4 leu2 his ade*	MC16 × 8MC16-1D

2. Recombinant plasmids[a]

Designation	Type of vectors	Yeast genes contained	Orginal recombinant DNA of component fragments
pYH3002	YEp	*GAL7 HIS3*	*λgt1Sc481* (St. John and Davis 1979), pYC1 (B. Hohn)
pYH3003	YEp	*GAL7 HIS3*	*λgt1Sc481*, pYC1
pYH3009	YCp	*GAL7 CEN4 TRP1 URA3*	*λgt1Sc481*, YCp19 (Stinchcomb et al. 1982)
pHG41	YEp	*GAL4 LEU2*	This study
pYH3010	YIp	*GAL4 LEU2*	pHG41, YIp32 (Botstein et al. 1979)
pYH3012	YEp	*GAL4 LEU2*	pHG41, YEp13 (Broach et al. 1979)
pYH3013	YEp	*GAL4 HIS3*	pHG41, pYC1
pYH3018	YEp	*GAL4 HIS3*	pHG41, pYC1
pYH3019	YEp	*GAL4 LEU2*	pHG41, YEp13
pYH3021	YEp	*GAL4 GAL7 HIS3*	pHG41, *λgt1Sc481*, pYC1
pYH3025	YCp	*GAL4 GAL7 CEN4 TRP1 URA3*	pHG41, *λgt1Sc481*, YCp19
pYH3026	YCp	*GAL4 CEN4 TRP1 URA3*	pHG41, YCp19
pYH3028	YRp	*GAL4 ARS1 TRI1 URA3*	pHG41, YRp16 (Stinchcomb et al. 1982)
pYH3035	YIp	*GAL4$^{\Delta}$ LEU2*	pHG41, YIp32
pYH3036	YEp	*GAL4 URA3*	pHG41, YEp24 (Botstein et al. 1979)
pYH3038	YEp	*GAL4$^{\Delta}$ HIS3*	pHG41, pYC1
pYH3039	YEp	*GAL4$^{\Delta}$ HIS3*	pHG41, pYC1
pYH3040	YEp	*GAL4$^{\Delta}$ HIS3*	pHG41, pYC1
pYH3032	YEp	*GAL4 GAL80 URA3*	pKK8001 (Nogi et al., in preparation), pHG41

[a] All the plasmids contain the pBR322 sequence, in which the *tet^r* gene is either replaced or inactivated

lacY) or LE392 (*metB hsdR supE supF gal lac trpR*) were used to prepare the recombinant plasmids. The yeast strains and the recombinant plasmids used are described in Table 1. A pool of recombinant plasmids that would contain 99% of the yeast genome was prepared by Mr. Susumu Okamoto of the University of Tokyo with YEp13 (Broach et al. 1979) as a vector and *S. cerevisiae* strain MT13 (*HO/HO HMRa/HNRa HMLα/HNLα ura3 lys1/lys1 his4/his4*) as a source of DNA: A partial *Sau*3A digest of MT13 DNA with an average size of 7.6 kb was ligated to the vector at a *Bam*HI site. The mixture of recombinant DNA was transferred to an *E. coli* K12 strain. About 3×10^4 ampicilin-resistant colonies obtained were pooled, from which plasmid DNA was purified. In 10 μl DNA solution 10^4–10^5 Leu$^+$ transformant colonies were obtained by the conventional method (Hinnen et al. 1978).

Media and Enzymes. To grow bacterial strains, L-broth (0.5% yeast extract, 1% Bacto tryptone, 1% NaCl) was used; plates included 1.5% agar and when necessary, 20 μg ampicilin/ml. YPD (2% Polypeptone, 1% yeast extract, 2% glucose, and 2% agar for plates) was used as the complete medium for yeast strains. Synthetic minimal media contained 0.7% yeast nitrogen base w/o amino acids (Difco, Detroit, USA), an appropriate amount of required amino acids or nucleic acid bases, and 2% sugar (glucose for SD, galactose for Sgal, and sucrose for Ssuc). EBGal plates contained 0.002% ethidium bromide, 2% peptone, 1% yeast extract, 2% galactose, and 2% agar. Restriction enzymes were obtained through commercial sources (Takarashuzo Co., Kyoto, Japan; Bethesda Research Laboratories Inc., Maryland, USA, or Boehringer-Mannheim, FRG). T4 DNA ligase and *E. coli* DNA polymerase were purchased from Takarashuzo Co.. Bovine pancreatic deoxyribonuclease was obtained from Worthington Biochemical Corp., Freehold, USA.

Preparation of DNA. Preparation of total DNA from yeast was carried out according to Davis et al. (1979) for recovery of the plasmid and according to Cryer et al. (1975) for determination of the plasmid copy number. The plasmid DNA was prepared by alkaline extraction (Birnboim and Doly 1979) from a plasmid-harboring *E. coli* strain grown in L-broth containing 20 μg ampicilin/ml.

Preparation of Crude Extract and Assay of the Galactose Enzymes. Cells were grown to a mid-exponential phase in either Sgal or Ssuc, collected by centrifugation, and disrupted by grinding with paste of alumina in an ice-cold mortar. Clear supernatant samples obtained by centrifugation of the disrupted cell samples at $18{,}000 \times g$ for 20 min in the cold were used for the galactose enzyme assay as described in Nogi and Fukasawa (1980).

As the carbon source for preparation of uninduced cells, sucrose was used instead of glycerol in the present work. The latter has been most commonly used in the regulation study for the absence of catabolite repression. However, glycerol is a poor carbon source in most minimal media. We had to use a minimal medium to avoid the segregational loss of a recombinant plasmid during the cell growth. Therefore we used sucrose as a compromise, because this sugar is not only an efficient carbon source in the minimal medium but exhibits a less repressional effect than glucose on the *GAL* cluster expression. In a preliminary experiment, a constitutive yeast (*gal80*) grown in Ssuc or in SD exhibited a galactokinase activity 75% or 2%, respectively, of the enzyme activity in the same yeast grown in Sgal.

Determination of the Plasmid Copy Number. Restriction endonuclease-cleaved total DNA was fractionated by horizontal agarose gel (1%) electrophoresis with 40 mM Tris-HCl buffer (pH 8.0) containing 5 mM sodium acetate and 1 mM Na_3EDTA, and the DNA fragments were transferred to nitrocellulose membrane by minor modifications of the procedures described by Southern (1975). As a probe DNA, the plasmid YIp1 (Botstein et al. 1979) carrying the *HIS3* gene was nick-translated with minor modifications according to Rigby et al. (1974) with ^{32}P-[α]-dATP (Ammersham, England, 3,000 Ci/mmol). The DNA immobilized on the membrane was hybridized with the ^{32}P-DNA probe at 42° C for 18 h in 0.9 M NaCl, 50 mM sodium phosphate (pH 7.0), 5 mM Na_2EDTA, 0.3% SDS, 100 µg/ml denatured fragmented salmon testis DNA, and 50% (v/v) deionized formamide. The plasmid and the chromosomal gene in the immobilized DNA were located by autoradiography and the corresponding bands were cut out from the nitrocellulose membrane. The radioactivity of the respective piece of membrane was determined by the liquid scintillation counter. The copy number of the plasmid per cell was estimated from the ratio of the radioactivity bound to the plasmid to the radioactivity bound to the chromosomal gene, after normalization of the size difference between the pBR322 and the *HIS*3-sequences.

Results

Isolation of a Plasmid Capable of Complementing a gal4 Mutation

Recessive mutations in the *GAL4* gene render the yeast galactose nonfermenting due to noninducibility of the *GAL* cluster (Douglas and Hawthorne 1966; St. John and Davis 1979). To isolate the *GAL4* gene, we searched for a recombinant plasmid that would convert a *gal4* mutant yeast to Gal^+ in a pool of recombinant molecules generated with the plasmid YEp13. The vector consists of pBR322, a part of 2 µm DNA sequence, and a yeast chromosomal fragment covering the *LEU2* gene. Thus the composite plasmid will replicate either in an *E. coli* cell conferring ampicilin and tetracycline-resistance, or in a yeast conferring the Leu^+ character if the yeast bears a *leu2* mutation. Screening of the desired plasmid was carried out through two steps for the following reason: We found that the Gal^+ character could not be used directly as the selection marker for the transformants because none of the yeast strains we tested will grow when embedded in the agar medium containing galactose as the sole carbon source.

At first, the yeast strain YK5-6B (*gal4*, *leu2*) was transformed with the pool of recombinant plasmids to select for Leu^+ colonies. About 10^4 Leu^+ colonies appeared on each of nine transformation plates. Transformant cells were then collected from the top agar which was crushed by passage through a stainless sieve. The Leu^+ cells from three plates were pooled and aliquots of each pool were spread on Sgal plates. All the plates yielded many Gal^+ colonies, from which four colonies were saved for further analysis. Total DNA was extracted from the chosen clone and used for transformation of *E. coli* strains. From the resultant ampicilin-resistant colonies, plasmids were extracted and transferred back to the strain YK5-6B. Transformants were selected as Leu^+ and then checked for Gal^+ on EBGal plates. Out of 22 independent plasmids tested, 17 yielded Leu^+ GAL^+ transformants. Those transformants segregated Leu^- Gal^- colonies at a frequency of about 20% of the total population after ten generations of growth in YPD. One of the plasmids, designated pHG41, was used for further analysis.

Localization of the DNA Fragment Capable of Complementing a gal4 Mutation on the Isolated Plasmid

To locate on the isolated plasmid (pHG41) the gene that will complement the *gal4* mutation, fragments generated by digestion with various restriction enzymes were fractionated and each fragment was inserted into an appropriate vector, as shown in Fig. 1. The recombinant plasmids were transferred to an appropriate recipient yeast bearing *gal4* and an additional marker, such as *leu2* or *his3*. Transformants were selected at first for the amino acid markers and then checked for galactose-utilization on EBGal plates. The results shown in Fig. 1 indicate an approximate localization of the gene in question within a 3.1 kb-fragment in between *Bam*HI and *Xba*I sites on the inserted DNA.

The Cloned Gene is Allelic to GAL4

If the *gal4*-complementing DNA fragment we isolated was in fact the *GAL4* gene itself, the DNA fragment should be integrated into the yeast chromosome at the *GAL4* locus by the homology. To test this idea, we first fractionated the 5.7 kb-fragment in between two *Hin*dIII sites from pHG41 (Fig. 1) and inserted it into an integrative vector YIp32 to generate pYH3010. The recombinant plasmid was then transferred to the *gal4* mutant (YK5-6B) and Leu^+ transformants were selected. All the Leu^+ colonies were Gal^+. Both phenotypes were stably inherited during mitotic growth in nonselecting conditions for many generations, suggesting integration of the recombinant molecule into a nuclear chromosome. Each of four independent transformants or the recipient strain YK5-6B was crossed with a Gal^+ Leu^+ strain (C5014-2B) and the resultant diploid strains were sporulated for tetrad analysis. If the DNA fragment capable of complementing *gal4* could be integrated

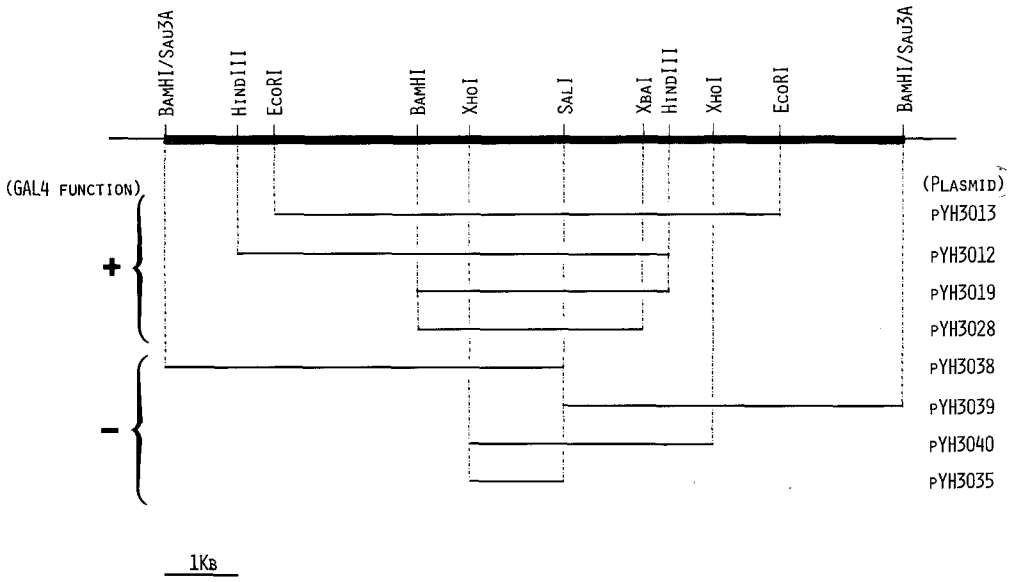

Fig. 1. Localization of the *GAL4* gene on the cloned DNA: The thick line in the upper part of the figure represents the inserted DNA in the isolated recombinant plasmid pHG41, and the thin line flanking the insert, the vector sequence. The vertical lines on the insert indicate the approximate positions of the cleavage sites of the indicated restriction enzymes. From the pHG41 DNA, the fragments shown as the thin lines under the insert were subcloned into appropriate vectors to yield the recombinant molecules whose designations are shown on the right (see also Table 1). The respective plasmid was introduced into a *gal4* mutant and studied for the *GAL4* character. Those plasmids that suppress the *gal4* phenotype are indicated by (+) while those who will not do so by (−) on the left

Table 2. Allelism test of the cloned gene

Gene	Cross[a]				
	I	II-1	II-2	II-3	II-4
GAL4	$30^+/28^-$	$60^+/5^-$[b]	$80^+/0^-$	$102^+/0^-$	$71^+/1^-$
LEU2	$30^+/28^-$	$45^+/20^-$	$58^+/22^-$	$73^+/29^-$	$52^+/20^-$
URA3	$26^+/32^-$	$27^+/38^-$	$35^+/45^-$	$44^+/58^-$	$32^+/40^-$
HOM3	$32^+/26^-$	$32^+/33^-$	$41^+/39^-$	$54^+/48^-$	$39^+/33^-$

[a] Cross I: C5014-2B/YK5-6B (*a ura3 arg4 hom3 his1*/α *gal4 his3 trp1 leu2*), Cross II-1 ~ 4: C5014-2B/YK5-6B [pYH3010]

[b] Four out of five Gal^- colonies were Leu^-, suggesting that the recombinant plasmid was lost in those segregants

into the chromosome at a locus other than *GAL4* in those transformants, Gal^- spores should be found among the segregants. In contrast, Gal^- spores should rarely be segregated out if the DNA fragment was integrated at the *GAL4* locus. The data are presented as a random spore analysis in Table 2 since less than 10% of the dissected asci yielded the complete tetrad in all the crosses. As is clear in the Table only a few Gal^- colonies were found among the segregants tested, which were derived from the diploid strains constructed by mating the transformants with the tester strain (Crosses II-1 ~ II-4). In contrast, Leu^- segregants were found in the same crosses at a ratio of approximately $3^+:1^-$; the result indicating that the *LEU2* gene was integrated at a locus other than the normal site, presumably near the *GAL4* locus in the transformants. The other markers tested (*URA3*, *HOM3*) segregated at a ratio of $1^+:1^-$ among those spore clones. In the control experiments (Cross I), all the markers segregated at a ratio of $1^+:1^-$. From these results, we conclude that the gene cloned into pHG41 was allelic to *GAL4*.

Gene Dosage Effect of GAL4 on the Expression of the GAL Cluster

Effect in the Uninduced State. When the fragment containing the *GAL4* gene was subcloned into a multi-copy vector (YEp; Botstein et al. 1979) and transferred into a yeast, we found that the host genes in the *GAL* cluster partially escaped the normal control and synthesized low levels of the *GAL* enzymes under the uninduced conditions (Table 3A). Thus all three enzymes for galactose metabolism were detectable even when the yeast was grown in sucrose medium as shown in Table 3A. Such an escape synthesis was not observed if the *GAL4* DNA was inserted into a single-copy vector designated YCp (Clarke and Carbon 1980; Stinchcomb et al. 1982). The copy number of the plasmid was directly determined in the plasmid-bearing cells grown under conditions similar to the experiment of Table 3: Total DNA was extracted from the exponential phase culture of pYH3036 or pYH3026-bearing yeast and analyzed by the procedures described in Materials and Methods. The result indicated that each haploid cell contains at least 14 copies of the YEp plasmid or approximately one copy of the YCp plasmid (Figs. 2, 3). Therefore the escape synthesis observed in the YEp-*GAL4* plasmid carriers may be attributed to the increased copy number of the *GAL4* gene.

In Table 3B, is shown the dosage effect of *GAL7* studied by a similar method: Gal-1-P uridylyl transferase encoded by *GAL7* on the multi-copy vector was synthesized at a low but significant level in the uninduced condition. The enzyme synthesis was unambiguously seen irrespective of the direction of insertion of the *GAL7* DNA into the vector, suggesting that the *GAL7* gene was transcribed from its own promoter. This enzyme synthesis was independent of the *GAL4* function because a similar level of enzyme activity

Table 3. Uninduced levels of *GAL* enzymes in yeast strains harboring various recombinant plasmids[a]

	Recombinant plasmid	Vector type	Host genotype[b]	Yeast genes on plasmid	Galacto-kinase[c]	Gal-1-P Uridylyl transferase[c]	UDPG-4 -epimerase[c]
A)	pYH3018	YEp	*gal4*	*GAL4*	0.09	0.23	0.19
	pYH3026	YCp	*gal4*	*GAL4*	<0.005	<0.01	<0.01
B)	pYH3002[d]	YEp	*gal7*	*GAL7*	<0.005	0.13	<0.01
	pYH3003[d]	YEp	*gal7*	*GAL7*	<0.005	0.14	<0.01
	pYH3009	YCp	*gal7*	*GAL7*	<0.005	<0.01	<0.01
C)	pYH3021	YEp	*gal4*	*GAL7·GAL4*	0.05	32.8	0.08
	pYH3025	YCp	*gal4*	*GAL7·GAL4*	<0.005	<0.01	<0.01

[a] Cells were grown to exponential phase in a synthetic medium containing sucrose as a carbon source and the nutrients required by the host yeast except histidine or tryptophan which was omitted to select for the carriers of the YEp-plasmid or of the YCp-plasmids, respectively

[b] The strains used were YK5-6B (α *gal4*, *trp1*, *his3*, *leu2*, *ade*) for the experiments of (a) and (b) or YH2-9D (α *gal7*, *trp1*, *his3*) for the experiments of (b)

[c] The enzyme activity was expressed as units per mg protein. One unit is defined as the amount of the enzyme that will convert one μmol of the respective substrate per h at 30° C

[d] The orientation of the *GAL7* sequence is different between pYH3002 and pYH3003

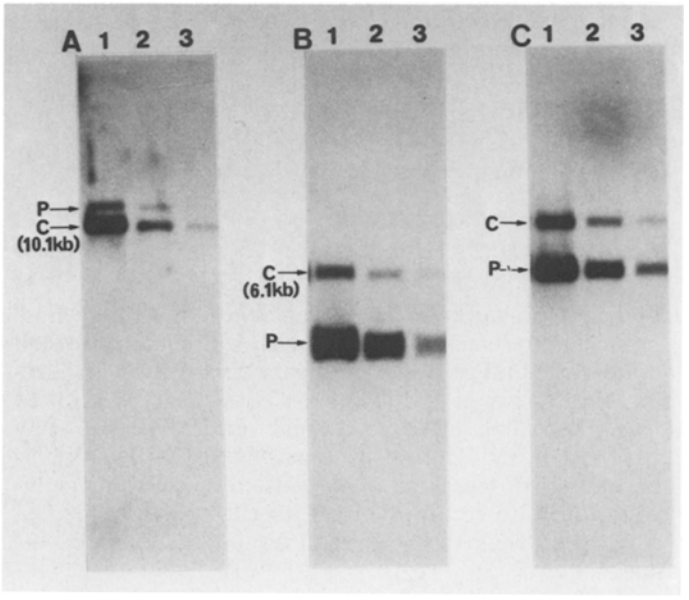

Fig. 2A–C. Determination of plasmid copy number in a cell: Total DNA extracted from a yeast bearing pYH3026 (**A**), pYH3036 (**B**) or pYH3032 (**C**) was digested with *Eco*RI for (**A** and **C**) or *Eco*RI and *Sal*I (**B**). The original digest of about 1 μg DNA (1), its threefold dilution (2), or ninefold dilution were fractionated by agarose gel electrophoresis and transferred to a nitrocellulose membrane. The bands of the plasmid (P) and *HIS3* (**C**) were located by hybridization with ^{32}P-labeled YIp1 DNA with a specific radioactivity of 7×10^8 dpm/μg. The plasmid contains the pBR322 sequence in which the 0.65 kb-fragment between *Eco*RI and *Sal*I was replaced with the yeast *HIS3* sequence (6.1 kb) flanked by these restriction sites (Botstein et al. 1979). The bands were cut out from the membrane and their radioactivities were determined in the liquid scintillation counter. The ratio of the radioactivity in the plasmid band to the radioactivity in the *HIS3* band was multiplied by a factor of 1.64 due to the size difference between the pBR322 sequence and the *HIS3* sequence in the probe DNA. The values obtained were 0.73, 14.1, and 6.6 for **A**, **B**, and **C**, respectively. The restriction maps of the recombinant plasmids used in these experiments are shown schematically in Fig. 3

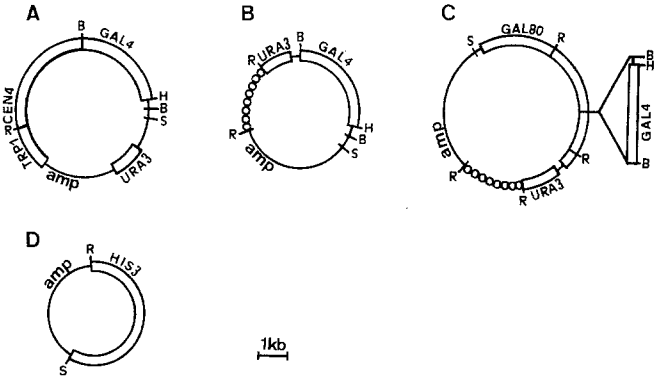

Fig. 3A–D. Organization of the recombinant plasmids used in the experiment of Fig. 2. **A** pYH3026; **B** pYH3036; **C** pYH3032; **D** YIp1 (Botstein et al. 1979). —— Represents pBR322DNA; ▭ represents yeast DNA; oooo represents 2 μ plasmid DNA. Some restriction endonuclease sites are indicated as follows: R = *Eco*RI, S = *Sal*I, B = *Bam*HI, H = *Hin*dIII. *CEN4* (the centromere sequence of chromosome IV) and the genes shown outside the plasmids roughly indicate their locations. The plasmid pYH3032 was constructed by inserting the *GAL4* sequence into pKK8001 bearing the *GAL80* gene (Nogi et al. in preparation)

was observed in a *gal4* mutant (Data not shown). We suggest that the *GAL7* gene and presumably *GAL1* or *GAL10* as well, is transcribed at a low rate without the normal *GAL4* protein, which could be visualized by the gene amplification.

If *GAL4* was inserted in addition to the *GAL7*-bearing plasmid, the constitutive synthesis of transferase was further pronounced, as shown in Table 3C. The implications of these results will be discussed below.

Effect in the Induced State. The dosage effect of the *GAL4* gene was also observed under the induced conditions, as shown in Table 4A. Thus a yeast strain carrying amplified *GAL4* copies exhibited two to threefold higher activity of

Table 4. Induced levels of *GAL* enzymes in yeast strains harboring various recombinant plasmids[a]

	Recombinant plasmid	Vector type	Host genotype	Yeast genes on plasmid	Galactokinase	Gal-1-P Uridylyl transferase	UDPG-4 -epimerase
A)	pYH3018	YEp	*gal4*	*GAL4*	20.5	58.6	18.1
	pYH3026	YCp	*gal4*	*GAL4*	8.5	17.3	5.8
B)	pYH3002	YEp	*gal7*	*GAL7*	63.1	229.5	24.8
	pYH3003	YEp	*gal7*	*GAL7*	55.1	203.7	25.1
	pYH3009	YCp	*gal7*	*GAL7*	62.5	47.7	36.5
C)	pYH3021	YEp	*gal4*	*GAL7·GAL4*	33.5	314.0	21.1
	pYH3025	YCp	*gal4*	*GAL7·GAL4*	14.4	67.2	12.2

[a] The experimental conditions were the same as in Table 3 except that galactose was used in place of sucrose as a carbon source in the growth medium. For other information see Table 3

Table 5. Dosage effect of *GAL80* and *GAL4*[a]

Recombinant plasmid	Yeast genes on plasmid	Galactokinase	Gal-1-P uridylyl transferase	UDPG-4--epimerase
A)				
pYH3036	*GAL4*	0.08	0.14	0.17
pYH3032	*GAL4·GAL80*	<0.005	<0.01	<0.01
B)				
pYH3036	*GAL4*	38.4	29.3	35.0
pYH3032	*GAL4·GAL80*	21.8	22.8	16.0

[a] Cells of the yeast strain YH5-2A (α *gal4 trp1 ura3 arg4 leu2 ade his*) were grown to exponential phase in a synthetic medium containing the required nutrients except uracil, and sucrose (A) or galactose (B) as a carbon source. For other information see Table 3

the *GAL* enzymes compared to that of the strain with a single gene copy of *GAL4*. The result suggested that the amount of *GAL4* protein would normally be a rate-limiting factor for the upper limit of the *GAL* cluster expression.

Table 4B demonstrates induced levels of the *GAL* enzymes synthesized either in a yeast with multiple copies of the *GAL7* gene or in a yeast with a single copy of that gene: The former strain synthesized four to fivefold more Gal-1-P uridylyl transferase than the latter strain. The increase in transferase activity was presumably due to the amplification of the *GAL7* gene. A similar situation was observed also in yeast strains carrying the *GAL4* and *GAL7* genes on a plasmid. Thus the yeast carrying both genes on the multi-copy plasmid synthesized more Gal-1-P uridylyl transferase than the yeast with those genes on the single-copy plasmid by a factor of about four (Table 4C).

Dosage Effect of the GAL80 Gene on the GAL4-Induced Escape Synthesis

Recently, we have isolated a yeast DNA fragment containing the other regulatory gene *GAL80* (Nogi et al. in preparation). Then we constructed a plasmid containing both *GAL80* and *GAL4* genes on a YEp-type vector (pYH3032; Fig. 3). The recombinant plasmid was transferred into a *gal4* yeast and the activity of the *GAL* enzymes was assayed compared to that of the yeast carrying the *GAL4*-bearing plasmid (pYH3036). As is clear from Table 5, constitutive synthesis of *GAL* enzymes in the *GAL4*-amplified yeast was completely suppressed if the *GAL80* gene was also amplified by the same vector. The result strongly suggests that the *GAL4*-induced escape synthesis is a consequence of the unbalanced copy number of two regulatory genes in a cell. The copy number of the pYH3036 was also determined as shown in Fig. 2. Although the value obtained was two times smaller than that of pYH3032, this would not affect the conclusion, because Johnston and Hopper (1982) have observed the escape synthesis in a yeast bearing YEp-*GAL4* whose copy number was estimated to be five to seven per cell.

Discussion

In the present work, we have isolated the *GAL4* gene, whose product is presumed to play a primary role in the expression of the structural genes for three enzymes involved in galactose metabolism in *Saccharomyces cerevisiae*. The assorted use of two kinds of stable plasmid vector, the multi-copy type (YEp) and single-copy type (YCp), has enabled us to study the dosage effect of the isolated gene without ambiguity; the YEp vector offers a yeast carrying the amplified *GAL4* gene, while the YCp vector furnishes a control experiment.

We have demonstrated that the amplification of *GAL4* in a cell leads to a partial escape of the *GAL* cluster expression from the normal repression. Similar experiments have been performed to obtain essentially the same results by Johnston and Hopper (1982). We have further shown that such an escape expression is completely suppressed by concomitant amplification of the other gene *GAL80*, whose product would negatively regulate the transcription of the *GAL* cluster. These results strongly suggest that a balance of two regulatory proteins in the cellular concentration is essential to maintain the repressed state of the *GAL* cluster genes. What the molecular nature of the *GAL4-GAL80* regulation could be is discussed below.

The recent findings by Perlman and Hopper (1979) and Matsumoto et al. (1980) have necessitated revision of the old model conceived by Douglas and Hawthorne (1966) who originally identified *GAL80* and *GAL4* as the regulatory genes for the *GAL* cluster. The latter workers thought that the *GAL80* product was the repressor of the *GAL4*

gene and that the *GAL4* product was the activator of the *GAL* cluster genes. Thus the *GAL80* product was presumed to act at the operator region of the *GAL4* gene and to be inactivated by the inducer. If this were the case, the *GAL4* gene should be expressed only in the presence of galactose. Instead, the experiments by the former workers strongly suggested that the *GAL4* product should be synthesized in the absence of galactose. (In fact, Laughon and Gesteland (1982) have directly demonstrated that the *GAL4* gene is transcribed constitutively.) Thus the revised model assumes that the *GAL80* product would hamper the action of the *GAL4* product itself under the uninduced conditions (see Perlman and Hopper 1979). Since both gene products are believed to be proteins, one can imagine that the two regulatory proteins form an inactive complex in the uninduced state and that the inducer binds the complex resulting in its dissociation to release the active *GAL4* protein. One of the features of this model would be that *GAL80* exhibits no direct interaction with the target gene. This hypothesis is called the 'Protein-protein interaction model' and referred to as Model A below.

Since no substantial evidence for the protein-protein interaction has been available, another model would also be possible which assumes that both regulatory proteins interact with the target gene at the control region (Guarente et al. 1982) and that the bindings of those proteins are mutually exclusive: In the uninduced state, the *GAL80* protein binds the control region, which would interfere with the binding of the *GAL4* protein. Upon induction, the inducer inactivates the *GAL80* protein resulting in its release from the control sequence, which in turn, allows the binding of the *GAL4* protein. It can be speculated that two binding sites on the control region are partially overlapping, or that the binding of one regulatory protein results in an alteration of the local chromatin structure, which hinders the binding of the other. This model is called Model B below.

Both of the above models assume that the promoters of the *GAL* cluster genes are inefficient unless the *GAL4* product binds their control regions. Thus either model would successfully explain the previous findings. For example, the dominant constitutive mutant *GAL4*^{c} (Douglas and Hawthorne 1966) would be assumed to produce an altered *GAL4* protein that is defective in binding to the *GAL80* protein in Model A. In Model B, the mutant protein would be assumed to exhibit a stronger affinity to the control sequence, so that *GAL80* fails to bind to it.

Model A would explain our experiments as follows: An excess amount of the *GAL4* protein in the *GAL4* gene-amplified yeast would titrate out the *GAL80* protein to give rise to free *GAL4* molecules which in turn, activate the transcription of the *GAL* cluster genes. This model however would not readily explain why the escape expression of the *GAL* cluster is partial. Based on Model B, we can explain the same results by assuming that an increased concentration of the *GAL4* protein in a cell leads to an increased probability in its binding to the control region to a limited extent. This model would adequately explain the fact that the escape is partial.

The third model has been proposed by Johnston and Hopper (1982) based on their most recent experiments: They have shown that different allelic states of *GAL80*, namely *GAL80*^{s} (dominant uninducible mutation; Douglas and Hawthorne 1972) and *gal80* (recessive constitutive mutation; Douglas and Hawthorne 1966), yield qualitatively different responses of the *GAL7* and *MEL1* genes in the *GAL4*-amplified yeast grown on glucose; the expression of *MEL1* is also known to be under the control of *GAL80* and *GAL4* (Kew and Douglas 1976). They have interpreted this result to mean that the *GAL80* product, mediating the signal of glucose repression, interacts directly with the regulatory regions of the respective genes with different affinities. From these considerations, they have hypothesized that both regulatory proteins bind the respective sites *as a complex* in the uninduced conditions. Induction thus involves relaxation of *GAL80* binding to *GAL4* and DNA, allowing the *GAL4* protein to function. The present experiments including the others have not been able to distinguish between these three models in a strict sense. Obviously direct experiments are needed to get an insight into the molecular mechanism involving *GAL4* and *GAL80*.

We have also demonstrated that the escape synthesis was remarkably enhanced if both *GAL7* and *GAL4* genes were amplified by a YEp vector: The transferase activity encoded by *GAL7* was 100 times higher in the yeast with the YEp bearing both genes (Table 3C) than in the yeast with the YEp bearing *GAL4* only (Table 3A). The difference observed in the enzyme level cannot be accounted for by the difference in the copy number of *GAL7* which would be less than 20-fold. It looks as if active *GAL4* protein exerts its action on the *GAL7* gene in the plasmid preferentially to those in the chromosome. It might also be possible that the *GAL80* protein binds the gene in the chromosome in preference to that gene in the plasmid. These ideas are now under investigation.

Acknowledgements. Our thanks are due to Dr. Barbara Hohn, Dr. Thomas P. St. John, Dr. Dan T. Stinchcomb, and Dr. Toshiaki Takahashi for the yeast strains or the plasmids in Table I, and to Mr. Susumu Okamoto for the yeast gene library. This work was supported in part by a grant (No. 57106009) from the Ministry of Education, Science and Culture.

References

Bassel J, Mortimer R (1971) Genetic order of the galactose structural genes in *Saccharomyces cerevisiae*. J Bacteriol 108:179–183

Beggs JD (1978) Transformation of yeast by a replicating hybrid plasmid. Nature 275:104–108

Birnboim HC, Doly J (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucl Acid Res 7:1513–1523

Botstein D, Falco SC, Stewart SE, Brennan M, Scherer S, Stinchcomb DT, Struhl K, Davis RW (1979) Sterile host yeasts (SHY): A eukaryotic system of biological containment for recombinant DNA experiments. Gene 8:17–24

Broach JR (1979) Galactose Regulation in *Saccharomyces cerevisiae*. The enzymes encoded by the *GAL7, 10, 1* cluster are coordinately controlled and separately translated. J Mol Biol 131:41–53

Broach JR, Strathern JN, Hicks JB (1979) Transformation in Yeast: Development of a hybrid cloning vector and isolation of the *CAN1* gene. Gene 8:121–133

Buttin G (1963) Mécanismes régulateurs dans la biosynthèse des enzymes du métabolisme du galactose chez *Escherichia coli* K12. J Mol Biol 7:164–182

Clarke L, Carbon J (1980) Isolation of a yeast centromere and construction of functional small circular chromosomes. Nature 287:504–509

Cryer DR, Eccleshall R, Marmur J (1975) Isolation of yeast DNA. In: Prescott DM (ed) Methods in cell biology, vol 12. Academic Press, New York, p 39–44

Davis RW, Thomas M, Cameron J, St. John TP, Scherer S, Padgett RA (1979) Rapid DNA isolations for enzymatic and hybridization analysis. Methods Enzymol 65:404–414

Douglas HC, Hawthorne DC (1964) Enzymatic expression and genetic linkage of genes controlling galactose utilization in *Saccharomyces*. Genetics 49:837–844

Douglas HC, Hawthorne DC (1966) Regulation of genes controlling synthesis of the galactose pathway enzymes in yeast. Genetics 54:911–916

Douglas HC, Hawthorne DC (1972) Uninducible mutants in the *gali* locus of *Saccharomyces cerevisiae*. J Bacteriol 109:1139–1143

Guarente L, Yocum RR, Gifford P (1982) A *GAL10-CYC1* hybrid yeast promoter identifies the *GAL4* regulatory region as an upstream site. Proc Natl Acad Sci USA 79:7410–7414

Hinnen A, Hicks JB, Fink GR (1978) Transformation of yeast. Proc Natl Acad Sci USA 75:1929–1933

Johnston SA, Hopper JE (1982) Isolation of the yeast regulatory gene *GAL4* and analysis of its dosage effects on the galactose/melibiose regulon. Proc Natl Acad Sci USA 79:6971–6975

Kew OH, Douglas HC (1976) Genetic co-regulation of galactose and melibiose utilization in *Saccharomyces*. J Bacteriol 125:33–41

Klar AJS, Halvorson HO (1974) Studies on the positive regulatory gene, *GAL4*, in regulation of galactose catabolic enzymes in *Saccharomyces cerevisiae*. Mol Gen Genet 125:203–212

Laughon A, Gesteland RF (1982) Isolation and preliminary characterization of the *GAL4* gene, a positive regulator of transcription in yeast. Proc Natl Acad Sci USA 79:6827–6831

Matsumoto K, Toh-e A, Oshima Y (1978) Genetic control of galactokinase synthesis in *Saccharomyces cerevisiae*: Evidence for constitutive expression of the positive regulatory gene *gal4*. J Bacteriol 134:446–457

Matsumoto K, Adachi Y, To-e A, Oshima Y (1980) 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. J Bacteriol 141:508–527

Nogi Y, Fukasawa T (1980) A novel mutation that affects utilization of galactose in *Saccharomyces cerevisiae*. Curr Genet 2:115–120

Perlman D, Hopper JE (1979) Constitutive synthesis of the *GAL4* protein, a galactose pathway regulator in *Saccharomyces cerevisiae*. Cell 16:89–95

Rigby PWJ, Dieckmann M, Rhodes C, Berg P (1974) Labeling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I. J Mol Biol 113:237–251

Segawa T, Fukasawa T (1980) Transcriptional units of GAL genes in *Saccharomyces cerevisiae* determined by ultraviolet light mapping. Curr Genet 2:223–228

Southern EM (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 98:503–517

St John TP, Davis RW (1979) Isolation of galactose-inducible DNA sequences from *Saccharomyces cerevisiae* by differential plaque filter hybridization. Cell 16:443–452

St John TP, Davis RW (1981) The organization and transcription of the galactose gene cluster of *Saccharomyces*. J Mol Biol 152:285–315

St John TP, Scherer S, McDonell MW, Davis RW (1981) Deletion analysis of the *Saccharomyces GAL* gene cluster. J Mol Biol 152:317–334

Stinchcomb DT, Mann C, Davis RW (1982) Centromeric DNA from *Saccharomyces cerevisiae*. J Mol Biol 158:157–179

Struhl K, Stinchcomb DT, Scherer S, Davis RW (1979) High-frequency transformation of yeast: Autonomous replication of hybrid DNA molecules. Proc Natl Acad Sci USA 76:1035–1039

Communicated by T. Yura

Received February 2, 1983