Expression of the *Saccharomyces cerevisiae* GAL7 Gene on Autonomous Plasmids

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Received 10 March 1984/Accepted 22 June 1984

We used a combination of cloned DNA fragments encoding the *GAL7* gene, yeast plasmid vectors, and chromosomal *gal7* deletions to characterize the in vivo transcription of the *GAL7* gene on autonomously replicating plasmids. Our results demonstrated that a plasmid-borne 3.1-kilobase DNA fragment containing the *GAL7* gene provides sufficient information to mimic the regulated expression of the chromosomal location. Normal expression of *GAL7* could occur in the absence of DNA encoding the functional genes of the *GAL* cluster region and was not altered when the gene was adjacent to other plasmid elements such as autonomously replicating sequences or centromeres. The chromosomal and single-copy centromeric plasmid locations of *GAL7* were indistinguishable in their response to growth conditions (induction by galactose, repression by glucose) and positive and negative regulatory factors (*GAL4* and *GAL80*). Increasing the gene dosage to more than 200 copies per cell resulted in constitutive expression of the *GAL7* mRNA; fully induced mRNA levels were increased more than 10-fold at these high gene dosages. When cells were shifted from noninducing to inducing conditions, the initial time of appearance and the rate of accumulation of *GAL7* mRNA were altered in cell populations containing multiple *GAL7* genes. The induction kinetics and final accumulation of the chromosomal *GAL10* mRNA were also affected by the presence of multiple copies of the *GAL7* gene; these results are consistent with a model involving limiting amounts of regulatory factors.

The conversion of exogenous galactose to glucose-1-phosphate in *Saccharomyces cerevisiae* is catalyzed by three inducible enzymes of the Leloir pathway: galactokinase (kinase; EC 2.7.1.6), α -D-galactose-1-phosphate uridylyl-transferase (transferase; EC 2.7.7.12), and uridine diphosphoglucose-4-epimerase (epimerase; EC 5.1.3.2). These enzymes are encoded by three tightly linked genes, *GAL7* (transferase), *GAL10* (epimerase), and *GAL1* (kinase), located near the centromere of chromosome II (2, 14). The *GAL7*, *GAL10*, and *GAL1* genes are tightly coregulated at the transcriptional level; each gene is a separate transcriptional unit (23, 46). The predominant transcripts are a 1.25-kilobase (kb) *GAL7* mRNA, a 2.25-kb *GAL10* mRNA, and 1.65- and 1.75-kb *GAL1* mRNAs (46). The *GAL1* and *GAL10* genes are transcribed from opposite DNA strands with approximately 600 base pairs of DNA separating their transcriptional initiation sites. The *GAL7* mRNA initiates approximately 650 base pairs from the 3' end of the *GAL10* mRNA and is transcribed from the same DNA strand as *GAL10* (35, 46).

The expression of the *GAL* gene cluster is altered by mutations in several other unlinked loci (1, 30, 34, 49). Two constitutively expressed genes, *GAL4* and *GAL80*, are primarily responsible for regulation (15, 23, 38). The *GAL4* gene product is thought to be a positive regulatory factor required for the activation of transcription. In the absence of inducer (galactose or a metabolite) the *GAL80* protein prevents activation by *GAL4* protein. In the presence of inducer the *GAL80* protein is no longer effective in preventing activation by *GAL4* protein, and under fully induced conditions expression of the structural genes is increased approximately 1,000-fold relative to the fully repressed state (reviewed in reference 37). Current models suggest that at least

one of the regulatory proteins binds sequences upstream from each gene. In the case of *GAL10* an upstream activating sequence has been located approximately 130 base pairs 5' to the transcriptional start site (19, 24a). It is not known whether the *GAL80* protein prevents transcriptional activation by binding directly to sequences within the promoter region or whether inactivation occurs by protein-protein interactions between *GAL80* and *GAL4* proteins.

To define the role of template structure and chromosomal position in the transcriptional regulation of the *GAL* gene cluster, we have introduced the *GAL7* gene into different recombinant plasmids. Transforming yeast strains containing deletions in the chromosomal *GAL7* gene with these plasmids has allowed us to study the effects of altering the position and copy number of the *GAL7* gene. Characterization of the behavior of these plasmids in terms of the transcriptional regulation occurring in vivo is a first step toward developing an in vitro reconstituted transcription system, using the autonomous plasmids isolated from *S. cerevisiae* as chromatin templates.

MATERIALS AND METHODS

Yeast and bacterial strains. Genotypes and sources of the yeast strains used are listed in Table 1. Only the relevant genotypes of these strains are presented in the text.

Media. Yeast strains were routinely grown and maintained in YPD medium (1% yeast extract [Difco Laboratories], 2% peptone [Difco], 2% glucose). YPE and YPG media contain 2% ethanol or 2% galactose, respectively. Yeast transformants were grown and maintained in defined synthetic complete (SC) media (27) lacking uracil (SC-ura), leucine (SC-leu), or uracil and tryptophan (SC-ura-trp). SC media contained 2% glucose (SCD), 2% galactose (SCG), 2% ethanol (SCE), or 3% glycerol plus 2% D,L-lactic acid (SCGL). Galactose, lactic acid, amino acids, and uracil were obtained from Sigma Chemical Co.

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TABLE 1. *S. cerevisiae* strains used in these studies

Strain	Genotype	Source
YNN13 (D585-11c)	<i>MATa lys1</i>	J. Fink
YNN86	<i>MATa arg4-17 his5-1 gal4-1</i>	J. Broach
YNN101 (NR23-2C)	<i>MATa arg GAL81-1 TSSgal4-3</i>	K. Matsumoto
YJJ4	<i>MATa gal7Δ102 lys1 trp1-289 ura3-52</i>	This work
YJJ13	<i>MATa gall gal7 gal10Δ108 lys1 trp1-289</i>	This work
YJJ27	<i>MATa ade5 gall gal7 gal10Δ108 his7-2 trp1-289 ura3-1 ura3-2</i>	This work
YJJ33	<i>MATa ade5 gal7 leu2-3 leu2-112 trp1-289 ura3-52</i>	This work
YJJ36	<i>MATa gall trp1-289 ura3-52</i>	This work
YJJ38	<i>MATa ade5 gal7Δ102 leu2-3 leu2-112 trp1-289 ura3-52</i>	This work
YJJ43	<i>MATa gal10 ura3-1 ura3-2</i>	This work
YJJ40	<i>MATa gal4-1 gal7Δ102 lys1 ura3-52</i>	This work
YJJ57	<i>MATa arg GAL81-1 TSSgal4-3 gal7Δ102 trp1-289 ura3-52</i>	This work
YJJ63 ^a	<i>MATa ade5 gal7Δ102 leu2-3 leu2-112 trp1-289 ura3-52 [cir^a]</i>	This work

^a YJJ38 was cured of endogenous 2μm as described by Dobson et al. (13) to create the YJJ63 strain. YJJ63 completely lacks 2μm sequences as judged by Southern (41) hybridization experiments.

Plasmids and plasmid constructions. The plasmid vectors used in this study were YRp17 (6), YCp19 (42), and pJDB219 (3) (Fig. 1). For simplicity, these plasmids will be referred to as YRp, YCp, and YEp in the body of this report. Plasmids YRp-Sc4811 and YRp-Sc4811' were constructed by ligating the 5.3-kb Sc4811 fragment (containing the entire *GAL7* gene and most of the 3' end of *GAL10* [46]) into the *EcoRI* site of YRp17. YCp-Sc4811 and YCp-Sc4811' were constructed by ligating the Sc4811 fragment into the *EcoRI* site of YCp19. Restriction mapping was used to determine the relative orientation of the inserted DNA with respect to the vector plasmids. Restriction endonucleases and DNA ligase were obtained from Bethesda Research Laboratories and New England Biolabs. The direction of transcription of *GAL7* is clockwise relative to the orientation of the vectors shown in Fig. 1. Plasmids containing *GAL7* inserted in the opposite (counterclockwise) orientation are denoted by the symbol (') following the plasmid name (e.g., YCp-Sc4811').

A 3.1-kb fragment, Sc731, containing the *GAL7* gene was obtained from Sc4811. After complete digestion with *PvuII* and partial digestion with *SaII*, the restriction fragments were made flush-ended with DNA polymerase I Klenow fragment (Bethesda Research Laboratories), followed by ligation to an excess of *BamHI* linkers (Collaborative Research). The Sc731 fragment was isolated from an agarose gel (16) after digestion with excess *BamHI*. YRp-Sc731 plus YRp-Sc731', YCp-Sc731 plus YCp-Sc731', and YEp-Sc731 plus YEp-Sc731' were constructed by ligating Sc731 into the unique *BamHI* site present in YRp, YCp, and YEp, respectively. Plasmids were isolated on a large scale by two

bandings in CsCl-ethidium bromide gradients (10) or on a small scale by the rapid alkaline extraction procedure (5).

Transformations and mitotic stability. *Escherichia coli* strains MC1066 (8) and BNN45 (46) were grown and transformed by standard methods (9). Yeast transformations were performed by the method of Hinnen et al. (22). The mitotic stability of yeast plasmids was determined by growing transformed strains under selection for *TRP1* and *URA3* to a density of 10⁷ cells per ml and plating identical dilutions on both YPD and SCD-ura-trp. At the same time, a portion of the culture was diluted 1,000-fold in liquid YPD. After 15 to 20 h of nonselective growth (8 to 12 generations), identical dilutions were plated on YPD and selective media. In some cases colonies growing on the YPD plate were also replicated onto selective medium. The mitotic stability was calculated from the fractional loss of plasmid markers as compared to the fraction of selectively grown cells originally containing plasmid markers.

Isolation of DNA and RNA. Total DNA was isolated from cells by using a minor modification of the method described by Hyman et al. (24). Total yeast RNA was isolated from cells in the early to mid-logarithmic phase of growth by minor modifications of the method described by Elder et al. (17). Occasionally these total nucleic acid preparations were also used for analysis of plasmid and genomic restriction fragments by digesting 50 μg of nucleic acid with RNase A (20 μg/ml) for 1 to 2 h before digestion with a 5- to 10-fold excess of restriction endonuclease.

Nick translation. DNA fragments were labeled with [α -³²P]CTP (~800 Ci/mmol; Amersham Corp.) essentially as described by Rigby et al. (39). In some nick translations the template was first cut with the appropriate restriction endonuclease and the digestion reaction mixture was added directly to the nick translation reaction. Nick-translated fragments were resolved on nondenaturing agarose gels, excised from the gel, and boiled for 10 min before being added to the hybridization solution.

DNA and RNA transfers and hybridizations. Total DNA (2 to 10 μg) was digested with a 5- to 10-fold excess of restriction endonuclease for 4 to 8 h before separation on 0.7% agarose gels. The transfer to nitrocellulose membranes (type HAHY; Millipore Corp.) was carried out as described by Davis et al. (11). Nitrocellulose filters were hybridized for 12 to 14 h at 42°C in the presence of 50% formamide. Unless stated otherwise, the final concentration of α -³²P-labeled probe (5 × 10⁷ to 10 × 10⁷ cpm/μg) was 0.1 to 0.2 μg/ml. After hybridization, the filters were washed for 1 h at 37°C in three changes of 10 mM sodium phosphate (pH 6.9)–0.1% sodium dodecyl sulfate, followed by two washes in the same buffer at 65°C for 1 h.

RNA samples (20 to 50 μg) were suspended in 50% formamide–6% formaldehyde–1× MOPS (20 mM morpholinopropanesulfonic acid, 5 mM sodium acetate, 1 mM EDTA, pH 7.0). Samples were incubated at 65°C for 15 min and loaded onto agarose gels (1.5% agarose, 6% formaldehyde, 1× MOPS). Gels were stained, destained, and photographed as described by McMaster and Carmichael (31). Gels were transferred as described by Maniatis et al. (28). Hybridization and washing conditions were the same as described above for DNA transfers.

Estimation of plasmid copy number and relative abundance of *GAL* transcripts. Autoradiographs or negatives of stained gels were scanned with an LKB scanning densitometer. For autoradiography, the correct exposure required for a linear film response was determined by including a set of standardized wedges of DE81 paper containing known amounts of

FIG. 1. Restriction map of the galactose cluster region (A) and yeast plasmid vectors (B). (A) Chromosomal deletions, *gal7Δ102* and *gal1,7,10Δ108*, originally described by St. John et al. (47), are denoted by cross-hatched areas shown above the wild-type sequence. The *GAL1*, *GAL7*, and *GAL10* transcripts are denoted by arrows. The two DNA fragments derived from the *GAL7* region, Sc4811 and Sc731, are shown below the wild-type sequence. (B) Thin lines represent pBR322 sequences, thick lines represent yeast DNA sequences, and open double lines represent 2μm plasmid sequences. YCp19 (42), YRp17 (6), and pJDB219 (3) are referred to as YCp, YRp, and YE, respectively, in the text. DNA fragments containing the *GAL7* gene (Sc4811 and Sc731) were inserted into the *EcoRI* (R) or *BamHI* (B) sites of these vectors as described in the text. Sites of cleavage by various restriction enzymes are denoted as follows: B, *BamHI*; Bg, *BglII*; K, *KpnI*; R, *EcoRI*; Pv, *PvuII*; S, *Sall*; X, *XbaI*.

radioactivity (36). In some experiments, labeled bands were cut from nitrocellulose filters and the radioactivity was measured by liquid scintillation counting. The results obtained by the two methods were in excellent agreement.

RESULTS

Expression of *GAL7* in transformed strains. The 5.3-kb Sc4811 *EcoRI* DNA fragment, clonally isolated originally by St. John and Davis (45), contains DNA corresponding to the entire 1.25-kb *GAL7* transcript. The additional 2.6 kb of upstream sequences also codes for all but the first 0.15 kb of the *GAL10* mRNA (Fig. 1A). To determine whether the Sc4811 fragment encodes sufficient information to express the *GAL7* function, the entire fragment was ligated into the single *EcoRI* site of two different yeast plasmids, YRp17 and YCp19 (Fig. 1B). Recombinant plasmids were constructed with both multicopy (YRp) and single-copy (YCp) plasmid

vectors to distinguish possible effects of gene dosage. In each recombinant plasmid the inserted Sc4811 fragment was recovered in both orientations relative to adjacent potential regulatory elements present in YRp (*TRP1 ARS1*) or YCp (*TRP1 ARS1* and *CEN4*). Yeast strain YJJ4 contains the *gal7Δ102* deletion (47) that removes 1.1 kb of DNA between the *XbaI* and *BglII* sites present in the *GAL7* gene; YJJ13 contains the *gal1,10,7Δ108* deletion (47) that removes the 5' region of *GAL7* and all of *GAL10* and *GAL1* (Fig. 1A). Neither of these deletion strains produces the 1.25-kb *GAL7* transcript. YJJ4 (*gal7Δ102*) and YJJ13 (*gal1,7,10Δ108*) were transformed to tryptophan prototrophy (*Trp⁺*) with YRp-Sc4811, YRp-Sc4811', YCp-Sc4811, and YCp-Sc4811'. The *Trp⁺* transformants of YJJ4 were directly tested for their Gal phenotype on SCG-ura indicator plates. All *Trp⁺* transformants tested were also *Gal⁺ Ura⁺*. The *Trp⁺* transformants of YJJ13 were tested for their Gal phenotype by complementation tests, using tester strains that permit continued selection

of plasmid markers (see Table 1). Diploids were tested for the ability to grow on SCG-trp indicator plates. By this criterion, all of the Trp⁺ transformants of YJJ13 were *gal10⁻ GAL7⁺*.

The mitotic stability of each of the plasmids was examined by using transformants of three *GAL* deletion strains, YJJ4, YJJ38, and YJJ27. The fractions of each cell population that remained phenotypically Ura⁺ Trp⁺ after growing under selection for uracil and tryptophan and after growing in YPD are shown in Table 2. These data show that the YRpSc4811 and YRpSc4811' transformed cells exhibit a broad range of both mitotic stability and the fraction of cells that are Trp⁺ Ura⁺ after selective growth. Typically, YRp17 is present in 5 to 30% of the cells after transformants are grown selectively in the absence of Ura or Trp and in 0.5% to 5% of the cells after 10 generations in YPD (24, 43, 44). The increased mitotic stability of some YRp transformants suggests that at least some of the plasmids are no longer replicating autonomously but have integrated into the host chromosome. The fraction of Ura⁺ Trp⁺ cells present after selective growth and the mitotic stability of the YCp-Sc4811 and YCp-Sc4811' transformed cells are more consistent with values expected for autonomous replication of centromere-containing plasmids (42).

Detection of integration events and determination of plasmid copy numbers. To determine whether expression of *GAL7* in the transformed cells was occurring from integrated forms or autonomously replicating forms of the plasmids containing *GAL7*, DNA from each of the transformed strains was isolated and digested with *Bam*HI, separated electrophoretically, transferred to nitrocellulose, and hybridized to nick-translated pBR322. The results obtained from this type of analysis (data not shown) demonstrated that YRp, YCp, YCp-Sc4811, and YCp-Sc4811' were present only as autonomously replicating forms. The YJJ38(YRp-Sc4811') trans-

formed population contained both integrated (18-kb band) and autonomous (12.3-kb band) forms of the plasmid. The 18-kb band is consistent with a recombination event occurring within the 5' flanking region of *GAL7*. The YJJ27(YRp-Sc4811) transformed population also contained both integrated (11-kb band) and autonomous forms of YRp-Sc4811. In these experiments, the large fraction of cells originally possessing plasmid markers (Ura⁺ Trp⁺) and the high mitotic stability of the YJJ38(YRp-Sc4811') and YJJ27(YRp-Sc4811) transformants presumably reflect the presence of integrated forms.

The DNA used for the analysis described above was also digested with *Eco*RI, fractionated by electrophoresis, immobilized on nitrocellulose, and probed with a nick-translated DNA fragment containing approximately equal amounts of DNA from both the *TRP1* *ARS1* and the *GAL7* regions (Fig. 2). Each of the strains indicated in Fig. 2 have two single-copy chromosomal *Eco*RI fragments that hybridize to the probe; these fragments are the 1.45-kb *trp1* *ARS1* fragment and the 4.1- or 2.2-kb fragments corresponding to the remnants of the *gal7* gene present in each deletion strain. By comparing the relative autoradiographic intensity or radioactivity present in these bands with that present in the bands produced by hybridization to plasmid-containing sequences (the YRp and YCp vectors as well as the Sc4811 insert of the recombinant plasmids), it is possible to estimate the plasmid copy number per haploid genome. The YCp plasmids are present at one to two copies per genome, whereas the YRp plasmids are present at six-tenths to four copies per genome. The YCp transformants are stable (typically 70 to 95% retain the plasmid after 8 to 12 generations of nonselective growth); therefore, these plasmids are present at approximately one to two copies per transformed cell. The mitotic instability of the YRp plasmids results in loss of these plasmids from a large fraction of the growing cell population. When the copy number per genome is normalized by the fraction of cells containing plasmid, these plasmids are found to be present at 3 to 20 copies per transformed cell.

Regulated transcription of the plasmid-borne *GAL7* gene. After a 90-min period of incubation in the presence of either 2% glucose or 2% galactose, portions of the same mid-log-phase cultures used for the isolation of DNA (Fig. 2) were also used for the isolation of RNA. Total RNA was analyzed as described in Materials and Methods. In the presence of galactose, each of the transformed strains contained amounts of *GAL7* mRNA that were equal to the amount obtained from induced D585-11c (data not shown). It was not possible to detect any hybridization to the Sc4811 probe when RNA was prepared from transformed strains incubated in the presence of 2% glucose. Although these results suggest that the Sc4811 sequence contains sufficient information for normal transcription and regulation, interpretation of the effects of gene dosage or adjacent regulatory elements is complicated by the presence of integrated forms of YRp-Sc4811 and YRp-Sc4811'. To reduce the frequency of homologous recombination, a smaller fragment, obtained from Sc4811, was subcloned into the *Bam*HI site of YRp and YCp (see Materials and Methods). These plasmids, YRp-Sc731, YRp-Sc731', YCp-Sc731, and YCp-Sc731', were used to transform YJJ38 and YJJ27. Complementation tests indicated that each transformant was *GAL7⁺*.

The transformed strains were grown to mid-log phase in SCE-ura-trp and each culture was divided into three parts. One part was used for the isolation of DNA and the other two parts were incubated in the presence of 2% glucose and 2% galactose for 90 min before isolation of RNA. RNA was

TABLE 2. Mitotic stability of plasmid-containing strains of *S. cerevisiae*^a

Plasmid	Host	% SC ^b	% YPD ^c
YCp19, YCp-Sc4811 or YCp-Sc4811'	YJJ4, YJJ27, YJJ38 or YJJ63	40-96	37-97
YCp-Sc731 or YCp-Sc731'	YJJ27	46-92	66-92
YCp-Sc731 or YCp-Sc731'	YJJ38	22-98	22-73
YRp17	YJJ27 or YJJ38	6.4-33	1.5-15
YRp-Sc4811 or YRp-Sc731 or YRp-Sc731'	YJJ4 or YJJ38	19-57	5-25 ^d
YRp-Sc4811' or YRp-Sc731'	YJJ27	20-25	4.8-20
YRp-Sc4811 or YRp-Sc731	YJJ27	11-83	21-86 ^e
YRp-Sc4811'	YJJ4 or YJJ38	24-95	9.7-94 ^e
pJDB219, YEp-Sc731, or YEp-Sc731'	YJJ63	88-100	80-92

^a The values listed were obtained from colonies originally isolated from a minimum of four independent transformations using each of the plasmids. A similar range of values was obtained if the transformed colonies were isolated from the same transformation experiment. In general, populations that were shown to contain integrated forms of the YRp plasmids also retained the plasmid in more than 50 to 70% of the cells after growth in selective or YPD media.

^b Values indicate the percentage of cells retaining plasmid markers (Trp⁺, Ura⁺, or Leu⁺) after growth in SC media lacking the appropriate nutrient.

^c Values indicate the percentage of cells retaining plasmid markers after 8 to 12 generations in YPD.

^d One out of the five cultures examined by Southern analysis (41) contained integrated forms of YRp-Sc4811.

^e In each group, two out of the three cultures examined by Southern (41) analysis contained integrated forms.

FIG. 2. Determination of plasmid copy number. The number of copies of the *GAL7* gene present in the transformed and untransformed cells was determined by cutting total DNA with *EcoRI*. After separation and transfer the DNA was probed with a nick-translated 2.0-kb *XbaI* fragment obtained from YRp-Sc4811. This fragment contains approximately equal amounts of DNA from the *GAL7* and *TRP1 ARS1* regions. The *EcoRI* fragments that hybridize with the probe are as follows: the YCp and YRp vectors (10.6 and 7.0 kb, respectively), the 5.3-kb Sc4811 fragment (either from the chromosome of D585-11c or from the recombinant plasmids), the remnants of the *GAL7* gene present in the chromosomes of YJ38 (*gal7Δ102*) and YJ27 (*gal1,7,10Δ108*) (4.1 and 2.2 kb, respectively), and the 1.45-kb fragment from the *trpI ARS1* region of the chromosome of each strain.

treated as described above and probed for the presence of *GAL10* mRNA and *GAL7* mRNA, using nick-translated Sc4811. Each of the strains transformed with the plasmids containing the Sc731 sequence accumulated *GAL7* mRNA only in the presence of galactose (Fig. 3). The fraction of Ura^+ Trp^+ cells present in the glucose- or galactose-pulsed cultures and the plasmid copy number were also measured (data not shown). The lower abundance of the *GAL7* mRNA in YJ38(YCp-Sc731) and YJ38(YCp-Sc731') (Fig. 3) can be attributed to the small fraction of cells containing these plasmids in this particular preparation. This result does not appear to be related to the properties of YCp-Sc731 or YCp-Sc731' since similar reductions in the plasmid copy number and level of *GAL7* mRNA do not consistently occur in other cultured YJ38 or YJ27 strains that have been transformed with these plasmids (see Table 2). These results also demonstrate that the strains transformed with YRp-Sc731 or YRp-Sc731' produce normal amounts of *GAL7* mRNA after induction with galactose even though only 18 to 20% of the cells have retained autonomously replicating forms of these plasmids (at copy numbers of 5 to 20 per Ura^+ Trp^+ cell). Hybridization of nick-translated Sc4811 to immobilized *BamHI*-plus-*EcoRI* DNA fragments (data not shown) demonstrated that only one of the transformants [YJ27(YRp-Sc731)] contained integrated forms of the plasmid. Additional experiments of this type have not detected integration of any of the other plasmids containing the Sc731 sequence.

Effect of regulatory mutations on transcription of plasmid and chromosomal *GAL* genes. The effect of several different allelic states of *GAL4* upon the transcription of *GAL7* and *GAL10* mRNAs was examined by RNA transfer and hybridization experiments. Two of the regulatory mutants, YNN86 (*gal4*) and YNN101 (*GAL81 TSSgal4*; this mutant is constitutive 25°C and noninducible at 37°C; see reference 29), were crossed with YJ4. Segregants containing the regulatory mutation and the *gal7Δ102* deletion were tested for the presence of *GAL10* mRNA before or after induction. The YJ40 strain (*gal4 gal7Δ102*) was not induced for transcription of *GAL10* mRNA after 90 min of incubation in 2% galactose (Fig. 4). The YJ57 strain (*GAL81 TSSgal4 gal7Δ102*) was constitutive at the permissive temperature (25°C in YPE) and not inducible at the nonpermissive temperature (37°C in YPG; Fig. 4). The YJ40 strain was transformed to Ura^+ with YRp-Sc4811. YJ40, YJ40(YRp-Sc4811), YJ4(YRp-Sc4811), YNN86, and D585-11c were grown to mid-log phase in SCE or SCE-ura-trp and pulsed for 90 min with 2% galactose. RNA was isolated from each of the cultures, treated as described above, and probed with nick-translated Sc4811 (Fig. 4). The YJ40(YRp-Sc4811), YJ40, and YNN86 strains are not inducible for either the *GAL7* or the *GAL10* transcript, indicating that the plasmid-borne *GAL7* gene also requires *GAL4* for expression. Similar studies with a *gal80 gal7Δ102* strain have demonstrated that a functional *GAL80* gene product is required for negative regulation of the autonomous *GAL* gene (data not shown).

The *GAL7* gene in a 2-μm-based vector. To determine the effects of further increases in copy number, the Sc731 fragment was ligated into the single *BamHI* site of YEp. The recombinant plasmids were used to transform YJ63 (*leu2 gal7Δ102 [cir^o]*). The Leu^+ transformants were isolated and transferred to SCG-leu indicator plates. Each of the Leu^+ transformants was also Gal^+ on the indicator plates.

FIG. 3. Regulated expression of *GAL7* from autonomous plasmids. Transformed cells were grown to an optical density at 600 nm of 1 (mid-log phase, $\sim 10^7$ cells per ml) in SCE-ura-trp. YJ38 and D585-11c were grown to the same density in SCE. One-half of each culture was made 2% galactose and the other half was made 2% glucose. The cultures were incubated at 30°C in a shaking water bath for 90 min. At the end of this time period total RNA was isolated, separated, and transferred as described in the text. The transferred RNA was probed with nick-translated Sc4811.

FIG. 4. Effects of regulatory mutations on *GAL7* gene transcription. Cultures were grown to mid-log phase in nonrepressing media (either YPE or SCE) and were then incubated in the presence of 2% galactose (lanes 1, 3, 5, 7, 9, and 10) or 2% glucose (lanes 2, 4, 6, and 8) for 90 min at 30°C except as indicated. Total RNA was isolated and processed as described in the legend to Fig. 3. The immobilized RNA was probed with nick-translated *Sc4811*. Lanes 1, 2, and 10 contain RNA from YJJ40(*gal7Δ102 gal4*), lanes 3 and 4 are RNA from D585-11c, lanes 5 to 8 are from YJJ57 (*gal7Δ102 GAL81 TSSgal4*), and lane 9 contains RNA from YJJ4. The RNA in lanes 5 and 6 was obtained from cells grown and pulsed at 25°C; lanes 7 and 8 contain RNA from cells grown at 25°C and pulsed at 37°C. RNA in lanes 9 and 10 were prepared from strains transformed with YRp-*Sc4811*.

YJJ63(YEp), YJJ63(YEp-*Sc731*), and YJJ63(YEp-*Sc731'*) were each grown to mid-log phase in SCD-leu, and total DNA was isolated. Because the rDNA repeat of the yeast chromosome is cleaved once by *KpnI* (4), digestion of total DNA with this enzyme results in a 9.06-kb fragment that is present at 100 to 140 copies per haploid cell (40, 50). Digestion with *KpnI* also cleaves each of the 2-μm-derived plasmids at a single site within the *LEU2* gene (see Fig. 1) to produce linear fragments that correspond to the size of each plasmid. After DNA was digested with *KpnI* and resolved on agarose gels, two prominent bands were present in each lane of the stained gel (Fig. 5A); the larger of the two bands corresponded to the linearized plasmid and the smaller band was the *KpnI* fragment of the rDNA repeat. The relative intensity of each of these bands was measured from densitometer tracings of the photographic negatives. After correcting for the different sizes of the restriction fragments, we estimated that these plasmids are two to four times more abundant than the rDNA and are present at 200 to 600 copies per genome. After growth in the absence of leucine, 88 to 100% of the transformants were *Leu*⁺. Approximately 80 to 92% of the transformants remained *Leu*⁺ after 12 generations in YPD (Table 2).

YJJ63(YEp), YJJ63(YEp-*Sc731*), and YJJ63(YEp-*Sc731'*) were grown to mid-log phase in SCGL-leu and treated as described in the legend to Fig. 5B. After incubation in the presence of 2% glucose for 90 min, YJJ63(YEp-*Sc731*) and YJJ63(YEp-*Sc731'*) contained detectable levels of *GAL7* mRNA (Fig. 5B; levels of *GAL10* mRNA do not decrease further on longer exposure to glucose [data not shown]). After growth in SCGL-leu, these two transformants had levels of *GAL7* mRNA that were about 30% of that produced by a fully induced wild-type strain; constitutive expression of the single-copy *GAL10* gene was also detectable under the same conditions. Incubation in the presence of 2% galactose resulted in an increased accumulation of *GAL7* mRNA to a level several times greater than the level present in fully

induced D585-11c (see below). However, the level of *GAL10* mRNA in the YEp-*Sc731* and YEp-*Sc731'* transformed cells was greatly reduced compared with wild-type levels.

Induction time course of *GAL7* mRNA from chromosomal and plasmid copies. The results described above indicate that

FIG. 5. Copy number of YEp-*Sc731* and YEp-*Sc731'* (A) and expression of *GAL7* from high-copy-number plasmids (B). (A) Total DNA from the indicated transformants was digested with *KpnI*, resolved on agarose gels, stained, and photographed. Densitometric scans were made from the photographic negative. The plasmid copy number was estimated by comparison of the sizes and relative intensities of the plasmid bands (approximately 12.9 kb for YEp and 16 kb for YEp-*Sc731* and YEp-*Sc731'*) to the 9.0-kb band produced by cleavage of the rDNA repeats (4). The rDNA repeat was assumed to be present in 100 to 140 copies per cell (40, 50). (B) Transformants were grown selectively in SCGL-leu. Upon reaching an optical density at 600 nm of 1, the cultures were divided into three aliquots. One aliquot was used immediately for isolation of RNA. Glucose or galactose were added to other aliquots to a final concentration of 2%, and the cultures were incubated for an additional 90 min at 30°C before isolation of RNA. The transferred RNA was probed with nick-translated *Sc4811*.

a two- to five-fold increase in the *GAL7* gene dosage in the cell population does not result in a similar increase in the amount of *GAL7* mRNA, whereas a >200-fold increase (YEp-*GAL7* plasmids) results in a constitutive level of expression of *GAL7* mRNA that is further increased after induction with galactose. To determine whether these results were due to different rates of *GAL7* mRNA accumulation, the strains indicated in Fig. 6A and B were grown in SCE, SCE-ura, SCGL, or SCGL-leu and incubated in 2% galactose for the indicated times. RNA was isolated and analyzed as described above. Amounts of *GAL7* and *GAL10* mRNA were measured at each time point and normalized by comparison to the *HIS3* mRNA, whose levels do not change under the conditions of these experiments (48). The first detectable *GAL7* mRNA was present at 4 min after induction of D585-11c and YJJ38(YCp-Sc4811) (Fig. 6A). We find, in agreement with the data of St. John and Davis (46), that *GAL7* mRNA appears in cells slightly before *GAL10* mRNA. The relative accumulation of *GAL7* and *GAL10* mRNAs at each time point was measured from densitometer tracings of the autoradiograms in Fig. 6A and B and are plotted in Fig.

7. The rates of induction of the *GAL7* and *GAL10* mRNAs of YJJ38(YCp-Sc4811) are indistinguishable from those of D585-11c. The rates of accumulation of the *GAL7* and *GAL10* mRNAs are altered in YRp-Sc4811 and YEp-Sc731 transformants. YJJ38(YRp-Sc4811) does not accumulate detectable levels of *GAL7* mRNA until 6 min after induction and fully induced levels are not reached until after 30 min. YJJ38(YRp-Sc4811) has a slower rate of accumulation of *GAL7* and *GAL10* mRNAs as compared to D585-11c, although the final accumulation is the same as fully induced D585-11c. The YJJ63(YEp-Sc731) transformant constitutively expresses *GAL7* mRNA at a level that is approximately 30% of the level of fully induced D585-11c (Fig. 6B). In these transformants the level of *GAL7* mRNA begins to increase at 4 min and continues to accumulate to a maximal level 30 min after induction. The level of constitutive expression of *GAL7* mRNA in YJJ63(YEp-Sc731) was subtracted from each of the indicated time points. In the YEp-Sc731 transformant, the rate of *GAL7* mRNA accumulation was approximately four times that observed in D585-11c. The maximum level of *GAL7* mRNA in YJJ63(YEp-Sc731) is approximately 13-fold

FIG. 6. Induction time course of *GAL7* and *GAL10* at low (A) and high (B) gene dosages. (A) Cultures were grown in SCE or SCE-ura. Upon reaching an optical density at 600 nm of 1, the cells were collected by centrifugation and were suspended in 1/20 volume of prewarmed SC or SC-ura. After taking a 1-ml sample for the initial time point, galactose was added (2%) and incubation was continued at 30°C in a shaking water bath. At 40 s before the indicated time points, 1-ml volumes of the cultures were centrifuged for 15 s and the supernatants were discarded. At the indicated times cell pellets were quickly frozen in a dry ice-ethanol bath. RNA was isolated, separated, transferred, and probed with nick-translated Sc4811. The symbols above the lanes refer to RNA extracted from D585-11c (W), YJJ38 transformed with YCp-Sc4811 (C) or YRp-Sc4811 (R), and untransformed YJJ38 (V). (B) Cultures of D585-11c, YJJ63, and YJJ63-YEp731 were grown in SCGL or SCGL-leu. All other conditions were the same as described in (A) except that the transferred RNA was hybridized with a total of 5 μ g of nick-translated Sc4811 (2×10^7 cpm/ μ g).

FIG. 7. Kinetics of *GAL* mRNA induction. Densitometer tracings of the autoradiograms in Fig. 6A and B were made, and the relative accumulation at each time point was normalized by comparison with the amount of *HIS3* mRNA. This *HIS3* mRNA was measured by reprobing the nitrocellulose membrane with DNA homologous to the *HIS3* coding region. Solid symbols represent accumulation of *GAL7* mRNA from the plasmid *GAL7* gene contained on YEp-SC731 (▲), YRp-Sc4811 (■), or YCp-Sc4811 (●). Accumulation of the *GAL10* mRNA from the chromosome of each of these transformed cell lines is shown at the bottom. The accumulation of mRNA from the chromosomal copies of the *GAL7* and *GAL10* genes in D585-11c is depicted by open symbols (○).

higher than fully induced D585-11c. In this exposure the *GAL10* mRNA was not detected until 30 min after induction; no further increases in the relative abundance of this transcript were detected after 30 min.

DISCUSSION

We have analyzed the role of chromosomal position and gene copy number on the transcriptional regulation of the *S. cerevisiae* *GAL7* gene. Qualitatively, the expression of the *GAL7* gene is unchanged when it is removed from its chromosomal location to a position on an autonomous plasmid. Proximity to a yeast centromere, autonomously replicating sequence, or another regulated gene does not alter the size of the *GAL7* mRNA, and its production is still dependent on external regulatory signals. Internal regulatory signals are also still necessary for regulated expression of the plasmid-borne *GAL7* gene; functional *GAL4* is required for induction and the *GAL80* gene product is necessary for repression. The regulated expression of *GAL7* mRNA does not depend upon the presence of functional copies of the

other genes of the cluster region (*GAL1* and *GAL10*), indicating that neither the normal metabolism of galactose to glucose-1-phosphate nor the deleted chromosomal sequences are required for the induction process. The three plasmid vectors we have used in these studies are maintained at different copy numbers in transformed cells. However, due to the imperfect segregation of these plasmids during mitosis (33), not every cell contains plasmids during growth in selective media. In reporting the plasmid copy numbers we have presented values that reflect the relative abundance of the plasmids with respect to chromosomal sequences (copies per genome) and with respect to the fraction of cells retaining the transformed phenotype (copies per transformed cell). Determination of these two parameters has enabled us to study the transcription of *GAL7* under conditions of defined gene dosage.

Cells transformed with recombinant plasmids derived from a centromeric vector (YCp) retain plasmid at one to two copies per transformed cell during growth in selective media. This population of cells is indistinguishable from wild-type cells with respect to the final accumulation of the *GAL7* and *GAL10* mRNAs. In cultures grown from cells transformed with recombinant plasmids derived from a replicating vector (YRp), the final accumulation of *GAL7* mRNA is also equivalent to the level produced in wild-type cells. This result has a complex interpretation: although the plasmids are present at three copies per genome, only about 15% of the cells contain the autonomously replicating form of the plasmid during growth in selective media. This suggests that the *GAL7* mRNA is overproduced in the small fraction of cells that have the plasmid. The overproduction is probably the consequence of increased *GAL7* gene dosage: cells that have the plasmid contain an average of 20 copies per transformed cell. Since the final accumulation is the same (see Fig. 3), this indicates a sevenfold overproduction of *GAL7* mRNA in those cells that contain the plasmids. Although we cannot completely rule out the possibility that overproduction is due to particular properties of the plasmid constructions (e.g., altered affinity for *GAL4* or *GAL80* gene products), this does not seem likely since each of the four different plasmids derived from YRp accumulate the same level of *GAL7* mRNA and each of the four plasmids derived from YCp (which is closely related to YRp) do not overproduce the mRNA.

The rate of accumulation of the *GAL7* mRNA after cells are shifted into galactose is indistinguishable if a single copy exists in the chromosome or on a recombinant centromere plasmid. When *GAL7* mRNA production is examined in the cell population containing the recombinant replicating plasmids (YRp), its initial appearance is delayed by 2 min after induction; the rate of induction is reduced relative to the single-copy case. Since approximately 15% of the cells each now contain 20 copies of the *GAL7* gene, the delayed appearance of the *GAL7* mRNA and the reduced rate of accumulation are consistent with a lack of factors necessary for induction. The multiple copies of the *GAL7* gene may be titrating already low concentrations of these factors. The transcription of the chromosomal copy of *GAL10* is affected in a similar fashion by the presence of multiple copies of *GAL7* on autonomously replicating plasmids.

To test the possibility that the aberrant induction kinetics of the *GAL7* and *GAL10* mRNAs were due to a titration of essential factors, we further increased the copy number of the autonomous *GAL7* gene by introducing it into cells on a yeast episomal plasmid. A recipient strain lacking 2 μ m plasmids was used to avoid possible artifacts resulting from

recombination with the homologous episomal sequences (12). This precaution also avoids possible competition for DNA replication factors and results in increased copy numbers (7). In addition, as observed by Erhart and Hollenberg, selection for the defective *LEU2* gene present on the vector gives a truly remarkable accumulation of plasmid (18); in this study we observed 200 to 600 copies per genome, representing 20 to 40% of the total DNA in the cell.

The response of cells to such high gene dosages of *GAL7* reveals several interesting features of the transcriptional regulatory mechanism. When cells are maintained on a nonrepressing carbon source, glycerol lactate, it is possible to detect constitutive expression of the multicopy *GAL7* gene and of the single-copy *GAL10* gene. This result is consistent with the observation made by Kirshman and Hopper (personal communication; In J. B. Hicks, A. Klar, J. N. Strathern, ed., *The Molecular Biology of Yeast*, abstr., p. 160, Cold Spring Harbor Press, Cold Spring Harbor, N.Y.) that multiple copies of the entire *GAL* cluster region result in constitutive expression of the multicopy *GAL7* transferase activity and the single-copy *MEL1* gene product. Our studies also show that growth on a repressing carbon source, glucose, reduces the mRNA level, but it is still elevated relative to control cells. Growth on the inducing substrate, galactose, stimulates a rapid 40-fold induction over the level on glycerol lactate. The final levels of *GAL7* mRNA are therefore about 13-fold higher when 200 to 600 copies are present and 7-fold higher when 10 to 20 copies are present relative to the single-copy case. This implies a saturation of the cells' ability to induce this RNA. The increase in the level and rate of *GAL7* mRNA accumulation occurs at the expense of transcription of the chromosomal copy of *GAL10*: *GAL10* mRNA is reduced in the presence of hundreds of *GAL7* genes to approximately 13% of its normal levels (Fig. 6B).

The results presented in this paper are in general agreement with the model proposed by Oshima (37); however, by introducing hundreds of copies of *GAL7* we have shown that it is possible to disrupt the normal mode of regulation. The constitutive expression of *GAL7* and *GAL10* is consistent with the possibility that high gene dosages of *GAL7* are capable of titrating negative regulatory factors. This also suggests that at least some of the *GAL4* gene product is no longer complexed with the *GAL80* gene product. We suggest that increasing the *GAL7* gene dosage by a factor of 100-fold or more may promote dissociation of the *GAL4*-*GAL80* protein complex, perhaps due to a low-affinity binding of *GAL80* protein to sequences present in the *GAL7* DNA. The copies of the *GAL7* and *GAL10* genes that are not associated with *GAL80* protein would be available for transcriptional activation by uncomplexed *GAL4* protein. We suggest that the reduced rate and final accumulation of *GAL10* mRNA in these cells is due to competition for limiting amounts of *GAL4* protein. A number of independent studies have suggested that fully induced expression of the *GAL* gene cluster is limited by the amount of *GAL4* protein normally present. Amplification of the *GAL4* gene results in the constitutive expression of *GAL7* or *GAL10* and a two- to threefold increase in the induced levels of the corresponding mRNAs or enzymatic activities as compared to induced wild-type levels (21, 25, 27).

Proving that the *GAL4* and *GAL80* gene products are DNA binding proteins which affect rates of transcription by RNA polymerase II requires a soluble in vitro transcription system. Attempts to develop such systems from yeast by using purified DNA templates have not been successful

(J. A. Jaehning, unpublished data; P. A. Weil, personal communication). Many recent experiments have indicated that functional templates for regulated transcription in vivo are supercoiled (20) and complexed with histone and nonhistone proteins as chromatin (32). In this work, we have characterized the in vivo transcription of different plasmids to determine their potential for use as chromatin templates for in vitro transcription studies. We have established two criteria for the suitability of a gene to function as a chromatin template. First, the gene must be present in a form that is transcribed and regulated in vivo in a manner that is indistinguishable from that which normally occurs when the gene is present as part of a yeast chromosome. Second, it must be possible to isolate a homogeneous chromatin form of the gene in sufficient quantities for biochemical characterization. The plasmids derived from YRp and YCp fulfill the first criterion; however, the relatively low copy number of these plasmids complicates isolation procedures. The plasmids derived from YE_p have been estimated to represent a significant percentage of the total cellular DNA and should provide useful quantities of plasmid chromatin. Although these plasmids are transcribed accurately, they are subject to aberrant regulation. Our current efforts are directed toward determining the fraction of plasmids that are actively transcribed and developing a host strain for the plasmids that can maximize transcription of multiple *GAL7* genes.

ACKNOWLEDGMENTS

We thank T. St. John and R. W. Davis for the clonally isolated *GAL7* gene and for the yeast *gal7* deletion strains. We also thank M. Johnston, T. Torchia, J. Hopper, and S. Johnston for discussing unpublished data and D. Shapiro, O. Uhlenbeck, J. Scott, T. Platt, and T. Fukasawa for many stimulating conversations during the course of this work.

This work was supported by Public Health Service grant R01-GM29118 to J.A.J. from the National Institutes of Health. S.M.B. was supported by Public Health Service grant 5T 32 GM07283 from the National Institutes of Health.

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