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Structure and Molecular Analysis of *RGR1*, a Gene Required for Glucose Repression of *Saccharomyces cerevisiae*

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An *RGR1* gene product is required to repress expression of glucose-regulated genes in *Saccharomyces cerevisiae*. The abnormal morphology of *rgr1* cells was studied. Scanning and transmission electron microscopic observations revealed that the cell wall of the daughter cell remained attached to that of mother cell. We cloned the *RGR1* gene by complementation and showed that the cloned DNA was tightly linked to the chromosomal *RGR1* locus. The cloned *RGR1* gene suppressed all of the phenotypes caused by the mutation and encoded a 3.6-kilobase poly(A)$^+$ RNA. The *RGR1* gene is located on chromosome XII, as determined by pulsed-field gel electrophoresis, and we mapped *rgr1* between *gal2* and *pep3* by genetic analysis. *rgr1* was shown to be a new locus. We also determined the nucleotide sequence of *RGR1*, which was predicted to encode a 123-kilodalton protein. The null mutation resulted in lethality, indicating that the *RGR1* gene is essential for growth. On the other hand, a carboxy-terminal deletion of the gene caused phenotypes similar to but more severe than those caused by the original mutation. The amount of reserve carbohydrates was reduced in *rgr1* cells. Possible functions of the *RGR1* product are discussed.

Glucose regulates the expression of many genes in *Saccharomyces cerevisiae* (for a review, see reference 5). One of them, the *SUC2* gene, which encodes invertase, is repressed by glucose (2, 3). Recently, we reported the isolation of a new mutation, *rgr1*, which affects expression of the *SUC2* gene (21). A recessive *rgr1-1* mutation which caused overexpression of mouse α-amylase under the control of the *SUC2* promoter was isolated, and the *RGR1* gene was found to be required for glucose repression. The *rgr1* mutation affected several cellular functions. Cells were resistant to glucose repression, temperature sensitive for cell growth, and sporulation deficient and showed abnormal cell morphology. Expression of the *SUC2* gene in *rgr1* strains was resistant to glucose repression, and *SUC2* expression was increased under glucose-derepressing conditions. In this report, we describe studies of the morphology of *rgr1* cells, the cloning and molecular analysis of the *RGR1* gene, and meiotic linkage analysis of *rgr1*. We constructed deletion mutations to determine the phenotypes of strains lacking a functional *RGR1* gene product and determined the nucleotide sequence of the gene. The *RGR1* gene affected accumulation of reserve carbohydrates.

MATERIALS AND METHODS

Strains and genetic methods. The strains of *S. cerevisiae* used in this study are listed in Table 1. All strains were derived from S288C. Crossing, sporulation, and tetrad analysis were carried out by standard genetic methods (23). The permissive and restrictive temperatures were 24 and 37°C, respectively. The phenotype of *pep3* strains was scored as described elsewhere (12). The transformation of yeast was performed by the LiOAc-method of Ito et al. (11). *Escherichia coli* HB101 and JM109 were employed as hosts for constructing and propagating plasmids. The transformation of *E. coli* was performed by the method of Hanahan (7).

Media. The basic culture medium used for *S. cerevisiae* was YPD medium containing 1% yeast extract (Difco Laboratories), 2% Bacto-Peptone (Difco), and 2% glucose (23). The synthetic medium was CSM medium containing 0.67% yeast nitrogen base without amino acids, 2% glucose, and amino acids as required (23). The media were solidified with 2% Bacto-Agar (Difco) for plates. Luria broth was used for the culture of *E. coli* and was supplemented with ampicillin for selection of the transformants as described previously (16).

Electron microscopic observation. Yeast cells were fixed with 3% glutaraldehyde for 4 h followed by postfixation with 2% osmium tetraoxide for 2 h at 4°C and subjected to scanning electron microscopic observation with an S-800 (Hitachi) instrument at an acceleration voltage of 20 kV. For transmission electron microscopy, the fixed cells were dehydrated and embedded in EPOK 812. Sections were poststained with 2% uranyl acetate and 1.5% lead salts (0.5% lead acetate, 0.5% lead citrate, and 0.5% lead nitrate) and observed with a JEOL 1200 EX instrument at an acceleration voltage of 80 kV.

Staining of nuclei. Yeast nuclei were stained with 4′,6′-diamidino-2-phenylindole according to the method of Sherman et al. (23) and examined by epifluorescence microscopy.

Staining of the chitin ring. The chitin ring was stained with calcofluor white (fluorescent brightener 28, Sigma Chemical Co.) according to the method of Roncero et al. (19) and examined by epifluorescence microscopy.

Preparation of DNA and RNA. Preparation of *E. coli* DNA, Southern hybridization, and Northern (RNA) hybridization were performed as described by Maniatis et al. (16). Yeast DNAs were prepared as described by Sherman et al. (23). Poly(A)-containing RNA was purified from logarithmically growing yeast cells by oligo(dT) cellulose chromatography as described previously (21). The S1 nuclease-mapping ex-

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TABLE 1. Yeast strains

Strain	Genotype	Source
AB972	*MATα CUP1 gal2 mal mel SUC2*	M. V. Olson
BJ490	*MATα pep3-12 trp1*	E. Jones
A192	*MATa aro7 can1 gal2 leu2 met14 ura3 SUC2*	A. Sakai
A192/G	*MATa aro7 can1 gal2::URA3 leu2 met14 ura3 SUC2*	This work
A318	*MATα aro7 can1 leu2 gal2 rgr1-1 ura3 SUC2*	This work
A319	*MATa aro7 can1 leu2 gal2 rgr1-1 ura3 SUC2*	This work
A365[a]	*MATa can1 gal2::URA3 pep3 trp1 ura3 SUC2*	This work
A475	*MATa can1 leu2 met14 gal2 rgr1-Δ2::URA3 trp1 ura3 SUC2*	This work
A476	*MATα aro7 can1 gal2 rgr1-Δ2::URA3 trp1 ura3 SUC2*	This work
A448	*MATα aro7 can1 gal2 trp1 ura3 SUC2*	This work
D9	*MATa/MATα aro7/ARO7 can1/can1 gal2/gal2 leu2/LEU2* MET14/*met14 trp1/trp1 ura3/ura3 SUC2/SUC2*	This work
D9/073-1[b]	*MATa/MATα aro7/ARO7 can1/can1 gal2/gal2 leu2/LEU2 MET14/met14 rgr1-Δ3::URA3/RGR1 trp1/trp1 ura3/ura3 SUC2/SUC2*	This work
D9/083-1[c]	*MATa/MATα aro7/ARO7 can1/can1 gal2/gal2 leu2/LEU2 MET14/met14 rgr1-Δ2::URA3/RGR1 trp1/trp1 ura3/ura3 SUC2/SUC2*	This work
D9/083-1-1A[d]	*MATa can1 leu2 met14 gal2 rgr1-Δ2::URA3 trp1 ura3*	This work
D9/083-1-1B[d]	*MATα aro7 can1 gal2 rgr1-Δ2::URA3 trp1 ura3*	This work
D9/083-1-1C[d]	*MATα can1 gal2 trp1 ura3*	This work
D9/083-1-1D[d]	*MATa aro7 can1 gal2 leu2 met14 trp1 ura3*	This work

[a] Constructed by several backcrosses of BJ490 to A192/G, which carried the *gal2::URA3* integration.
[b] Constructed by transformation of D9 with a *Bgl*II/*Hin*dIII fragment of pYUK073 (representative of D9/073-1, -2, and -3).
[c] Constructed by transformation of D9 with a *Dra*I fragment of pSAK083 (representative of D9/083-1 and -2).
[d] Tetrads germinated by sporulation of the diploid strain D9/083-1.

periment was performed as follows. A 397-base-pair (bp) *Eco*RI-*Dra*II *RGR1* DNA fragment, which carried 288 bp of the upstream region and 109 bp of the coding region, was isolated from pSAK065 and labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase. The labeled DNA was digested with *Xba*I, and the 295-bp fragment, which carried 186 bp of the upstream region and 109 bp of the coding region of *RGR1*, was isolated by agarose gel electrophoresis and used as a probe. Poly(A)$^+$ RNA (10 μg) was hybridized at 42°C with the ^{32}P-labeled probe as described previously (21). The mixture was treated with S1 nuclease (500 U/ml) for 30 min. DNA fragments protected from digestion were analyzed by electrophoresis through a standard DNA sequencing gel.

Cloning the *RGR1* gene. The genomic DNA from S288C was partially digested with *Sau*3AI, and the DNA fragments were cloned into YEp213 (23). DNA from this library was used to transform A319 to leucine prototrophy. The transformants were collected and suspended in a CSM−Leu medium and grown with shaking at 24°C for 2 days. Most of the clumpy cells were removed by allowing the cell suspension to sit undisturbed for 2 min. The supernatant fluid was recovered and inoculated into fresh CSM−Leu medium. This process was repeated three times. From the third supernatant fluid, Leu$^+$ transformants were recovered by plating onto selective plates. Twenty transformants were tested for growth on CSM−Leu plates at 37°C, and two were temperature resistant. Plasmid DNAs were recovered from those transformants, and the two were identical. This plasmid was called pSAK034.

Subclones. pSAK041, pSAK044, pSAK050, and pSAK051 (Fig. 1) are subclones of pSAK034 in the vector YCp50 (20). pSAK090 was a subclone in YEp213 which had the same insert DNA as pSAK050. pSAK035 was a subclone in YIp5. All of the other plasmids shown in Fig. 1 were subclones in pUC19. pSAK083 was constructed by deletion of a 1.1-kbp *Xba*I fragment which contained the carboxy-terminal region of the *RGR1* gene and insertion of the 1,565-bp *Nru*I-*Sma*I fragment of YIp5 containing *URA3* with *Xba*I linkers. pYUK073 was constructed by deleting *Xba*I fragments from pSAK050 and inserting the 1,565-bp *URA3* fragment with *Xba*I linkers.

Sequence analysis. Restriction fragments were cloned into pUC18 and pUC19. The nucleotide sequence was determined by the method of Sanger et al. (22) with a 7-deaza-Sequenase kit (United States Biochemicals) and [α-^{35}S]-dCTP (Dupont, NEN Research Products). All of the DNA sequences presented (see Fig. 3) were determined on both strands.

Pulsed-field gel electrophoreses. Sample preparation was performed by the agarose block method of Carle and Olson (1). Each sample gel was washed with 1 ml of 10 mM Tris hydrochloride (pH 8.0)–0.1 mM EDTA at 30°C for 2 h and subjected to pulsed-field gel electrophoresis. Pulsed-field gel electrophoreses were performed as described by Carle and Olson (1) (orthogonal-field alternation gel electrophoresis) and Chu et al. (4) (contour-clamped homogeneous electric field), with a switching time of 80 s at 180 V for 24 h.

Construction of the disruption strains of *RGR1*. Alleles *rgr1-Δ2::URA3* and *rgr1-Δ3::URA3* were constructed by one-step gene replacement by the method of Rothstein (20). Plasmids pSAK083 and pYUK073 were digested with restriction enzymes to release fragments of *S. cerevisiae* DNA carrying the *URA3* insertion. These fragments were used to transform the diploid strain D9 to uracil prototrophy. Transformants in which one copy of the *RGR1* gene was disrupted were identified by Southern blot analysis (16) of genomic DNA. Ura$^+$ diploids carrying each disruption were sporulated and subjected to tetrad analysis to recover haploid segregants carrying the disruption.

Invertase assay. Invertase activity was assayed as described previously (21). Cells were grown to the mid-log phase in medium containing 5% glucose and used as glucose-repressed cells. One unit of the activity was expressed as 1 μmol of glucose released.

Estimation of reserve carbohydrates. Glycogen determinations were made by inverting a plate of cells over iodine vapors. Quantitative measurements of glycogen and

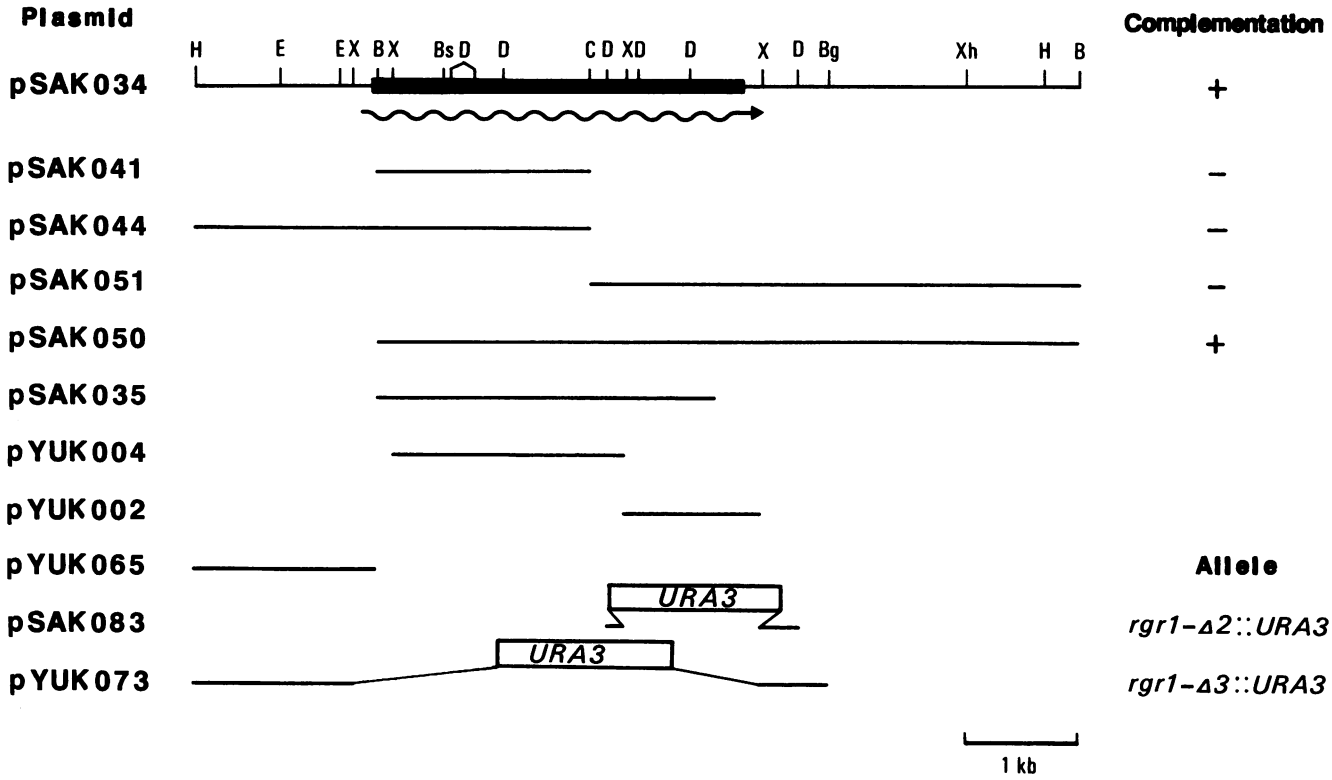

FIG. 1. Restriction maps of *RGR1* clones. Plasmids are described in the text. The wavy arrow indicates the direction and approximate position of transcription of the *RGR1* RNA, and the closed box designates the open reading frame. The allele designations of the chromosomal *rgr1* mutations constructed with each plasmid are indicated. Abbreviations: B, *Bam*HI; Bg, *Bgl*II; Bs, *Bst*EII; C, *Cla*I; D, *Dra*I; E, *Eco*RI; H, *Hin*dIII; X, *Xba*I; Xh, *Xho*I; kb, kilobase.

trehalose were made according to the method of Lillie and Pringle (14). Protein concentration was determined by the method of Lowry et al. (15) with bovine serum albumin as a standard.

RESULTS

Abnormal cell morphology of *rgr1* mutant. As we reported previously, the morphologies of the *rgr1* strains (both haploid and homozygous diploid) were highly abnormal and the *rgr1* cells showed clumpiness even at a permissive temperature (21). Scanning electron microscopy showed that the cell walls of daughter cells remained attached to those of mother cells (Fig. 2). As a result, the *rgr1* strains showed chain-forming phenotypes and grew as grapelike clusters. The number of bud scars of the *rgr1* cells was estimated as approximately 6% of that of wild-type cells (Table 2). Calcofluor staining revealed that a chitin ring was present around the bud neck, and staining of nuclei with 4′,6′-diamidino-2-phenylindole showed that each cell had a single nucleus (data not shown). Ultrathin-section observation indicated that the cell wall of the daughter cell remained fused to that of the mother cell (Fig. 2).

Cloning the *RGR1* gene. An *rgr1 leu2* strain (A319) was transformed with a genomic library cloned in the multicopy vector YEp213 (23). A plasmid that complements *rgr1* should confer a nonclumpy phenotype and temperature-resistant growth (Ts^+). Selection was for nonclumpy cells, which were then screened for the Ts^+ phenotype as described in Materials and Methods. Plasmid DNA was recovered from two transformants, and both of them were identical. The restriction map of the DNA is shown in Fig. 1. In order to test the linkage of the cloned DNA to the *RGR1* locus, an integrative plasmid, pSAK035 (Fig. 1), was constructed. We transformed A448 (*RGR1 ura3*) with pSAK035 DNA digested with *Bst*EII. Two Ura^+ transformants were crossed to A319 (*rgr1 ura3*). Tetrad analysis of the resultant diploid showed that the Ura^+ and Ts^+ phenotypes cosegregated 2:2 in 24 tetrads, demonstrating that pSAK035 had integrated into the genome at a site tightly linked to the *RGR1* locus.

To localize the *RGR1* gene within the cloned DNA, we constructed subclones pSAK041, pSAK044, pSAK050, and pSAK051 (Fig. 1) in a centromeric plasmid vector YCp50 (23) and tested their ability to complement *rgr1* by transforming strain A319 (*rgr1 ura3*). Five Ura^+ transformants for each plasmid were examined. Only pSAK050 complemented the *rgr1* mutation, and the plasmid suppressed all of the phenotypes caused by the *rgr1*.

Sequence and analysis of the *RGR1* gene. The nucleotide sequence of the *RGR1* gene was determined and found to contain an open reading frame of 3,246 bp (1,082 amino acids) that could encode a 123,340-dalton protein. The nucleotide sequence and the predicted amino acid sequence are shown in Fig. 3. The predicted *RGR1* protein was hydrophobic, and six possible weak membrane-spanning regions were detected by using the computer program of Kyte and Doolittle (13). The *RGR1* protein did not have significant homology with any proteins in the National Biomedical Research Foundation and GenBank databases. To identify the RNA encoded by *RGR1*, poly(A)-containing

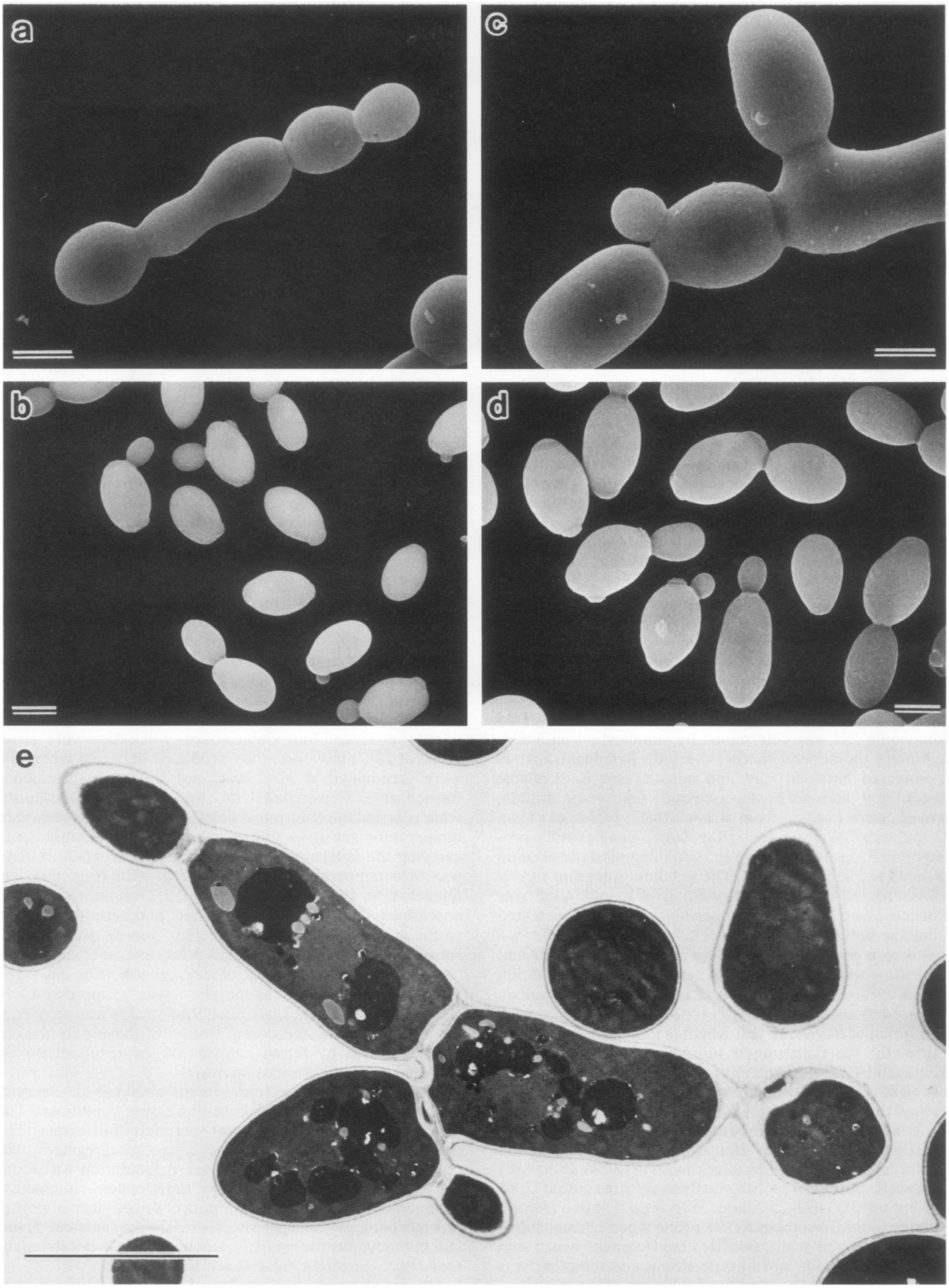

FIG. 2. Scanning (a to d) and transmission (e) electron microscopic observation of *rgr1* cells. Haploid yeast strains A319 (*rgr1-1*) and A448 (*RGR1*) were grown in YPD medium at 24°C. (a) A319 cells showing a diploid budding pattern (magnification, ×3,000); (b and d) A448 cells (magnification, ×3,000 and ×4,000, respectively); (c) A319 cells showing a haploid budding pattern (magnification, ×4,000); (e) A319 cells (magnification, ×15,000). Bars, 5 μm.

TABLE 2. Number of bud scars[a]

Strain	Relevant genotype	No. of cells observed	No. of buds observed	Scars per cell
A448	*RGR1*	312	360	1.15
A319	*rgr1-1*	432	32	0.074

[a] Estimated from the scanning electron micrographs.

RNA was analyzed by Northern hybridization. Probes were prepared from plasmids pYUK002 and pYUK004 (Fig. 1). In each case, an approximately 3.6-kilobase RNA was detected (data not shown). The complementation data imply that this RNA was encoded by *RGR1*. The approximate position of the *RGR1* RNA relative to the map of the cloned DNA is indicated in Fig. 1. A nuclease S1-mapping experiment showed that initiation sites of *RGR1* transcripts were −11, −26, and −36 (data not shown).

The *RGR1* gene is located on chromosome XII. By a series of backcrosses, *rgr1* was found not to be tightly linked to any markers tested. We then localized the *RGR1* gene to a particular chromosome by using a pulsed-field gel electrophoretic system (contour-clamped homogeneous electric field) followed by Southern hybridization with the *RGR1* gene as a probe. Both the *RGR1* probe and the rDNA probe clearly hybridized to the same DNA band corresponding to chromosome XII (Fig. 4). Orthogonal-field alternation gel electrophoresis blots with the same *RGR1* probe, in which chromosome XII was not resolved, showed no hybridization to any band but strong hybridization to the well (data not shown). These results support the localization of the *RGR1* gene on chromosome XII.

Genetic map position of *rgr1*. The map position of *rgr1* on chromosome XII was determined by standard tetrad analysis. Among the several markers tested, significant linkage was detected between *rgr1* and *pep3* (Table 3). Linkage between *rgr1* and *gal2* was assessed. Our yeast strains, however, were derived from strain S288C, which carries a *gal2* mutation. We subcloned the *GAL2* gene (kindly provided by J. F. Tschopp) into YIp5 and integrated the plasmid into the *GAL2* locus in A192. The resultant integrant (A192/G) was crossed several times with BJ491, and A365 was constructed as a *GAL2*::*URA3 pep3* strain. Then, we tested the linkage between *rgr1* and *GAL2*::*URA3* (Table 3). *rgr1* mapped to a position 20.9 centimorgans distal to *gal2* and 18.5 centimorgans centromeric to *pep3*. There is no mutation reported at this map position; *rgr1* is therefore a previously unreported locus.

Disruption of *RGR1*. To test whether *RGR1* is essential for cell growth, we constructed null alleles. pYUK073 was constructed by deleting the entire *RGR1* gene (from nucleotides −186 to 3448; Fig. 3) by digestion with *Xba*I and inserting the 1,565-bp *URA3* fragment with *Xba*I linkers (Fig. 1; see Materials and Methods). A wild-type diploid strain D9 (*ura3/ura3*) was transformed to Ura$^+$ with the DNA fragment generated by digesting pYUK073 with *Bgl*II and *Hin*dIII. Southern blotting analysis of three stable Ura$^+$ transformants (D9/073-1, -2, and -3) showed that two *Hin*dIII fragments hybridized to an *RGR1* probe when chromosomal DNA was digested with *Hin*dIII. One fragment was 7 kbp and was also found in a wild-type strain, and the other was a 5.1-kbp fragment generated by integration of the disrupted gene at the *RGR1* locus (Fig. 5a; only D9/073-1 data are shown). D9/073-1, -2, and -3 are therefore heterozygous for *RGR1* with the genotype of *RGR1*/*rgr1*-Δ*3*::URA3. These strains were sporulated and dissected to separate the four haploid meiotic spores that were allowed to germinate on a YPD plate at 24°C. Of 12 dissected asci, 8 produced only two spores and the other 4 produced only one spore able to form colonies (Fig. 5b; only D9/073-1 data are shown), and these colonies were all Ura$^-$ (data not shown). Microscopic observation showed that the spores which were not able to form colonies stopped growing after several buddings. D9/073-1 cells were transformed with YEp213, pSAK090, and pSAK034 to Leu$^+$, and the transformants were sporulated and dissected. Dissected asci of the YEp213 transformants produced only two spores able to form colonies, and they were all Ura$^-$. On the other hand, dissected asci of the pSAK090 and pSAK034 transformants produced four spores able to form colonies, and Ura$^-$ and Ura$^+$ colonies were segregated 2:2 (data not shown). These results suggest that the *RGR1* gene is essential for cell growth.

Next, we constructed a carboxy-terminal (C-terminal) deletion of *RGR1*. A C-terminal DNA sequence (from nucleotides 2239 to 3448; from amino acid 747 to the end of the C-terminal region, including some untranslated sequence; Fig. 3) was deleted by digesting pSAK034 with *Xba*I and inserting the 1,556-bp *URA3* fragment with *Xba*I linkers at this site (pSAK083, Fig. 1). The wild-type diploid strain D9 was transformed with the DNA fragment generated by digesting pSAK083 with *Dra*I. Southern blot analysis of genomic DNA from two stable Ura$^+$ transformants (D9/083-1 and -2) showed two fragments which hybridized to a *RGR1* probe when the DNA was digested with *Bgl*II and *Cla*I. A 2.1-kbp fragment was also found in a wild-type strain, and a 2.5-kbp fragment resulted from integration of the C-terminal deletion of the *RGR1* gene (Fig. 6a). The heterozygous diploid strains were sporulated, and dissected asci were analyzed. Most of the dissected asci (21 out of 25 tetrads) produced four viable spores when they were germinated at 24°C; however, they produced only two when they were germinated at 37°C (data not shown). The colonies formed at 37°C were all Ura$^-$, indicating that the spores which carried the C-terminal deletion of the *RGR1* gene were temperature sensitive for germination. The mutant strain carrying the C-terminal deletion of *RGR1* (*rgr1*-Δ*2*::*URA3*) was also temperature sensitive for growth (Fig. 6b). The temperature sensitivity segregated 2:2 in all tetrads and cosegregated with the *URA3* marker. Southern blot analysis of the genomic DNA from the four spores of one tetrad showed that the *rgr1*-Δ*2*::*URA3* deletion cosegregated with the temperature sensitivity of cell growth (Fig. 6b). This temperature-sensitive phenotype was suppressed by pSAK090. One of the *rgr1*-Δ*2*::*URA3* strains was crossed to a wild-type strain, and cosegregation of these two markers was confirmed by tetrad analysis of the resultant diploid followed by Southern blot analysis.

Four spores of one tetrad were assayed for secreted invertase activity under glucose-repressing conditions. Two spores showed glucose-resistant secretion of invertase. This glucose repression resistance also cosegregated with *rgr1*-Δ*2*::*URA3* (Table 4). The *rgr1*-Δ*2* mutation was found to be recessive to the wild-type *RGR1* allele. It failed to complement *rgr1-1* for temperature sensitivity, abnormal cell morphology, and glucose repression resistance. A diploid homozygous for *rgr1*-Δ*2*::*URA3* failed to sporulate, and *rgr1-1*/*rgr1*-Δ*2*::*URA3* also failed to sporulate.

Amount of reserve carbohydrates. During the linkage analysis, we found that compared with the wild type, the *rgr1* strain showed reduced staining with iodine vapor, suggesting that the *rgr1* mutant strain contains less glycogen. The

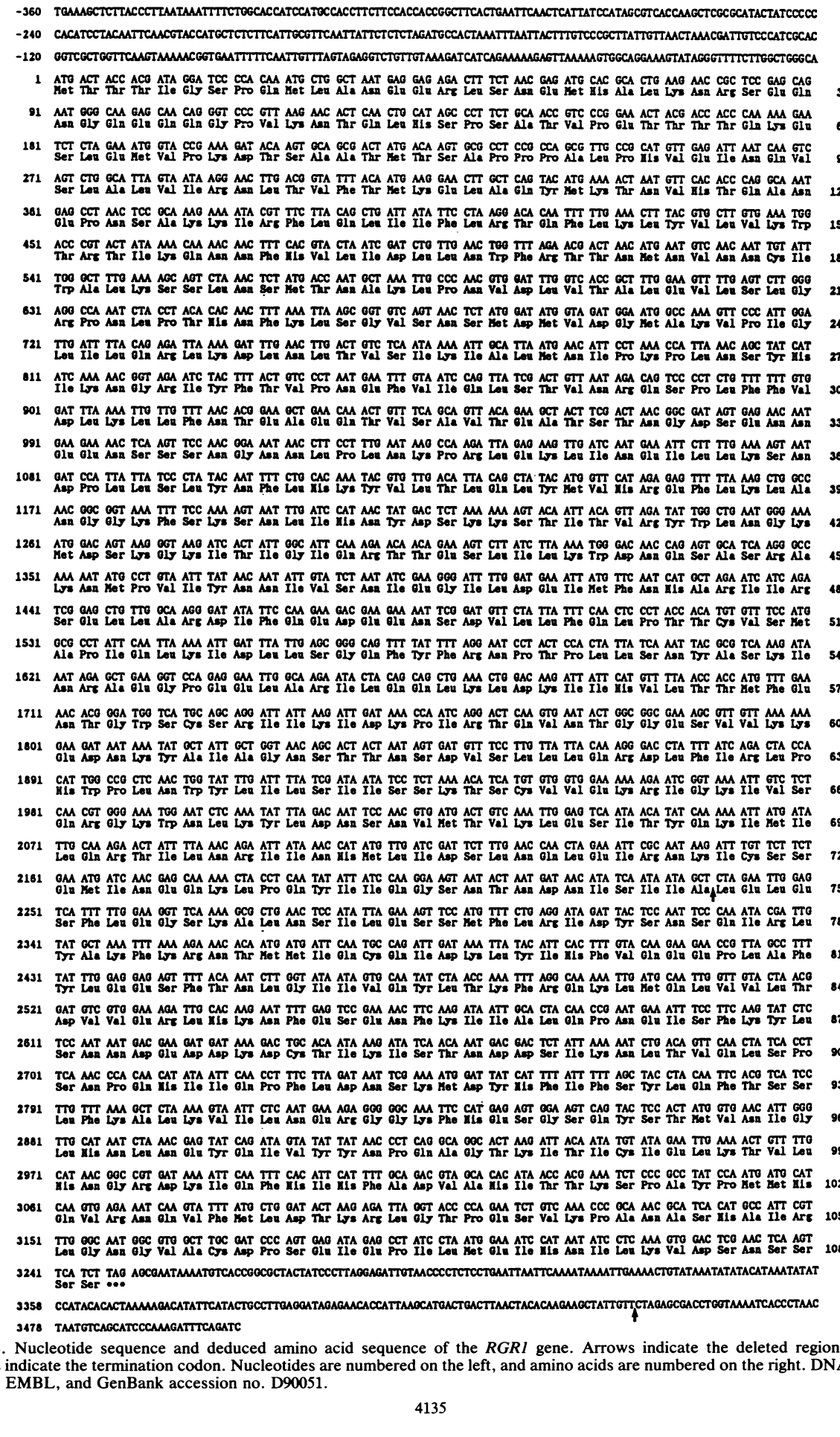

FIG. 3. Nucleotide sequence and deduced amino acid sequence of the *RGR1* gene. Arrows indicate the deleted region of *rgr1*-Δ*2*. Asterisks indicate the termination codon. Nucleotides are numbered on the left, and amino acids are numbered on the right. DNA Data Bank of Japan, EMBL, and GenBank accession no. D90051.

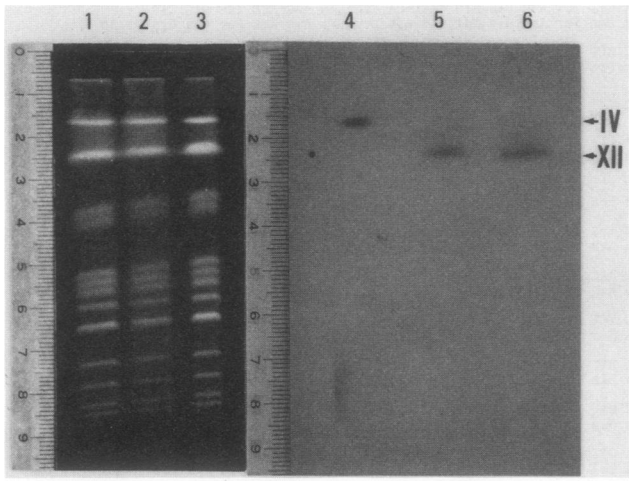

FIG. 4. Physical mapping of *RGR1* to chromosome XII. Yeast chromosomal DNA of strain AB972 was separated by using a contour-clamped homogeneous electric field apparatus (see Materials and Methods) followed by Southern hybridization. Lanes 1 to 3, Ethidium bromide staining; lanes 4 to 6, corresponding autoradiographs. Lane 4, Hybridization with a ^{32}P-labeled *TRP1* gene probe (1.45-kbp *Eco*RI fragment); lane 5, hybridization with a ^{32}P-labeled *RGR1* gene probe (1.1-kbp *Xba*I fragment of pYUK002); lane 6, hybridization with a ^{32}P-labeled rDNA gene probe (1.7-kbp *Stu*I fragment of 25S rDNA). Roman numerals on the right indicate chromosome numbers. The units on the left are centimeters.

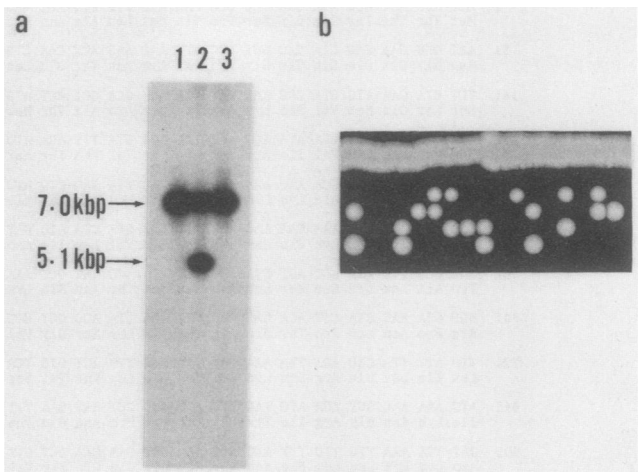

FIG. 5. Complete deletion of *RGR1* gene. (a) Southern blotting analysis of the null allele of *RGR1* gene. Total cellular DNA was prepared from the wild-type diploid strain D9 (*RGR1*/*RGR1*), the heterozygous null diploid strain D9/073-1 (*RGR1*/*rgr1-Δ3*::*URA3*), and the wild-type haploid strain A448 (*RGR1*). DNA digested with *Hin*dIII was electrophoresed and hybridized with a ^{32}P-labeled 7-kbp *Hin*dIII fragment of pSAK034. Lane 1, D9; lane 2, D9/073; lane 3, A448. The 7.0- and 5.1-kbp bands correspond to fragments of the wild-type allele of *RGR1* and the null allele *rgr1-Δ3*::*URA3*, respectively. (b) Dissection of haploid progeny derived from D9/073-1. D9/073-1 diploid cells were sporulated, and tetrads were dissected by micromanipulation. The four spores from individual asci are aligned vertically. The spores were allowed to germinate on a YPD plate at 24°C for 12 days.

reduced staining with iodine vapor and the *rgr1* (both *rgr1-1* and *rgr1-Δ2*::*URA3*) mutations cosegregated 2:2 in more than 50 tetrads. The amounts of storage carbohydrates (glycogen and trehalose) were measured. Data on the amounts of glycogen and trehalose are presented in Table 5. Both reserve carbohydrates in the *rgr1* cells were reduced to approximately 40% of amounts in *RGR1* cells.

DISCUSSION

We examined the abnormal cell morphology of *rgr1* cells by electron microscopy. The cell walls of the daughter cells of *rgr1* cells remained fused to those of mother cells. This can explain why the *rgr1* cells showed a chain-forming phenotype and grew as a grapelike cluster. The fewer bud scars in *rgr1* compared with *RGR1* cells (Table 2) is consistent with the cell wall fusion phenotype of the mutant strain. The clumpiness phenotype of the *rgr1* cells is also consistent with the above morphological characteristics. In *S. cerevisiae*, each new bud of a haploid cell arises adjacent to the bud of the previous cell cycle, whereas **a**/α diploid cells bud at the opposite pole (6, 9). The orientation of the bud emergence in haploid *rgr1* cells is a mixture of both haploid and diploid types. The *rgr1* mutation therefore also affects the orientation of bud emergence. Several genes which affect cell morphology and budding pattern are known. Among them, a mutation in the *CDC4* gene causes a multibud phenotype similar to that caused by *rgr1*. This phenotype of *cdc4* cells is, however, shown only when the cell cycle is arrested at a restrictive temperature (8, 18). Differing from the *rgr1* cells, a single nucleus is present in the mother cell of arrested *cdc4* cells and the resultant daughter buds have no nuclei (8). The cell wall fusion phenotype is also observed in *cdc3*, *cdc10*, *cdc11*, and *cdc12* mutants at the restrictive temperature (8). It is interesting to note that the *cdc24* mutation, allelic to *cls4* (17), causes a random budding pattern even at a permissive temperature (18). Even though the *rgr1* mutant cells did not show cell cycle phenotypes, they showed all of the morphological abnormalities associated with these *cdc* mutations. The role of the *RGR1* and these *CDC* genes in cell morphology remains to be clarified.

TABLE 3. Genetic mapping of *rgr1-1* on chromosome XII

Gene pair	No. of ascus type[a]:			Map distance (cM)[b]
	PD	NPD	T	
rgr1-pep3	56	0	33	18.5
rgr1-gal2	30	1	12	20.9[c]

[a] Abbreviations: PD, parental ditype; NPD, nonparental ditype; T, tetratype.

[b] Genetic map distances in centimorgans (cM) were calculated by the following equation: cM = [(T + 6NPD)/2(PD + NPD + T)] × 100.

[c] This map distance was relatively inaccurate since the *gal2* locus had *GAL2*-YIp5 integrated.

We cloned the *RGR1* gene and localized it on the right arm of chromosome XII, 20.9 centimorgans distal to *gal2* and 18.5 centimorgans centromeric to *pep3* (Table 3). Because there is no mutation reported at this map position, *rgr1* is a new locus.

The nucleotide sequence of the *RGR1* gene contains a 3,246-bp open reading frame (Fig. 3). The 3,600-nucleotide poly(A)-containing RNA corresponds to the size expected from the nucleotide sequence. The predicted 123-kilodalton *RGR1* protein showed no significant homology to any other protein in databases we have searched.

A null allele of *RGR1* (*rgr1-Δ3*) showed a lethal phenotype, indicating that the *RGR1* gene is essential for cell

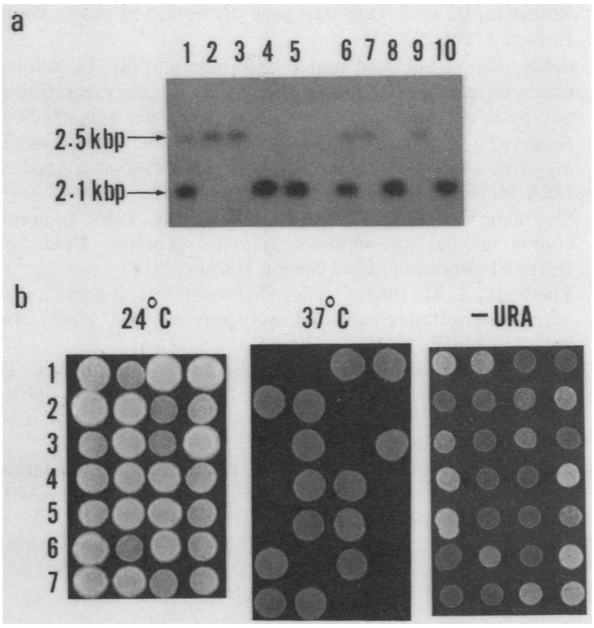

FIG. 6. C-terminal deletion of *RGR1* gene. (a) Southern blotting analysis. Total cellular DNA digested with *Bgl*II and *Cla*I was electrophoresed and hybridized with a ^{32}P-labeled 2.1-kbp *Bgl*II-*Cla*I fragment of pSAK034. Lane 1, D9/083-1 (*RGR1*/*rgr1*-Δ*2*::*URA3*); lane 2, D9/083-1-1A (*rgr1*-Δ*2*::*URA3*); lane 3, D9/083-1-1B (*rgr1*-Δ*2*::*URA3*); lane 4, D9/083-1-1C (*RGR1*); lane 5, D9/083-1-1D (*RGR1*); lane 6, D9/083-1-1A × A448 diploid; lanes 7 to 10, haploid progeny of one tetrad derived from D9/083-1-1A × A448 diploid. (b) The *rgr1*-Δ*2* mutation caused temperature-sensitive growth. The haploid cells from seven tetrads of D9/083 diploid cells germinated at 24°C were suspended in water and spotted on plates and incubated—from left to right, on a YPD plate at 24°C, on a YPD plate at 37°C, and on a CSM−Ura plate at 24°C. The four spores from individual asci are aligned horizontally.

growth (Fig. 5). A carboxy-terminal deletion (*rgr1*-Δ*2*) allele of *RGR1* caused pleiotropic effects similar to, but more severe than, those caused by the *rgr1-1* mutation. These include temperature-sensitive growth, resistance to glucose repression, abnormal morphology, and sporulation deficiency. This C-terminal deletion was recessive to and complemented by the *RGR1* gene.

The amounts of reserve carbohydrates in *rgr1* cells were reduced to 40% of the levels in *RGR1* cells. It is well known that the enzymes catalyzing the degradation of reserve carbohydrates, glycogen phosphorylase and trehalase, are activated by cyclic AMP (cAMP)-dependent phosphorylation (10, 24–26). It will be interesting to investigate whether the reduction in amounts of reserve carbohydrates in *rgr1* cells is associated with an elevated level of intercellular cAMP. We have in fact recently found that the intercellular cAMP level in *rgr1* cells was higher than that in wild-type cells (unpublished data). Our working hypothesis is that the expression of the *SUC2* gene is controlled by *SNF* proteins, which in turn are regulated by intercellular cAMP levels through the *RAS*-cAMP pathway. The *RGR1* protein may affect the expression of the *SUC2* gene through the interaction with the *RAS*-cAMP pathway.

TABLE 4. Glucose repression-resistant secretion of invertase caused by C-terminal deletion of *RGR1*

Strain[a]	Relevant genotype	Growth at 37°C[b]	Invertase activity (U/min per mg of dry weight)
D9/083-1A	*rgr1*-Δ*2*	−	2.2
D9/083-1B	*rgr1*-Δ*2*	−	1.8
D9/083-1C	*RGR1*	+	<0.1
D9/083-1D	*RGR1*	+	<0.1

[a] Yeast cells were grown in the presence of 5% glucose (see Materials and Methods).
[b] +, Growth; −, no growth.

TABLE 5. Glycogen and trehalose content

Strain	Relevant genotype	Amt (μg/mg of protein)[a] of:	
		Glycogen	Trehalose
A448	*RGR1*	73	1,000
A475	*rgr1*-Δ*2*	30	433

[a] Amounts of glycogen and trehalose were expressed as micrograms of glucose liberated on hydrolysis of the polysaccharide.

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