

# Systematic Identification of the Genes Affecting Glycogen Storage in the Yeast *Saccharomyces cerevisiae*

IMPLICATION OF THE VACUOLE AS A DETERMINANT OF GLYCOGEN LEVEL\*<sup>§</sup>

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**At the onset of nutrient limitation, the yeast *Saccharomyces cerevisiae* synthesizes glycogen to serve as a carbon and energy reserve. We undertook a systematic survey for the genes that affect glycogen accumulation by taking advantage of the strain deletion set generated by the *Saccharomyces* Genome Deletion Project. The strain collection analyzed contained some 4600 diploid homozygous null deletants, representing ~88% of all viable haploid disruptants. We identified 324 strains with low and 242 with elevated glycogen stores, accounting for 12.4% of the genes analyzed. The screen was validated by the identification of many of the genes known already to influence glycogen accumulation. Many of the mutants could be placed into coherent families. For example, 195 or 60% of the hypoaccumulators carry mutations linked to respiratory function, a class of mutants well known to be defective in glycogen storage. The second largest group consists of ~60 genes involved in vesicular trafficking and vacuolar function, including genes encoding 13 of 17 proteins involved in the structure or assembly of the vacuolar ATPase. These data are consistent with our recent findings that the process of autophagy has a significant impact on glycogen storage (Wang, Z., Wilson, W. A., Fujino, M. A., and Roach, P. J. (2001) Antagonistic controls of autophagy and glycogen accumulation by Snf1p, the yeast homolog of AMP-activated protein kinase, and the cyclin-dependent kinase Pho85p. *Mol. Cell. Biol.* 21, 5742–5752). Autophagy delivers glycogen to the vacuole, and we propose that the impaired vacuolar function associated with ATPase mutants (*vma10* or *vma22*) results in reduced degradation and subsequent hyperaccumulation of glycogen. *Molecular & Cellular Proteomics* 1: 232–242, 2002.**

One attractive feature of the budding yeast *Saccharomyces cerevisiae* as an experimental organism has been its ease of genetic manipulation, including the ability to perform genetic screens by which yeast strains with a phenotype of interest

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can be recognized. Experimentally, the next phase involves identification of the gene(s) responsible, a task that can be time-consuming and sometimes non-trivial. The *S. cerevisiae* genome contains some 6,200 open reading frames (ORFs),<sup>1</sup> and these have been disrupted systematically in the *Saccharomyces* genome deletion project (1). The availability of the resulting set of deletion strains, each carrying a deletion of one specific ORF, permits a totally different type of screen or survey for genes linked to a particular phenotype. Most importantly, the survey is systematic. The first report of using a partial strain deletion set analyzed growth rates in rich and minimal medium (1). Recently, an analysis of the rapamycin sensitivity of 2,216 haploid disruptants was reported (2), and a systematic study of fluid phase endocytosis using around 700 strains generated by the European Functional Analysis Network has been conducted (3). The first truly genome-wide screen of a defined null mutant collection was reported by Ni and Snyder (4) who analyzed over 4000 strains in a study of polarized growth. Information is also available about viability, based on analysis of the deletion strain set, and is available from the *Saccharomyces* Genome Database (5) ([genome-www.stanford.edu/Saccharomyces](http://genome-www.stanford.edu/Saccharomyces)). We report here the use of a specific metabolic end point, the ability to store glycogen, as the basis for a screen of ~4600 homozygous diploid mutants to identify genes that affect glycogen accumulation.

Glycogen serves as a reserve of glucose. Its accumulation is initiated under conditions of nutrient limitation, such as the approach to stationary phase in liquid culture. Limitation for carbon, nitrogen, phosphorous, or sulfur all act as triggers for increased glycogen synthesis (6). Our laboratory has been interested in glycogen as an example, in mammals and yeast, of a compound whose synthesis and utilization is under complex and intricate controls linked to the intracellular energy state, as well as the nutritional status of the environment (see Ref. 7 for a review). Synthesis of glycogen requires the activities of glycogenin, a self-glucosylating initiator protein (encoded by *GLG1* and *GLG2*; see Ref. 8), glycogen synthase (*GSY1* and *GSY2*; see Ref. 9), which catalyzes bulk synthesis, and the branching enzyme (*GLC3*; see Ref. 10), which intro-

<sup>1</sup> The abbreviations used are: ORF, open reading frame; PMSF, phenylmethylsulfonyl fluoride.

duces the branches characteristic of the mature polysaccharide. Glycogen breakdown requires glycogen phosphorylase (Gph1p; see Ref. 11) and debranching enzyme (Gdb1p; see Refs. 12 and 13) or, under certain conditions, glucoamylase (Sga1p; see Ref. 14). However, the enzymes of glycogen metabolism are under a variety of transcriptional and post-translational controls, and so genes encoding a number of other proteins affect glycogen accumulation (for a review, see Ref. 7). For example, the cyclic AMP pathway controls both gene expression and phosphorylation of key proteins (15–17). Starvation, as sensed by the Tor pathway, stimulates glycogen accumulation (18). Signaling through the Snf1p and Pho85p protein kinases has antagonistic effects on glycogen storage (19). The genes mentioned above have been identified by a combination of conventional biochemical and genetic approaches, and there is no guarantee that all relevant genes have been found or any indication as to how many genes affect glycogen storage. The systematic survey described in this work indicated that 566 of ~4600 strains from the homozygous diploid release of the deletion library had glycogen levels that differed from wild type. Of these genes, a surprising fraction, about 10%, had functions related to vesicular trafficking or vacuolar function.

#### MATERIALS AND METHODS

**Strains and Media**—The homozygous diploid deletion series (BY4743 strain background) was purchased from Research Genetics. The yeast deletion series comprises a set of mutants where each open reading frame has been disrupted from start to stop codon, and a *kanMX* marker cassette (conferring resistance to the antibiotic G418) has been inserted. The library of deletions was supplied frozen in 96-well microtiter plates. Each well contained 200  $\mu$ l of YPD (2% peptone, 2% glucose, 1% yeast extract) medium supplemented with G418 (200  $\mu$ g/ml) and 15% glycerol. For keying, contamination checks and orientation purposes, two wells per plate contained only medium. One empty well defines the bottom left hand corner, and the other serves as an identifier. Complete details regarding series construction and availability can be found at [sequence-www.stanford.edu/group/yeast\\_deletion\\_project/deletions3.html](http://sequence-www.stanford.edu/group/yeast_deletion_project/deletions3.html) and [www.resgen.com/products/YEASTD.php3](http://www.resgen.com/products/YEASTD.php3). The deletion set contained 4,639 different strains, of which 82 were flagged by Research Genetics as quality-control failures. Excluding these, the collection is of 4,557 strains that correspond to 88% of all possible viable mutants. Additionally, we constructed strains derived from BY4741 and BY4742, which are also available from Research Genetics and are the MAT $\alpha$  and MAT $\alpha$  parents, respectively, of BY4743. A polymerase chain reaction strategy (20) was used to generate *apg1::URA3* in BY4741 and *vma10::LEU2* in BY4742. These strains were mated, and tetrad dissection was performed to isolate *apg1::URA3 vma10::LEU2* double mutants of both mating types. The genotypes of the strains isolated were MAT $\alpha$  *his3 leu2 met15 lys2 apg1::URA3 vma10::LEU2* and MAT $\alpha$  *his3 leu2 met15 lys2 apg1::URA3 vma10::LEU2*. These MAT $\alpha$  and MAT $\alpha$  strains were then crossed to generate a diploid strain.

**Growth Conditions**—For analysis, the master plates were thawed, and the cells were resuspended by pipetting up and down using a 12-channel automatic pipette. A 96-pin microplate replicator was used to transfer an aliquot from each well to a fresh microtiter plate containing 200  $\mu$ l of appropriate medium per well. To assess glycogen accumulation, SC medium (0.67% yeast nitrogen base, 0.079%

Complete Supplement Mix (Bio101 Inc.), 2% glucose) was used. Growth on YPG medium (2% peptone, 1% yeast extract, 3% glycerol) was used to assess respiratory competence. For glycogen accumulation studies, plates were prepared in duplicate. The parental wild-type strain (BY4743) was inoculated into well H1 on each plate to serve as an internal control. Plates were incubated at 30 °C for 48 h without shaking.

**Genomic Survey for Aberrant Glycogen Accumulation**—The 54 microtiter plates that constitute the deletion series were analyzed for both glycogen accumulation and ability to grow using glycerol as a carbon source. Preliminary studies with strains known to over- or underaccumulate glycogen were used to optimize the growth and staining conditions. Growth of cells in the microplate format was slower than in standard liquid culture in shaking flasks, likely because of a lack of aeration. Thus, to obtain enough cells for analysis of glycogen content by iodine staining, we grew cultures for 48 h. Cells were resuspended by pipetting up and down using a 12-channel automatic pipette. The cell suspensions were then transferred to a 96-well vacuum manifold (Bio-Dot microfiltration unit; Bio-Rad) and filtered onto a nitrocellulose membrane. The membrane was removed and stained for 2 min by exposure to iodine vapor, a time that was found to be suitable for uniform and reproducible results. Each membrane was photographed using a digital camera to create a permanent record, and cultures that stained either more or less intensely than wild type were scored (Fig. 1). Images were downloaded from the camera to an Apple Macintosh G4 microcomputer and processed with Adobe PhotoShop LE. In each case, duplicate plates were grown, harvested, and stained to control for any variability in staining intensity, and only wells that gave the same result on both plates were scored in the final tally. Growth in glycerol was assessed by visual inspection of plates and comparison to the congeneric wild-type strain.

**Mutant Identification**—The deleted gene responsible for the aberrant glycogen phenotype in each case was identified by reference to a spreadsheet compiled from data available at [www.resgen.com/products/YEASTD.php3](http://www.resgen.com/products/YEASTD.php3) where a text file detailing the ORF deletion present in each well of each microtiter plate can be found. Information from the *Saccharomyces* Genome Database (5) ([genome-www.stanford.edu/Saccharomyces](http://genome-www.stanford.edu/Saccharomyces)) and from the YPD database (21, 22) ([www.proteome.com/databases/index.html](http://www.proteome.com/databases/index.html)) was used to pair ORF numbers with gene names and functional properties where known.

**Enzymatic Determination of Glycogen**—For quantitative determination of glycogen levels, cells were grown for 24 h to early stationary phase ( $\sim 1 \times 10^8$  cells/ml) in 10 ml of SC medium at 30 °C. The cell density was checked by counting using a hemocytometer. An aliquot (1 ml) of culture was removed, and the cells were collected by centrifugation (14,000  $\times g$ , 1 min, 4 °C). The supernatant was aspirated, and the cell pellet was immediately frozen on dry ice and stored at –80 °C until use. The culture was maintained for another 24 h, and a second sample (48 h time point) was taken as described. Cell pellets were thawed by addition of 200  $\mu$ l of 20% (w/v) KOH and boiled in a water bath for 1 h. The solution was neutralized by addition of HCl and processed as described previously (23). The data shown are the mean of at least two independent determinations performed in duplicate.

**Determination of Glycogen Synthase Activity**—Glycogen synthase was assayed in extracts prepared from yeast cells by lysis with glass beads using the method of Thomas *et al.* (24) as described previously (25). Phosphorylation of glycogen synthase converts the enzyme into a less active form that requires the presence of the allosteric activator glucose 6-phosphate to elicit full activity. Thus, the ratio of activity without and with glucose 6-phosphate (–/+ glucose 6-phosphate activity ratio) is an index of the phosphorylation state of the enzyme with high values indicating that dephosphorylated and active glycogen synthase predominates.

**Induction of Autophagy, Microscopy, and Image Capture**—To in-

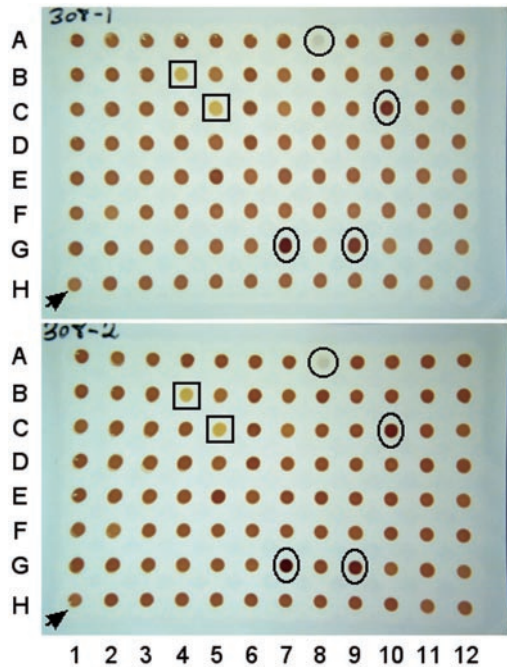


FIG. 1. Screen for glycogen accumulation using a 96-well microtiter plate format. Cells were cultured in 96-well microtiter plates and collected by filtration onto nitrocellulose membranes. The nitrocellulose membranes were stained by exposure to iodine vapor to assess glycogen accumulation, and wells staining either more (ovals) or less (squares) intensely than the well containing the wild-type strain (arrowheads) were scored. The empty keying well, which identifies the plate number in the library, is marked with a circle.

duce autophagy, cells were grown to late logarithmic phase in YPD medium and then starved for nitrogen as described by Takeshige *et al.* (26) either in the presence or absence of 1 mM phenylmethylsulfonyl fluoride (PMSF). For analysis of *vma10*, *vma22*, and *apg1 vma10* mutants, cells were grown to logarithmic phase or to saturation in SC medium. Cells were examined under a Nikon Microphot-FXA microscope, equipped with Nomarski optics, using a  $\times 60$  oil-immersion objective at a magnification of  $\times 1200$ . Images were captured using a Pulnix TM-745 digital camera and the public domain NIH Image 1.62 software (developed at the United States National Institutes of Health and available on the Internet at [rsb.info.nih.gov/nih-image/](http://rsb.info.nih.gov/nih-image/)) running on a Macintosh G4 microcomputer. Movies were prepared and edited using QuickTime Pro 5.0.1 software from Apple Computer.

RESULTS

Overview—The strain deletion set was surveyed, in duplicate, for glycogen accumulation using iodine staining of cells harvested after growth in microtiter plates and filtration through nitrocellulose filters, as described under “Materials and Methods” (see Fig. 1). In addition, the ability to grow on glycerol was monitored. The rationale for including this assay was the well established observation that cells unable to grow using non-fermentable carbon sources cannot store glycogen, usually because of respiratory defects in what are termed petite mutants (27, 28). In this way, we could place mutants in this category, even if the corresponding gene had no known function.

TABLE I  
Gene families

The mutants that were identified in the screen were assigned to various different families. For details of all family members and annotations, see Supplemental Material.

Description	Total number	Glycogen phenotype	
		Low	High
Mitochondrial or respiratory	207	195	12
Vesicular transport or vacuolar function	58	16	42
Carbohydrate metabolism	12	3	9
Amino acid metabolism	10	1	9
Adenine metabolism	4	0	4
Inositol metabolism	7	6	1
Miscellaneous metabolism	22	10	12
Protein kinases	14	6	8
Protein phosphatases	8	5	3
WD-40 repeat proteins	7	3	4
Other signaling	10	4	6
Ubiquitination	3	1	2
Cytoskeleton	5	2	3
Transport, pore proteins	8	1	7
Sporulation	4	1	3
Transcription, RNA processing	35	17	18
Small ribosomal subunit	12	0	12
Chromatin, DNA structure	18	7	11
Known function, miscellaneous	22	7	15
Unknown function, little, or nothing known	100	39	61
Total	566	324	242

From the 4557 strains examined, we recovered 242 with high glycogen and 324 with low glycogen so that a total of 12.4% of the gene deletions influenced glycogen. We attempted to cluster the mutants into logical families (Table I). In some cases the families are clearly meaningful, with the presence of multiple genes of related function reinforcing the validity of their identification in the survey. In other instances, we grouped proteins according to biochemical function, such as “protein kinases,” where common biological functions were not obvious. A large group of 100 mutants, representing almost 18% of the genes identified, corresponded to ORFs of unknown function, about which little or nothing is known. Thirty-five genes involved in general transcription or RNA processing were identified as were 18 that had to do with DNA structure or maintenance of structural integrity. Our thought is that the mutations in these strains are likely to be only very indirectly linked to glycogen metabolism. Glycogen storage is in part a response to stress, which may be an aspect of the phenotype of these groups of mutants. Such could also be true for the genes encoding small ribosomal subunits, all 12 of which were associated with glycogen hyperaccumulation. Because of the large number of mutants identified, it is not possible to give a thorough description of each (refer to the Supplemental Material for a table containing all of the strains identified and all of our annotations).

A number of mutants were chosen for additional analyses. The selection was made to represent several of the gene families of Table I, together with some subjective choices (Table II). The further analyses included assessment of glyco-



TABLE II  
Further analysis of selected deletion mutants isolated in the screen

The amount of glycogen and the glycogen synthase activities at 24 and 48 h are normalized to the values obtained with wild-type cells at the same time points. For the qualitative determinations of glycogen on YPD and SC plates, wild-type cells were scored as (+). The activity ratio of glycogen synthase in the wild type strain at the 24-h time point was  $0.26 \pm 0.02$  ( $n = 6$ ). ND indicates no determination could be made.

	Original call	Glycogen (iodine) on		Glycogen (enzymatic)		Glycogen synthase		Activity ratio (24 h)
		YPD	SC	24 h	48 h	24 h	48 h	
Protein kinases								
<i>KIN1</i>	Low	+/-	+/-	0.88	2	1.5	0.97	0.11
<i>PTK2</i>	Low	+/-	+	0.91	2.4	1.3	0.4	0.15
<i>RIM15</i>	Low	-	-	0.27	0.41	1	0.45	0.07
<i>CTK1</i>	High	+	+++	ND	7.4		0.43	
<i>DBF2</i>	High	+	++	2.1	4.2	0.82	0.92	0.23
<i>NPR1</i>	High	+	+	1.6	0.73	1	0.72	0.04
<i>TOR1</i>	High	+	+++	1.4	3.4	1.1	1	0.22
<i>YDL025C</i>	High	+	++	0.79	1	1.8	0.52	0.15
<i>YDR247W</i>	High	+	++	1.1	1.7	0.83	1.4	0.28
<i>YNL099C</i>	High	+	+	0.9	1.9	1	0.64	0.18
<i>YOL045W</i>	High	+	++	1.1	3.3	0.76	1.8	0.36
<i>YPL150W</i>	High	++	++	1.5	5.8	1.2	1.8	0.18
Protein phosphatases								
<i>PIG2</i>	Low	+	+/-	0.55	0.26	1.6	0.98	0.09
<i>RTS1</i>	High (very)	+++	+++	2	8.5	1.1	1.3	0.17
<i>YCR079W</i>	High	+	+++	1.6	3.6	1.3	1.4	0.17
WD-40 repeat proteins								
<i>YAR003W</i>	Low	+	++	0.51	0.27	1.1	0.4	0.18
<i>YPL247C</i>	High	+	++	1.4	0.53	1.3	0.87	0.17
<i>YKL121W</i>	High	+	++	1	0.96	1.2	0.74	0.19
<i>YOL087C</i>	High	+	+	0.59	1.72	1.6	0.7	0.21
<i>YOL138C</i>	High (very)	++	+++	0.79	0.52	1.1	0.68	0.55
Vesicular transport and vacuolar function								
<i>BST1</i>	High (very)	+	++	2.1	1.3			
<i>SEC22</i>	High (very)	++	++	2.1	1.3			
<i>VID21</i>	High (very)	+++	+++	6.5	6.8	2	2	0.22
<i>VID22</i>	High (very)	+	+	2.2	2.8			
<i>VMA10</i>	High (very)	++	+	1.8	5.7			
<i>VMA22</i>	High (very)	+	+	1.2	3.3			
<i>VMA3</i>	High	++	+	ND	3.5			
Inositol metabolism								
<i>INO1</i>	Low	+/-	+/-	0.35	0.63	1.2	0	0.04
<i>IPK1</i>	Low	+/-	++	0.6	1.5	1.2	1.4	0.09
Carbohydrate metabolism								
<i>RPE1</i>	High (very)	++	++	1	1.1	0.89	1.2	0.12
<i>YHR204W</i>	High (very)	++	+	1.2	1.6	1.7	1.9	0.09
Others								
<i>ADO1</i>	High (very)	+++	+++	1	5	1.6	1.7	0.31
<i>GIS4</i>	Low	++	+/-	0.71	1.3	1.3	1.9	0.09

gen levels as judged by iodine staining of colonies on either YPD or SC plates, enzymatic determination of glycogen in liquid cultures, and measurement of glycogen synthase activity. These secondary measures of glycogen usually reproduced the original screening result, especially noting that yeast accumulate generally much less glycogen when grown in rich as opposed to synthetic medium. Thus, a number of mutants scored as high glycogen on the original survey with synthetic medium were wild type when grown on YPD plates. A notable exception is provided by some of the vacuolar and vesicular trafficking mutants (see below). Of the mutants selected for further study, few exhibited very great changes in total glycogen synthase activity. Glycogen synthase phosphorylation, and activation state, can be monitored via the

-/+ glucose 6-phosphate activity ratio, lower values correlating with greater phosphorylation (see "Materials and Methods"). Deletion of genes implicated in direct phosphorylation of glycogen synthase, such as *PHO85*, leads to elevated activity ratio. Of the genes tested, only one, a WD-40 repeat protein of unknown function, had a significantly elevated activity ratio, making it a candidate to be a constituent of a glycogen synthase kinase.

*Genes Implicated Previously in Glycogen Storage*—A number of the genes that were isolated in the screen have been implicated previously in the control of glycogen accumulation (Table III), which we view as a validation of the screening methodology. In an earlier genetic screen for aberrant glycogen accumulation, Cannon *et al.* (29) had characterized eight

TABLE III  
Genes isolated by the screen and that had been implicated previously in glycogen accumulation

Gene	Glycogen phenotype	Description	Reference
<i>GAC1</i>	Low	Regulatory subunit for protein serine/threonine phosphatase Glc7	33
<i>GLC3</i>	Low	$\alpha$ -1,4-glucan branching enzyme	29
<i>GLC8</i>	Low	Modulator of protein serine/threonine phosphatase Glc7p	29
<i>GSY2</i>	Low	Major isoform of glycogen synthase	9
<i>IRA2 (GLC4)</i>	Low	GTPase activating protein for Ras1p and Ras2p	29
<i>PIG2</i>	Low	Protein interacting with Gsy2p; possible regulatory subunit for the protein serine/threonine phosphatase Glc7p	35
<i>RIM15</i>	Low	Serine/threonine protein kinase; positive regulator of <i>IME2</i> expression and sporulation	40
<i>SNF1 (GLC2)</i>	Low	Serine/threonine protein kinase essential for derepression of glucose-repressed genes	29
<i>GDB1</i>	High	Glycogen debranching enzyme	13
<i>GPH1</i>	High	Glycogen phosphorylase	11
<i>PFK1</i>	High	Phosphofructokinase $\alpha$ subunit	32
<i>REG1</i>	High	Regulatory subunit for protein phosphatase Glc7p, required for glucose repression	38
<i>TPS1 (GLC6)</i>	High	Trehalose 6-phosphate synthase	29

so-called *glc* mutants and identified the corresponding genes. Five *GLC* genes were found in our screen (Table III). Of the three remaining *glc* mutants, two were not represented in the deletion series (*GLC7* is essential, and *GLC5/IRA1* was missing) and one, *glc1/ras2*, was present but had wild-type levels of glycogen in this genetic background under the growth conditions used.

Genes encoding four enzymes involved directly in glycogen metabolism were identified. Strains defective for the degradative enzymes, glycogen phosphorylase, Gph1p, and debranching enzyme, Gdb1p, were hyperaccumulators. The strain lacking *GSY2*, which encodes the predominant isoform of glycogen synthase, had low glycogen storage as did the strain lacking the glycogen branching enzyme encoded by *GLC3*. The *PFK1* and *TPS1* genes encode components of phosphofructokinase and the trehalose synthase complex, respectively. Both *pfk1* and *tps1* mutations result in elevated intracellular glucose 6-phosphate (30, 31). It is probable that the increased glucose 6-phosphate levels bypass the phosphorylation control of glycogen synthase resulting in deregulated, hyperactive glycogen synthase (see for example Ref. 32).

Four putative or actual targeting/regulatory subunits for the type 1 protein phosphatase Glc7p were identified. Of these, the best characterized is Gac1p, a protein that targets Glc7p to dephosphorylate and activate glycogen synthase (33, 34). The *GLC8* gene encodes a protein related in sequence to mammalian I-2, a constituent of a cytosolic form of type I protein phosphatase (29). The putative Glc7p targeting protein Pig2p was isolated first in a two-hybrid screen for proteins that interacted with yeast glycogen synthase (35). Previous studies had not revealed any substantial role for Pig2p in glycogen accumulation, but this may reflect differences in the strain backgrounds used. The fourth protein phosphatase non-catalytic subunit isolated was Reg1p, which Tu and Carlson (36) had shown to physically associate with Glc7p and which had previously been implicated in glucose repres-

sion of gene expression (37). Our laboratory had also isolated *reg1* mutants as suppressors of the *glc7-1* glycogen accumulation defect (38). The role of *SNF1* in controlling glycogen synthase is well established and is thought to result both from a negative control of the phosphorylation of glycogen synthase (17) and a positive control of autophagy, which normally preserves glycogen (39). Strains lacking *snf1* are therefore defective in glycogen storage. Likewise, the PKA pathway has long been known to play a role in glycogen accumulation, with PKA negatively regulating transcription of *GSY2* and other enzymes of glycogen metabolism (15–17). Rim15p is proposed to function downstream of PKA, as a regulator of entry into stationary phase, and *rim15* mutants had been reported to have low levels of glycogen and the other storage carbohydrate, trehalose (40).

**Genes Required for Respiratory Growth**—Of the 324 mutants with reduced glycogen stores, 60% of the disruptions were in genes that were either known to be required for respiratory growth (e.g. those encoding components of the mitochondrion) or were found in the screen to be unable to grow with glycerol as a carbon source. Many mutants that fail to utilize non-fermentable carbon sources actually synthesize glycogen (28), but this glycogen is used to fuel growth upon exhaustion of glucose, because, unlike wild-type cells, respiratory mutants cannot oxidize the ethanol produced in the initial growth phase. It is noteworthy that we found 11 mutants that were unable to grow using glycerol but had increased glycogen. It is possible that these mutants are defective in glycogen breakdown and therefore might be worthy of future study.

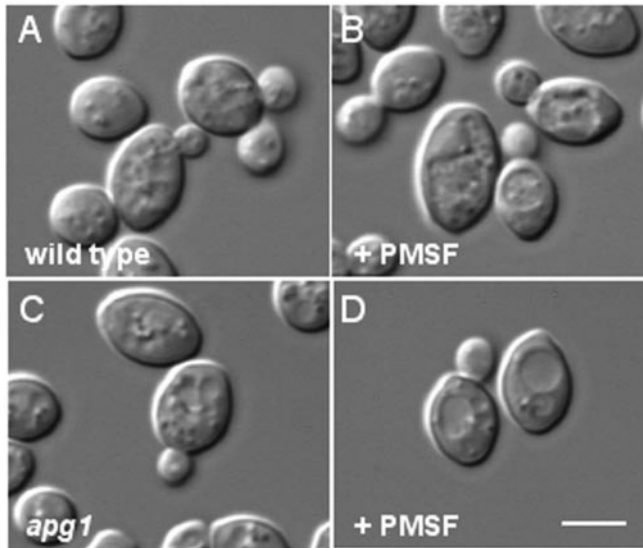
**Other Protein Kinases and Phosphatases**—Protein kinases linked to glycogen accumulation have the potential to be involved in regulatory pathways, like Snf1p or Rim15p, or the direct phosphorylation of metabolic enzymes. Thirteen conventional protein Ser/Thr kinases were recovered in the screen, six with reduced glycogen (including the *snf1* and *rim15* mutants discussed above) and seven with increased glycogen. Of those

TABLE IV  
Genes identified in the screen and that have roles in vesicle trafficking or vacuole function

Vesicular transport and vacuolar function			
Gene	Glycogen	ORF	Comments
<i>DID4</i>	Low	YKL002W	Vacuolar protein sorting
<i>DOA4</i>	Low (very)	YDR069C	Ubiquitin C-terminal hydrolase
<i>PEP12</i>	Low	YORO36W	tSNARE
<i>PEP3</i>	Low	YLR148W	Vacuolar protein sorting and required for vacuole biogenesis
<i>PEP7</i>	Low	YDR323C	Vacuole inheritance and vacuole protein sorting
<i>SNF7</i>	Low	YLR025W	Glucose derepression and protein sorting
<i>VID28</i>	Low	YIL017C	Vacuolar import and degradation
<i>VPS20</i>	Low	YMR077C	Similarity to Snf7p
<i>VPS28</i>	Low	YPL065W	Protein transport from prevacuolar endosome
<i>VPS33</i>	Low	YLR396C	Vacuolar protein sorting
<i>VPS34</i>	Low (very)	YLR240W	Phosphatidylinositol 3-kinase required for vacuolar protein sorting
<i>VPS4</i>	Low	YPR173C	Vacuolar protein sorting
<i>VPS45</i>	Low	YGL095C	Protein of the Sec1p family
<i>VPS53</i>	Low	YJL029C	Protein sorting in the late Golgi
<i>ANP1</i>	High	YEL036C	Retention of glycosyltransferases in the Golgi
<i>APL3</i>	High	YBL037W	$\alpha$ -adaptin, in clathrin-associated protein (AP) complex
<i>APL6</i>	High	YGR261C	$\beta$ -adaptin, in clathrin-associated protein (AP) complex
<i>APM3</i>	High	YBR288C	Medium subunit, clathrin-associated protein (AP) complex
<i>APS3</i>	High	YJL024C	Small subunit of the clathrin-associated protein (AP) complex
<i>ARL3</i>	High (very)	YPL051W	Member of the arf-sar family
<i>AUT1</i>	High	YNR007C	Required for autophagy
<i>AUT7</i>	High	YBL078C	Protein of autophagosomes
<i>BFR1</i>	High	YOR198C	Involved in secretion and nuclear segregation
<i>BST1</i>	High (very)	YFL025C	Negatively regulates COPII vesicle formation
<i>ERV14</i>	High	YGL054C	Protein of ER-derived vesicles
<i>LST4</i>	High	YKL176C	Trafficking of nitrogen-regulated permeases
<i>MSB3</i>	High	YNL293W	GTPase activating protein for Sec4p
<i>PIB2</i>	High	YGL023C	Related to Vps27p, Pep7p, Fab1p and Pib1p; unknown function
<i>PMR1</i>	High	YGL167C	Ca <sup>2+</sup> -transporting P-type ATPase of Golgi membrane
<i>RTG2</i>	High	YGL252C	Protein involved in inter-organelle communication
<i>RUD3</i>	High	YOR216C	Hydrophilic protein involved in vesicle docking
<i>SCJ1</i>	High (very)	YMR214W	Homolog of <i>E. coli</i> DnaJ
<i>SEC22</i>	High (very)	YLR268W	Synaptobrevin (v-SNARE) homolog
<i>SEC66</i>	High	YBR171W	Component of ER protein-translocation subcomplex
<i>SSA2</i>	High	YLL024C	Member of the HSP70 family
<i>SYS1</i>	High	YJL004C	Multicopy suppressor of ypt6 (vesicular transport)
<i>VID21</i>	High (very)	YDR359C	Involved in vacuolar import and degradation
<i>VID22</i>	High (very)	YLR373C	Targeting of fructose-1,6-bisphosphatase to Vid vesicles
<i>VID31</i>	High	YKL054C	Involved in vacuolar import and degradation
<i>VPS29</i>	High	YHR012W	Vacuolar protein sorting
<i>YBR077C</i>	High (very)	YBR077C	Interacts with Mvp1p (protein sorting to vacuole); unknown function
<i>YCR044C</i>	High (very)	YCR044C	Manganese homeostasis; localizes to vacuole membrane
<i>YGR071C</i>	High (very)	YGR071C	Related to Vid22p; unknown function
<i>YJL151C</i>	High (very)	YJL151C	Interacts with Vam7p (morphogenesis of the vacuole); unknown function
<i>YPT7</i>	High	YML001W	GTP-binding protein; protein transport between endosome-like structures
Components of vacuolar ATPase			
<i>VMA16</i>	Low (very)	YHR026W	V0 subcomplex, no growth on glycerol
<i>VMA5</i>	Low	YKL080W	V1 subcomplex, no growth on glycerol
<i>RAV2</i>	High	YDR202C	Regulation of ATPase assembly
<i>VMA1</i>	High (very)	YDL185W	V1 subcomplex
<i>VMA10</i>	High (very)	YHR039C-B	V1 subcomplex
<i>VMA12</i>	High	YKL119C	Assembly factor
<i>VMA13</i>	High	YPR036W	V1 subcomplex
<i>VMA21</i>	High	YGR105W	Assembly factor
<i>VMA22</i>	High (very)	YHR060W	Assembly factor
<i>VMA3</i>	High	YEL027W	V0 subcomplex
<i>VMA4</i>	High	YOR332W	V1 subcomplex
<i>VMA6</i>	High	YLR447C	V0 subcomplex
<i>VPH1</i>	High	YOR270C	V0 subcomplex

kinase mutants with elevated glycogen, none was associated with a significant increase in the glycogen synthase activity ratio, as would be predicted for a glycogen synthase kinase. An additional protein kinase of the phosphatidylinositol 3-/phos-

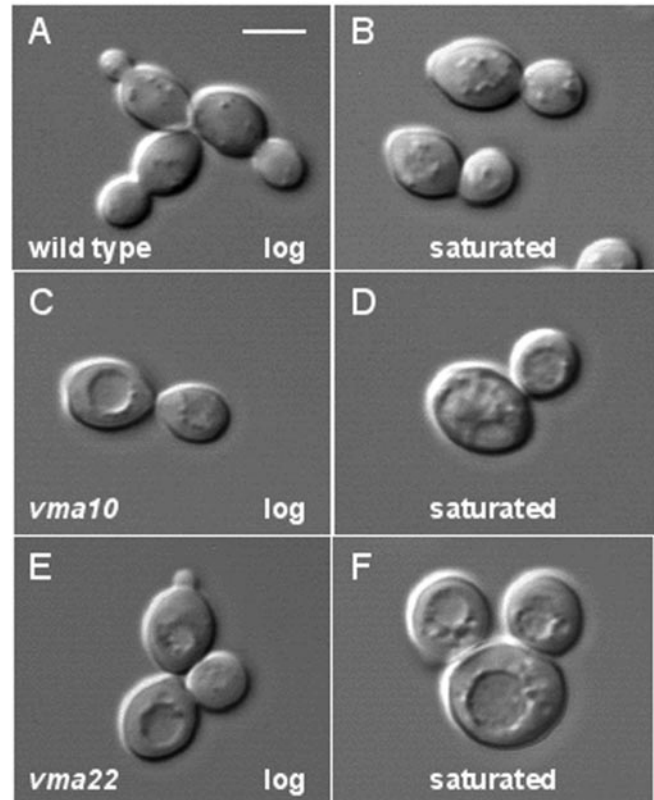
phatidylinositol 4-kinase family was identified, namely Tor1p. The strain with a *tor1* mutation hyperaccumulated glycogen, consistent with the known effect of rapamycin treatment, to cause increased glycogen synthesis (18).



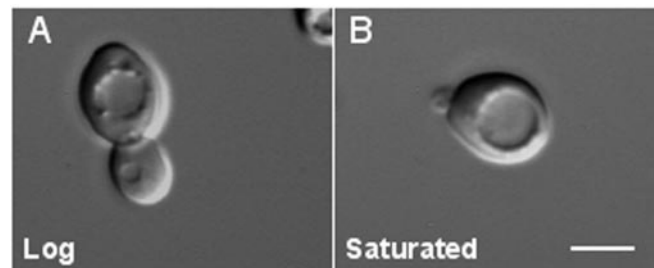
**FIG. 2. Comparison of the vacuoles of wild-type and *apg1* mutant cells subjected to nitrogen starvation.** The wild-type strain, BY4743 (panels A and B), and an *apg1* mutant (panels C and D) in the BY4743 background were grown to late logarithmic phase in YPD medium. The cells were then transferred to nitrogen starvation medium (26), and either the protease inhibitor PMSF dissolved in isopropanol (panels B and D) or isopropanol (panels A and C) was added. Incubation was continued for 4 h, and the cells were examined under a microscope equipped with Nomarski optics. The scale bar represents 5  $\mu$ m.

In addition to the Glc7p-associated subunits described above, several other protein phosphatase subunits were identified. The deletion mutant of *RTS1*, which encodes a homolog of the mammalian B' regulatory subunit of type 2A protein phosphatases (41), was found to have greatly increased glycogen. Although type 2A phosphatases had been implicated previously in glycogen storage (42–44), this is the first indication of a role for *RTS1* in the process. Deletion of *YCR079W*, which encodes a protein phosphatase of the type 2C family (45), also caused increased glycogen. Ycr079p has been little studied, and its cellular role is unknown, although deletion of *YCR079W* has been reported to increase the sensitivity of the cell to caffeine, which could indicate a link to the cyclic AMP pathway (46).

**Genes Implicated in Vacuole Function or Vesicle Transport**—The second largest family of genes identified has to do with vesicular trafficking or vacuolar function (Table IV). Approximately 10% of the mutants with aberrant glycogen levels fall into this category. This result would have been quite perplexing but for recent work in our laboratory indicating that yeast defective for autophagy are unable to maintain their glycogen stores even though the polysaccharide is synthesized normally (39). Autophagy, in response to starvation signals, is a process whereby cytosol and organelles are engulfed to become autophagosomes that are delivered to the vacuole for the recycling of constituents (reviewed in Refs. 47 and 48). Therefore, a variety of defects in the formation or



**FIG. 3. Accumulation of autophagic bodies in the vacuole of *vma10* and *vma22* mutants.** The wild-type strain BY4743 (panels A and B) and *vma10* (panels C and D) or *vma22* (panels E and F) deletion mutants in the BY4743 background were grown in SC medium. Aliquots were removed in the logarithmic phase of growth (panels A, C, and E) and again when the cultures were saturated (panels B, D, and F). The cells were examined under a microscope equipped with Nomarski optics. The scale bar represents 5  $\mu$ m.



**FIG. 4. The vacuoles of *apg1 vma10* mutants fail to accumulate autophagic bodies.** A homozygous diploid strain in which both the *APG1* and *VMA10* genes were deleted was grown in SC medium. Aliquots were removed during the logarithmic phase of growth (panel A) and at saturation (panel B). The cells were examined under a microscope equipped with Nomarski optics. The scale bar represents 5  $\mu$ m.

delivery of autophagosomes or in vacuolar function could be linked with glycogen storage. Correspondingly, genes with a wide variety of specific roles were detected, and it is not simple to rationalize in detail the results for every individual gene.

A clearly defined subfamily in this category involves com-



TABLE V  
Glycogen accumulation by various yeast strains

The results shown are the mean  $\pm$  S.E. for three independent determinations.

Strain	$\mu\text{g}$ Glycogen ( $10^7$ cells) $^{-1}$
apg1	7.82 $\pm$ 1.15
vma10	19.6 $\pm$ 1.93
apg1 vma10	8.58 $\pm$ 0.06

ponents of the vacuolar H<sup>+</sup>-ATPase that is responsible for acidification of the vacuole (reviewed in Ref. 49). This complex comprises some 13 different gene products plus at least four factors that participate in its assembly. Of these 17 different genes, deletion mutants in 13 were recovered in the screen, 11 with high and two with low glycogen. Deletion mutants in the remaining genes (*VMA2*, *VMA7*, *VMA8*, and *VMA11*) were present in the deletion series but were scored as wild type. The two mutants that had low glycogen (*vma5* and *vma16*) were the only two of the 17 that failed to grow with glycerol as a carbon source, suggesting a respiratory defect that could explain the low glycogen levels. It has been reported that a characteristic of the *vma* class of mutants is their inability to grow using non-fermentable carbon sources (50), but presumably there must be strain to strain variation, because most of the *vma* mutants studied here did grow on glycerol. These ATPase genes represent an obvious functional cluster, making a robust link with glycogen storage.

Although the exact mechanistic link between autophagy and glycogen storage is not completely understood, our previous studies (39) led us to two conclusions. First, autophagy provides a source of intermediates and energy in stationary phase, and its absence may promote premature utilization of cytosolic glycogen. Second, autophagy delivers glycogen to the vacuole where it is actually protected from this cytosolic utilization and is degraded by vacuolar enzymes such as Sga1p. Ohsumi and colleagues (26) developed an assay for autophagy in which yeast growing in rich medium are transferred to a starvation medium, totally lacking nitrogen, and also containing PMSF. The rationale is that starvation will induce autophagy, and the PMSF will inhibit vacuolar proteases thus preventing degradation of autophagosomes delivered to the vacuole. Wild-type cells transferred to starvation medium in the presence of PMSF have full, active vacuoles compared with cells not exposed to PMSF (Fig. 2). This difference is hardly evident in the still shot but is very obvious if the cells are viewed over time when undegraded autophagic bodies can be seen moving rapidly within the confines of the vacuole (see supplemental video for Fig. 2). *APG1* encodes a protein kinase that is necessary for autophagy, and analysis of *apg1* mutants using the starvation assay reveals empty vacuoles, evident even in the still shot. When monitored over time, there is no motion within the vacuole, but particles can be seen moving outside of the vacuole (see supplemental video for Fig. 2).

We monitored by microscopy two of the mutants defective in the vacuolar ATPase, *vma10* and *vma22*, during growth on SC medium in liquid culture. At the stage of exponential growth, the *vma10* and *vma22* mutants had more clearly defined vacuoles than wild-type cells (Fig. 3), but typically the level of movement within the vacuole was similar (see video for Fig. 3). A small fraction, 5%, of the mutants had large and full vacuoles, however (not shown). After 24 h, most of the mutants cells,  $\sim$ 60%, had large well defined vacuoles inside of which an accumulation of particles can be seen in rapid motion (see supplemental video for Fig. 3). In fact, these vacuoles resembled the wild-type cells subjected to the autophagy assay in the presence of PMSF (Fig. 2). Autophagy is presumably induced normally upon entry to stationary phase (26, 39), but degradation of autophagic bodies is impaired, because the vacuole cannot acidify because of a defective ATPase (51). A double mutant that lacked both *apg1* and *vma10* (Fig. 4) resembled the *apg1* mutant rather than the *vma10* mutant, as has been reported by Nakamura *et al.* (51). Thus, with autophagy impaired, the inability of the *vma* mutants to degrade autophagic bodies is not in evidence. Interestingly, analysis of the glycogen content revealed the same epistatic relationship, with glycogen accumulation in the double mutant resembling that of *apg1* mutants rather than *vma10* mutants (Table V). Therefore, the elevated glycogen storage associated with *vma10* and *vma22* mutants is most likely explained by hyperaccumulation of vacuolar glycogen.

#### DISCUSSION

The availability of the gene deletion strain collection is symbolic of an era in which not only information but also reagents are available on a genomic scale. In the present case, the entire strain collection occupies only 54 microtiter plates so that, even without investment in robotic instrumentation, many types of phenotype scan can be performed with relative ease (for example, see the work of Ni and Snyder (4) or Wiederkehr *et al.* (3)). It is of interest to compare the approach described in this study with conventional genetic screens. Advantages of the genomic scan are severalfold. One is simplicity. Once a phenotype is scored, gene identification is by reference to a database. Nonetheless, if an important conclusion would be based on a single member of the deletion set, it would obviously be prudent to confirm that the phenotype can be reproduced by an independent gene knock-out. This is necessary as part of ongoing quality control as the deletion set becomes used by the yeast research community. Another advantage is that the screen is systematic and does not depend fundamentally on chance; coverage of the genome with mutations is defined. As has been stated by Ni and Snyder (4), there are several disadvantages to this new mode of screening. First, it is limited to studying loss of function mutations. Second, in the simplest approach, essential genes are obviously not included, and for the homozygous diploid set, nor are genes required for mating. Also, because the



duplication of the yeast genome (52) has resulted in some pairs of genes of very high sequence identity, no attempt was made to target both genes of the pair, and so not all are represented. A related point, which applies equally to conventional screens, is that any genes that have redundant functions are unlikely to be detected. Finally, some gene deletions are simply missing from the series at this time. Nonetheless, the collection currently includes ~88% of viable mutants and is a valuable resource for this new approach to genetic screening.

As described under “Results,” many genes known already to affect glycogen storage were identified, providing strong validation of the screen. Some genes that were expected did not surface in the screen. One of the most notable was *PHO85*, which we know from our previous work is involved in the direct regulation of glycogen synthesis (19, 53). In the genetic background of the strain that we use in most of our work, deletion of *PHO85* causes a substantial hyperaccumulation of glycogen. However, in the present screen, the *pho85* mutant was scored wild type for glycogen. The explanation is the difference in strain backgrounds, because we have found that, in some strains, loss of *pho85* causes activation of glycogen synthase but no increase in glycogen accumulation.<sup>2</sup> It is likely that *PHO85* has other connections to the control of glycogen storage.<sup>3</sup> Lee *et al.* (54) also noted that in some strains *PHO85* deletion does not result in glycogen hyperaccumulation.

With our level of knowledge of yeast glycogen metabolism and its control prior to our screen, one would have had difficulty justifying more than 30 genes that affect glycogen storage if respiratory or mitochondrial mutants were excluded. These would have been the genes responsible for glycogen and related metabolism, direct regulators like protein kinases and phosphatases, or genes involved in known regulatory pathways such as those involving cyclic AMP, *SNF1* and glucose repression, *PHO85*, and the Tor pathway. Therefore, the first notable feature of our results is the large number of genes identified in the screen, which is 362 even if the respiratory mutants are excluded. Of these, 100 have unknown function. It is likely that, as biological functions are assigned, these genes will for the most part fit into existing categories, and we may have already defined most of the biologically important families. Given the large number of novel mutations identified here as affecting glycogen storage compared with the small number known previously to influence this process, it is of interest to ask whether the previously identified mutants were those that conferred the strongest glycogen accumulation phenotype. To a certain extent this may be true, because *reg1* and *pfk1* mutants, which had previously been shown to have a high level of glycogen, were among the mutants that stained most intensely with iodine in

the present screen. However, several of the newly identified mutants, such as *rts1*, *vid21*, and *ado1*, stained just as strongly as *reg1*.

The second striking aspect of the results was that, based on the 35 strains where we followed up with glycogen synthase assays, a large fraction of the changes in glycogen storage did not correlate with the glycogen synthase activity ratio, which indexes its phosphorylation and activation state. Part of the explanation is our current appreciation that not only synthesis but also the ability to retain glycogen is a key determinant of glycogen storage (39). This conclusion, based on recent analysis of *SNF1* and its relationship to autophagy, is reinforced by the results of this study.

The most interesting gene category to emerge is that defined by the genes involved in vesicular trafficking and vacuolar function. This is a family where there is a reasonably good connection with the process of autophagy, which we have recently linked to glycogen storage (39). We have proposed that defective autophagy leads to premature depletion of glycogen stores. The mechanism is not fully understood, but at least two points can be made. First, in the absence of autophagy, the cell has lost an important source of intermediary metabolites and may be forced to use other reserves, like glycogen, at an earlier stage than normal. Second, there is a vacuolar glycogen pool (26), and we hypothesized that vacuolar glycogen is protected from cytosolic degradation until late into stationary phase (39). Thus, many of the genes identified with low glycogen in this group likely reflect some defect in autophagy.

Within the vesicular/vacuolar category, the H<sup>+</sup>-ATPase genes are especially interesting. First, the fact that such a large fraction of the ATPase genes was netted in the screen makes the case very strongly that this vacuolar function is linked to glycogen levels. It has been reported that elimination of any one of the subunits cripples ATPase function, consistent with our identifying genes coding for several ATPase subunits. Without its ATPase, the vacuole cannot acidify and function normally. In particular, the degradative enzymes of the vacuole, including the vacuolar glycosidase Sga1p, will not be fully active (51, 55). Impaired vacuolar function removes a source of intermediates and energy in stationary phase, and one might have predicted a premature utilization of glycogen from the cytosol, much as is seen for those mutants defective in autophagy (39). The fact that defective ATPase correlated with a hyperaccumulation of glycogen is thus best explained by a stabilization of the vacuolar pool. This proposal is also consistent with the observation that the elevated glycogen in a *vma10* mutant is eliminated if autophagy is also impaired by mutation of *APG1*. In conclusion, the study suggests a significant role for the vacuole in the maintenance of yeast glycogen stores.

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<sup>2</sup> M. A. Fujino and P. J. Roach, unpublished observations.

<sup>3</sup> W. A. Wilson, Z. Wang, M. A. Fujino, and P. J. Roach, unpublished observations.

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