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

IMPLICATION OF THE VACUOLE AS A DETERMINANT OF GLYCOGEN LEVEL

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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 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),¹ 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). 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-

¹ 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, glucosamylase (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 all 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 µl of YPD (2% peptone, 2% glucose, 1% yeast extract) medium supplemented with G418 (200 µg/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/Saccharomyces and from the YPD database (21, 22) 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) and from the YPD database (21, 22) (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 (~1 × 10⁸ 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 × 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 µl 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-
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 /H11003 oil-immersion objective at a magnification of /H11003. 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/) 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.

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-
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 ± 0.02 (n = 6). ND indicates no determination could be made.

| Protein kinases          | Original call | Glycogen (iodine) on | 24 h | 48 h | Glycogen synthase | 24 h | 48 h | Activity ratio (24 h) |
|-------------------------|---------------|----------------------|------|------|------------------|------|------|----------------------|
| KIN1                    | Low           | +/−                  | 0.88 | 2    | 1.5              | 0.97 | 0.11 |
| PTK2                    | Low           | +/−                  | 0.91 | 2.4  | 1.3              | 0.4  | 0.15 |
| RIM15                   | Low           | −                    | 0.27 | 0.41 | 1                | 0.45 | 0.07 |
| CTK1                    | High          | +                    | ND   | 7.4  | 0.43             |      |      |
| DBF2                    | High          | +                    | 2.1  | 4.2  | 0.82             | 0.92 | 0.23 |
| NPR1                    | High          | +                    | 1.6  | 0.73 | 1                | 0.72 | 0.04 |
| TOR1                    | High          | +                    | 1.4  | 3.4  | 1.1              | 1.1  | 0.22 |
| YDL025C                 | High          | +                    | 0.79 | 1    | 1.8              | 0.52 | 0.15 |
| YDR247W                 | High          | +                    | 1.1  | 1.7  | 0.83             | 1.4  | 0.28 |
| YNL099C                 | High          | +                    | 0.9  | 1    | 0.64             | 0.18 |      |
| YOL045W                 | High          | +                    | 1.1  | 3.3  | 0.76             | 1.8  | 0.36 |
| YPL150W                 | High          | +                    | 1.5  | 5.8  | 1.2              | 1.8  | 0.18 |
| Protein phosphatases    |               |                      |      |      |                  |      |      |
| PIG2                    | Low           | +                    | 0.55 | 0.26 | 1.6              | 0.98 | 0.09 |
| RTS1                    | High (very)   | +++                  | 2    | 8.5  | 1.1              | 1.3  | 0.17 |
| YCR079W                 | High          | +                    | 1.6  | 3.6  | 1.3              | 1.4  | 0.17 |
| WD-40 repeat proteins   |               |                      |      |      |                  |      |      |
| YAR0033W                | Low           | +                    | 0.51 | 0.27 | 1.1              | 0.4  | 0.18 |
| YPL247C                 | High          | +                    | 1.4  | 0.53 | 1.3              | 0.87 | 0.17 |
| YKL121W                 | High          | +                    | 1    | 0.96 | 1.2              | 0.74 | 0.19 |
| YOL087C                 | High          | +                    | 0.59 | 1.72 | 1.6              | 0.7  | 0.21 |
| YOL138C                 | High (very)   | +++                  | 0.79 | 0.52 | 1.1              | 0.68 | 0.55 |
| Vesicular transport and vacuolar function |               |                      |      |      |                  |      |      |
| BST1                    | High (very)   | +                    | 2.1  | 1.3  |                  |      |      |
| SEC22                   | High (very)   | ++                   | 2.1  | 1.3  |                  |      |      |
| VID21                   | High (very)   | +++                  | 6.5  | 6.8  | 2                | 2    | 0.22 |
| VID22                   | High (very)   | +                    | 2.2  | 2.8  |                  |      |      |
| VMA10                   | High (very)   | +                    | 1.8  | 5.7  |                  |      |      |
| VMA22                   | High (very)   | +                    | 1.2  | 3.3  |                  |      |      |
| VMA3                    | High          | +                    | ND   | 3.5  |                  |      |      |
| Inositol metabolism     |               |                      |      |      |                  |      |      |
| INO1                    | Low           | +/−                  | 0.35 | 0.63 | 1.2              | 0    | 0.04 |
| IPK1                    | Low           | +/−                  | 0.6  | 1.5  | 1.2              | 1.4  | 0.09 |
| Carbohydrate metabolism |               |                      |      |      |                  |      |      |
| RPE1                    | High (very)   | ++                   | 1    | 1.1  | 0.89             | 1.2  | 0.12 |
| YHR204W                 | High (very)   | ++                   | 1.2  | 1.6  | 1.7              | 1.9  | 0.09 |
| Others                  | High (very)   | +++                  | 1    | 5    | 1.6              | 1.7  | 0.31 |
| GIS4                    | Low           | +                    | 0.71 | 1.3  | 1.3              | 1.9  | 0.09 |

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
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 GLCS/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 repression 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
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-/phosphatidylinositol 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).

| Gene   | Glycogen | ORF           | Comments                                      |
|--------|----------|---------------|-----------------------------------------------|
| DID4   | Low      | YKL002W       | Vacuolar protein sorting                      |
| DOA4   | Low (very)| YDR069C       | Ubiquitin C-terminal hydrolase                |
| PEP12  | Low      | YOR036W       | tsNARE                                        |
| PEP3   | Low      | YLR148W       | Vacuolar protein sorting and required for vacuole biogenesis |
| PEP7   | Low      | YDR323C       | Vacuole inheritance and vacuole protein sorting |
| SNF7   | Low      | YLR025W       | Glucose derepression and protein sorting      |
| VID28  | Low      | YDL017C       | Vacuolar import and degradation               |
| VPS20  | Low      | YMR077C       | Similarity to Snf7p                           |
| VPS28  | Low      | YPL065W       | Protein transport from prevacuolar endosome   |
| VPS33  | Low      | YLR396C       | Vacuolar protein sorting                      |
| VPS34  | Low (very)| YLR240W       | Phosphatidylinositol 3-kinase required for vacuolar protein sorting |
| VPS4   | Low      | YPR173C       | Vacuolar protein sorting                      |
| VPS45  | Low      | YGL095C       | Protein of the Sec1p family                   |
| VPS53  | Low      | YJL029C       | Protein sorting in the late Golgi             |
| ANP1   | High     | YEL036C       | Retention of glycosyltransferases in the Golgi|
| APL3   | High     | YBL037W       | α-adaptin, in clathrin-associated protein (AP) complex |
| APL6   | High     | YGR261C       | β-adaptin, in clathrin-associated protein (AP) complex |
| APM3   | High     | YBR288C       | Medium subunit, clathrin-associated protein (AP) complex |
| APS3   | High     | YJL024C       | Small subunit of the clathrin-associated protein (AP) complex |
| ARPL3  | High (very)| YPL051W       | Member of the arf-sar family                  |
| AUT1   | High     | YNR007C       | Required for autophagy                        |
| AUT7   | High     | YBL078C       | Protein of autophagosomes                     |
| BFR1   | High     | YOR198C       | Involved in secretion and nuclear segregation |
| BST1   | High (very)| YFL025C       | Negatively regulates COPII vesicle formation  |
| ERV14  | High     | YGL054C       | Protein of ER-derived vesicles                |
| LST4   | High     | YKL176C       | Trafficking of nitrogen-regulated permeases   |
| MSB3   | High     | YNL293W       | GTPase activating protein for Sec4p            |
| PIB2   | High     | YGL023C       | Related to Vps27p, Pep7p, Fab1p and Pib1p; unknown function |
| PMR1   | High     | YGL167C       | Ca2++-transporting P-type ATPase of Golgi membrane |
| RTG2   | High     | YGL252C       | Protein involved in inter-organelle communication |
| RUD3   | High     | YOR216C       | Hydrophilic protein involved in vesicle docking |
| SCJ1   | High (very)| YMR214W       | Homolog of E. coli DnaJ                       |
| SEC22  | High (very)| YLR268W       | Synaptobrevin (v-SNARE) homolog               |
| SEC66  | High     | YBR171W       | Component of ER protein-translocation subcomplex |
| SSA2   | High     | YLL024C       | Member of the HSP70 family                    |
| SYS1   | High     | YJL004C       | Multicopy suppressor of ypt6 (vesicular transport) |
| VID21  | High (very)| YDR359C       | Involved in vacuolar import and degradation   |
| VID22  | High (very)| YLR373C       | Targeting of fructose-1,6-bisphosphatase to Vid vesicles |
| VID31  | High     | YKL054C       | Involved in vacuolar import and degradation   |
| VPS29  | High     | YHR012W       | Vacuolar protein sorting                      |
| YBR077C| High (very)| YBR077C       | Interacts with Mvp1p (protein sorting to vacuole); unknown function |
| YCR044C| High (very)| YCR044C       | Manganese homeostasis; localizes to vacuole membrane |
| YGR071C| High (very)| YGR071C       | Related to Vid22p; unknown function           |
| YJL151C| High (very)| YJL151C       | Interacts with Vam7p (morphogenesis of the vacuole); unknown function |
| YPT7   | High     | YML001W       | GTP-binding protein; protein transport between endosome-like structures |

Components of vacuolar ATPase

| Gene    | Glycogen | ORF           | Comments                                      |
|---------|----------|---------------|-----------------------------------------------|
| VMA16   | Low (very)| YHR026W       | V0 subcomplex, no growth on glycerol          |
| VMA5    | Low      | YLR080W       | V1 subcomplex, no growth on glycerol          |
| RAV2    | High     | YDR202C       | Regulation of ATPase assembly                 |
| VMA1    | High (very)| YDL185W       | V1 subcomplex                                |
| VMA10   | High (very)| YHR039C-B     | V1 subcomplex                                |
| VMA12   | High     | YKL119C       | Assembly factor                               |
| VMA13   | High     | YPR036W       | V1 subcomplex                                |
| VMA21   | High     | YGR103W       | Assembly factor                               |
| VMA22   | High (very)| YHR060W       | Assembly factor                               |
| VMA3    | High     | YEL027W       | V0 subcomplex                                |
| VMA4    | High     | YDR332W       | V1 subcomplex                                |
| VMA6    | High     | YLR447C       | V0 subcomplex                                |
| VPH1    | High     | YOR270C       | V0 subcomplex                                |
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/H11032 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 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-
ponents of the vacuolar H\(^+\)-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 degradation 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 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).

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

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, ~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
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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. It is likely that PHO85 has other connections to the control of glycogen storage. 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⁺-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.

Acknowledgments—We thank Drs. Mark Goebl and Ron Wek for many helpful discussions.

2 M. A. Fujino and P. J. Roach, unpublished observations.
3 W. A. Wilson, Z. Wang, M. A. Fujino, and P. J. Roach, unpublished observations.
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