The salt-instigated protein expression of *Saccharomyces cerevisiae* during growth in either 0.7 or 1.4 m NaCl was studied by two-dimensional polyacrylamide gel electrophoresis. The 73 protein spots that were identified as more than 3-fold responsive in 1.4 m NaCl were further grouped by response class (halometric, low-salt, and high-salt regulation). Roughly 40% of these responsive proteins were found to decrease in expression, while at higher magnitudes of change (>8-fold) only induction was recorded. Enolase 1 (Eno1p) was the most increasing protein by absolute numbers per cell, but not by -fold change, and the enzymes involved in glycerol synthesis, Gpd1p and Gpp2p, were also induced to a similar degree as Eno1p. We furthermore present evidence for salt induction of glycerol dissimilation via dihydroxyacetone and also identify genes putatively encoding the two enzymes involved; dihydroxyacetone kinase (DAK1 and DAK2) and glycerol dehydrogenase (YPR1 and GCY1). The GPD1, GPP2, GCY1, DAK1, and ENO1 genes all displayed a halometric increase in the amount of transcript. This increase was closely linked to the salt-induced rate of protein synthesis of the corresponding proteins, indicating mainly transcriptional regulation of expression for these genes. A consensus element with homology to the URS sequence of the ENO1 promoter was found in the promoters of the GPD1, GPP2, GCY1, and DAK1 genes.

The physiological response of *Saccharomyces cerevisiae* to hypertonic stress has for long been a subject of study (1). The main response has been shown to be an increased production and accumulation of glycerol, as a compatible solute, following the external osmotic pressure up to molar intracellular concentrations (2). This increased production is believed to be caused mainly by an enhanced activity of glycerol-3-phosphate dehydrogenase (3) encoded by two genes, the osmoreponsive *GPD1* (4) and *GPD2* (5), the latter of which seems to be involved in regulation of cytoplasmic redox balance. The induction of *GPD1* has been shown to occur at the level of transcription (5, 7). The second enzyme in the pathway to glycerol is glycerol-3-phosphatase, recently shown to be encoded by two genes, the constitutively expressed *GPP1* and the osmotically induced *GPP2* (8), also reported as *RHR2* and *HOR2*, respectively (9). The induction of these genes is, at least partly, dependent on a functional HOG pathway involving homologs of human mitogen-activated protein kinases (10), which is coupled to two putative osmosensor proteins, Slnlp (11) and Sho1p (12), the signals from which converge at Pbs2p. In addition to the genes involved in glycerol production, there are other genes reportedly regulated via the HOG path, such as *CTT1* (13), *HSP12* (14), and three adjacent genes including an aldehyde dehydrogenase, the expression of which was maximal at 0.3 m NaCl (15).

However, there is increasing evidence that other signaling paths are of importance for osmotic regulation of gene expression. *DDR48* is induced maximally by 1 m NaCl, with very little induction below 0.5 m, independent of *HOG1* (15), and most of the seven *HOR* genes were induced, albeit to a lower degree, even in a *HOG1* disruptant (9). The ENA1 gene, encoding a Na-pump, is regulated via Hog1p, calcineurin, and protein kinase A acting in concert (16, 17), implying that combinations of these mechanisms are responsible for the osmotic regulation of gene expression in yeast.

It was previously reported that *S. cerevisiae* strain Y41 (ATCC 38531) displayed largely transient changes in protein expression during adaptation to 0.7 m NaCl (18). The transient protein response during adaptation was further substantiated, since very few proteins were found to display expression changes in this strain during growth (19). From the responses observed, it was suggested that a decrease of the glyceraldehyde-3-phosphate dehydrogenase activity together with an increase in GPD activity is required for increased production of glycerol.

The aim of the present study was to identify responsive proteins during growth in 1.4 m NaCl medium and furthermore to group these proteins into response classes, presumably caused by differences in interaction of signaling pathways acting at the respective promoters. The classification of response type can then be used to select genes with similar patterns of expression for comparative promoter studies. During the course of this work we found evidence for a salt-induced dissimilation of glycerol via dihydroxyacetone, as has been indicated previously for *Zygosaccharomyces rouxii* (20) and *Debaryomyces hansenii* (21). On the basis of homology to known proteins from other organisms, we suggest candidate genes for the two enzymatic steps involved.

**EXPERIMENTAL PROCEDURES**

Organism, Media, and Growth Conditions—*S. cerevisiae*, strain SKQ2n (ATCC 44827; genotype: *a/a, ade11/+*, *+ade2/+his3*) or Y41 (ATCC 38531) was used for all experiments. Glucose concentration was 20 g/liter, but apart from this, media and growth conditions were the same as described previously (22). The defined YNB medium was sup-

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1 Ansell, R., Granath, K., Hohmann, S., Thevelein, J. M., and Adler, L. (1994) *EMBO J.*, in press.
implemented with appropriate amounts of sodium chloride where indicated. Growth was monitored as optical density at 610 nm (OD$_{610}$).

Labeling and Harvest—At an OD$_{610}$ of 0.5 (5 x 10$^6$ cells/ml) the cultures were labeled with 150 µCi of [$^{35}$S]methionine (15 µCi/µl, >1000 Ci/mmol, S1515, Amersham Inc.) for 30 min before harvest as described previously (22).

Preparation of Protein Extract—Protein extraction was performed, as described previously (23), by vortexing with glass beads and boiling with SDS/mercaptoethanol added before nuclease treatment. 20 µg of protein was lyophilized and dissolved in 10 µl of urea-containing sample buffer, all of which was applied on the first dimension gels. Protein concentration and amount of incorporated [$^{35}$S]-methionine in the extract was determined as described previously (23). Extracts from 0 m NaCl cultures contained 3–4 µg of protein/µl of extract and approximately 170,000 dpm/µg protein, while the corresponding values for salt-grown cells were 2–2.5 µg/µl and approximately 100,000 dpm/µg, respectively.

Two-dimensional Polyacrylamide Gel Electrophoresis—Two-dimensional PAGE$^2$ was run on an Investigator$^®$ system using a modified procedure of Garrels (24) with all chemicals and equipment supplied by Oxford Glycosystems. First dimension acrylic gels were 4% T, 2.6% C (Duracryl 0.8% bisacrylamide, ELCR 2DC 010), 9.5M urea, 2% oxiglycerol, and amount of incorporated [$^{35}$S]-methionine in the extract was determined as described previously (23). Extracts from 0 m NaCl cultures contained 3–4 µg of protein/µl of extract and approximately 170,000 dpm/µg protein, while the corresponding values for salt-grown cells were 2–2.5 µg/µl and approximately 100,000 dpm/µg, respectively.

Two-dimensional Polyacrylamide Gel Electrophoresis—Two-dimensional PAGE$^2$ was run on an Investigator$^®$ system using a modified procedure of Garrels (24) with all chemicals and equipment supplied by Oxford Glycosystems. First dimension acrylic gels were 4% T, 2.6% C (Duracryl 0.8% bisacrylamide, ELCR 2DC 010), 9.5M urea, 2% (v/v) Nonidet P-40, and 5.8% (v/v) of a 40% (w/v) amylolyte pH 3–10 stock solution (ELCR 1DC 110). The gels were prefocused until a voltage of 1500 V had been reached, prior to sample application with a maximum of 20 µg of protein (2 µg/ml in sample buffer). Focusing was run for 18,500 V-h overnight at room temperature (20–23°C). The isoelectric focusing gels were equilibrated for 2 min in SDS buffer (containing 3% (w/v) SDS, 50 mM dithiothreitol, 0.3 M Tris base, 0.075 M Tris-HCl, and 0.01% (v/v) bromophen blue) before mounting on the SDS-PAGE slab gels. These second dimension acrylic gels were 10% T, 2.1% C (Duracryl 0.65% bisacrylamide; ELCR 2DC 070) containing 0.1% (w/v) SDS, 0.37 M Tris base, and 0.27 M Tris-HCl. Gels were cast at room temperature, and samples were run on a vertical system at 20°C (Investigator; Oxford Glycosystems) with gels fully submerged in running buffer for efficient running. The running buffer was 25 mM Tris base, 0.1% (w/v) SDS, and 192 mM glycine with the upper tank containing approximately 2 liters of 2 x concentrated running buffer. Electrophoresis in the second dimension was performed at limiting power of 16,000 megawatts (maximum voltage 500 V) per gel for about 5 h until the dye front reached the bottom of the gel. Immediately after the run, the gels were dried on filter paper without any previous fixation. In all cases the gel surface was covered by plastic film during the drying process.

Scanning and Computerized Data Analysis—Dried, analytical two-dimensional PAGE gels were exposed to image plates, which were subsequently scanned in a PhosphorImager (Molecular Dynamics) with a pixel size of 176 x 176 µm. All image files thus produced were processed in the PDQuest two-dimensional analysis program (PDI; Protein and DNA Imageware Inc.) version 4.1. In brief, gel scans were subjected to background subtraction and smoothing to produce a synthetic gel image on which spot detection was performed (22). Quantitative analysis was combined with a log Student’s t test to produce sets containing the proteins that change significantly and by the specified factor.

Identification of Glycerol Dehydrogenase—NADP$^+$-specific glycerol dehydrogenase from Aspergillus niger was obtained from Sigma (product number C2909). The protein was dissolved in SDS buffer (0.3% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 10% (v/v) glycine in 50 mM Tris buffer) and boiled for 5 min. Extract equivalent to 5 µg of protein was subsequently applied on an SDS-PAGE gel of 15% acrylamide utilizing the same chemicals as the two-dimensional PAGE second dimension gels. Gels were stained with Coomassie Blue, and protein bands were trypsin-digested as described (22).

Identification of Protein Spots—Unknown spots were identified by the methodology described previously (22), using preparative two-dimensional PAGE electrophoresis, in-gel trypsin digestion, separation of generated peptides on high pressure liquid chromatography, and N-terminal sequencing. Homology searches were then performed using the BLAST program to screen all public yeast sequences.

M, and pI Axis—The molecular weight (M) and isoelectric point (pI) axis was constructed using values taken from the latest update of the database.

$^2$The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; FUN gene, gene of previously unknown or only putative function; GLD, glycerol dehydrogenase; MES, 4-morpholineethanesulfonic acid.

$^3$The latest update of the YPD data base can be found on the World Wide Web at http://quest7.proteome.com/.

**FIG. 1.** Representative growth curves for SKQ2n in YNB medium with 2% (w/v) glucose as a carbon source and with NaCl added to 0 m (open squares), 0.7 m (filled circles), and 1.4 m (open triangles). The dashed line indicates an OD$_{610}$ of 0.5 at which 150 µCi of $^{35}$S-labeled methionine was added for 30 min before harvest.

YPD data base. The proteins used were, in order of decreasing M$, \text{Met1p} (p95,7/6.25), \text{Ssa2p} (p69.3/4.9), \text{Sch1p} (p66.5/5.23), \text{Lys5p} (p49.8/5.18), \text{Eno2p} (p46.8/5.82), \text{Act1p} (p41.7/5.39), \text{Isp1p} (p32.2/5.46), and \text{YKL056c} (p18.7/4.44).

Enzyme Assays—Assays for dihydroxyacetone kinase and glycerol dehydrogenase (GLD) were performed as described earlier (25). Extract for the assay was made from cells of 250 ml of YNB medium with 0 or 1.4 M NaCl grown to an OD$_{610}$ of 0.5. These cells were washed in 10 ml of ice-cold 20 mM MES buffer, pH 6.5, and subsequently disrupted by vortexing for 4 x 30 s with the addition of 0.6 g of acid-washed glass beads and 600 µl of the MES buffer.

Determination of protein concentration was performed as described previously (23). The dihydroxyacetone assay was performed in 0.1 M imidazole, pH 7.5, 10 mM 2,2'-dipyridyl, 0.1 mM NADPH or NADH, an appropriate amount of extract, and 4 mM dihydroxyacetone as substrate, approximately 2 units of glycerol-3-phosphate dehydrogenase (Boehringer Mannheim), and an appropriate amount of extract. The reaction was started by the addition of ATP to 10 mM final concentration. The oxidation of NADH was monitored at 340 nm, and the slope was normalized for protein amount to yield the specific activity in units defined as 1 nmol of NADH/min being oxidized per mg NAD$^+$.

Attempts at measuring GLD activity were made with the following buffers: (i) 0.1 M K$_2$CO$_3$, pH 9, with 10 mM 2,2'-dipyridyl, 0.1 mM NADPH or NADH, an appropriate amount of extract, and 4 mM dihydroxyacetone to start the reaction, (ii) 0.1 M imidazole, 10 mM 2,2'-dipyridyl, 0.1 mM NADPH or NADH, 1 mM KCN, 20 mM MgCl$_2$, and 4 mM dihydroxyacetone as substrate, approximately 2 units of glycerol-3-phosphate dehydrogenase (Boehringer Mannheim), and an appropriate amount of extract. The reaction was started by the addition of ATP to 10 mM final concentration. The oxidation of NADH was monitored at 340 nm, and the slope was normalized for protein amount to yield the specific activity in units defined as 1 nmol of NADH/min being oxidized per mg NAD$^+$.

**RESULTS**

Salt-dependent Growth Curve Phenotype—After inoculation of fresh medium with an overnight culture of SKQ2n (ATCC 5545). The obtained values were then normalized by division with the corresponding value for ACT1.

**Northern Blotting**—Total RNA was isolated from cells grown and harvested as described for protein extracts, and running and blotting procedures were as described previously (5) with identical ACT1 and GPD1 probes also used (kindly provided by R. Ansell). For probing the other genes, the following oligonucleotides were used (all of which were obtained from Life Technologies Ltd): GPP1, 5'-TGT GGT CAA AGG CAG ATC GAG GAT GGC-3'; DAK1, 5'-TTG TTC CAT TGA GAT TCT GAT GAT ATC CTA A-3'; DAK2, 5'-TTG TTC CAT TGA GAT TCT GAT GAT ATC CTA A-3'; DAK3, 5'-TTT CCT GGT AGC AGG GAG TAT CCA CCA C-3'; YPR1, 5'-GCC ATT ATT TAG TCT GTC TCT AAG AAA AGG TCT AAG GAT TAT GTC TCT GAT GAT ATC CTA A-3'. The bands were quantified in the ImageQuant program (Molecular Dynamics) after scanning on a PhosphorImager (Molecular Dynamics 422E). The obtained values were then normalized by division with the corresponding value for ACT1.
44827), a lag phase followed, the length of which depended on the NaCl concentration (Fig. 1). For 0 and 0.7 M NaCl medium there was no significant difference, with growth resuming after about 1 h. Inoculation into 1.4 M, however, was stress of a higher magnitude, and the adaptation phase lasted for approximately 6 h. A salt dependence of the growth rate was also observed with generation times of 1.7, 2.7, and 6.6 h for 0, 0.7, and 1.4 M NaCl, respectively. [35S]methionine was added at an OD610 of 0.5 (indicated by the dashed line in Fig. 1), which was more than one generation before growth arrest in the diauxic shift. As can be further seen, the density at which cell growth was arrested, due to exhaustion of glucose, was strongly related to the medium composition (Fig. 1), with control cells reaching an optical density of approximately 6.6, 0.7 M cells reaching an OD610 of 3.4, and 1.4 M grown cells arresting at an OD610 of about 1.4. Strain Y41 (ATCC 38531) displayed similar generation times as SKQ2n irrespective of salinity (data not shown).

Gel Running and Protein Identifications—Protein extracted from the harvested cells was applied on isoelectric focusing gels, which were run overnight and subsequently mounted on top of SDS-PAGE gels for the second dimension. Gels were dried and exposed to PhosphorImager plates to produce a digital image of the protein pattern (Fig. 2). Many protein spots on these gels were previously identified by N-terminal sequencing of in-gel trypsin-generated peptides (19, 22). Amino acid sequences of seven previously unidentified protein spots are presented in this study (Table I). Four of these are genes of previously unknown or only putative function (FUN genes), and the expression of all seven, except YKL056c, was affected by the medium salinity. The Mr and pI of these identified proteins is shown on two-dimensional gels from 0 and 1.4 M NaCl (Fig. 2), and the locations on the gel are furthermore available on a two-dimensional PAGE yeast database, which is continuously being updated as identifications proceed. The Mr and pI of the 73 responsive proteins described above, with quantifications at 0 and 1.4 M NaCl, expressed as ppm of total protein, is presented together with identity where possible (Table II). The more dominant proteins often have “satellite spots” of slightly different pI values, but the reason for the appearance of these spots is not known. Such “satellites” are indicated when location and expression data suggests identity (Table II).

The GPP1 gene, coding for the constitutive form of glycerol-3-phosphatase (8), has also been identified in another study, as a homolog of GPP2/HOR2, under the name RHR2 (9). However, there was a conflict regarding which of two alternative start codons is actually utilized. We therefore sequenced the N terminus of the purified enzyme and were thereby able to

| Mr  | pI | Amino acid sequence | Residues in protein | Gene assigned          |
|-----|----|---------------------|---------------------|-----------------------|
| 62.3| 6.0| AVNFQ               | 394–399             | CTT1                  |
| 59.6| 5.5| AFHD                | 75–78               | YPL061W (ALD6)        |
| 59.5| 5.2| EMGEVEY             | 481–487             |                      |
| 29.4| 4.7| XXYGVLBP*           | 104–112             | SC9745_2 (DAK1)       |
| 27.7| 4.8| IYXAFANG          | 159–167             | NAB1A/YST1*           |
| 27.9| 5.5| XYYQRQ(E/D)P1      | 27–35               |                      |
| 25.5| 5.7| EAYVNPVIAL         | 134–145             |                      |
| 18.7| 4.4| FDPNDSELLSDA       | 6–17                | YKL056c               |

* Underlined amino acids are unique to DAK1.

Sequence does not discriminate between the two genes, identification is from the predicted Mr and pI and from Garrels (6). YST1 and YST2 are alternative names for NAB1A and NAB1B, respectively (28).

N-terminal sequence from purified Gpp1p. Position in the protein sequence is from the cDNA of the RHR2 gene (9), and XX should be TT.

4 This data base can be found on the World Wide Web at http://yeast-3dpage.gmm.gu.se.
confirm the shorter of the two reading frames as the correct one. We furthermore found that the initial methionine was missing. The predicted Mr and pI of this shorter form (27.9 and 5.43, respectively) also correlates better with the apparent Mr and pI of the identified Gpp1p on two-dimensional gels (p27.9/5.5).

Quantification of Changes in Protein Expression—The individual protein spots on the two-dimensional gels were quantified, normalized to the total dpm found in valid spots on the respective gel, and analyzed for statistically significant changes.

A primary selection was made for proteins changing in quantity by at least 3-fold, going from 0 to 1.4 M NaCl, and this yielded 73 responding protein spots (Table II). From a plot of the number of proteins against -fold change it could be seen that the magnitude of change was generally greater for the increasing proteins (Fig. 3). Proteins displaying a salt-dependent expression change of less than 3-fold are hereafter only discussed in the cases where the two-dimensional identity is known (Table III). 30 of the 73 responsive proteins were found to be repressed, and these are indicated by circles on the pattern from the control, 0 M NaCl, cells (Fig. 2A). Arrows point to the three proteins repressed by a 6-fold criterion. On a gel of extract from 1.4 M NaCl-grown cells (Fig. 2B) are similarly shown the 43 proteins induced more than 3-fold by this salinity; the 13 arrows indicate proteins induced more than 6-fold.

Inspection of the growth curves from the different salinities shows that harvest of the cells in 1.4 M NaCl was only one to two generations from the cessation of fermentative growth, and many of the changes observed could thus be argued to be due to this fact, since the transition phase at glucose depletion is accompanied by massive changes in protein expression (26, 27). Cells were therefore labeled and harvested at an earlier stage of the growth curve as a control, and from two-dimensional gels of these it was evident that the proteins reported as responsive also here displayed salinity-dependent changes (data not shown).

Classification of Salt-responsive Proteins—It was evident

| Response class | M_r  | pI | Quantity 0 M | Quantity 1.4 M | Gene name | Response class | M_r  | pI | Quantity 0 M | Quantity 1.4 M | Gene name |
|----------------|------|----|-------------|----------------|-----------|----------------|------|----|-------------|----------------|-----------|
| Halometric increase | 15.3 | 6.0 | 954 | 3722^* | Halometric decrease | 16.9 | 5.2 | 867 | 275 |
| 16.5 | 5.7 | 230 | 1055^* | 16.6 | 6.1 | 137 | 645^* | 19.4 | 5.8 | 176 | 1017^* |
| 23.0 | 5.9 | 439 | 1581^* | GPP2 | 25.3 | 4.6 | 166 | 624^* | 29.2 | 5.4 | 301 | 1287^* |
| 37.5 | 5.9 | 143 | 917 | 40.8 | 5.4 | 120 | 403^* | 45.7 | 6.0 | 146 | 1209^* |
| 46.1 | 5.4 | 297 | 966^* | GDP1 | 59.5 | 5.2 | 411 | 1492^* | DAK1 |
| High-salt increase | 30.7 | 5.4 | 201 | 638 | Halometric decrease | 16.9 | 5.2 | 867 | 275 |
| 31.2 | 5.2 | 113 | 365 | 37.9 | 5.9 | 136 | 789 | 16.8 | 6.1 | 101 | 325^* |
| 33.1 | 5.8 | 122 | 746 | 48.9 | 6.3 | 3390 | 15,190 | ENO1 | 49.2 | 6.2 | 341 | 1110^* |
| Low-salt increase | 20.0 | 5.0 | 92 | 590 | High-salt decrease | 85.7 | 6.2 | 3343 | 887^* |
| 22.5 | 5.9 | 64 | 348 | 33.8 | 6.1 | 663 | 2330 | 37.5 | 5.9 | 143 | 917 |
| 25.1 | 5.0 | 79 | 286 | 29.1 | 6.1 | 31 | 208 | 32.0 | 5.3 | 40 | 312 |
| 32.4 | 5.7 | 61 | 479 | 35.1 | 5.8 | 80 | 308 | 37.1 | 5.2 | 71 | 260 |
| Unclassified increase | 38.2 | 5.7 | 58 | 505 | Unclassified decrease | 25.7 | 5.0 | 370 | 88 |
| 39.1 | 5.3 | 59 | 226 | 39.3 | 5.5 | 59 | 342 |
| 46.2 | 5.5 | 50 | 234 | 47.6 | 6.2 | 67 | 222 |
| 49.1 | 6.5 | 90 | 492 | 50.4 | 5.4 | 37 | 131 |
| 50.9 | 5.5 | 58 | 205 | 56.8 | 6.7 | 1160 | 436^* |
| 52.7 | 5.7 | 12 | 123 | 53.1 | 5.7 | 38 | 841 |
| 59.9 | 5.6 | 69 | 233 | 62.3 | 6.0 | 74 | 420 | CTT1 |
| 70.7 | 5.9 | 16 | 230 | 71.0 | 5.8 | 13 | 318 |
from the quantitation of individual proteins that several different patterns of regulation could be discerned, as shown in a blowup of a portion of the gels centered around an $M_r$ of 55 and a pI of 5.5 and demonstrating the regulation of three selected proteins in SKQ2n, from 0, 0.7, and 1.4 M NaCl, and Y41 from 1.4 M NaCl (Fig. 4). A ratio, $Q$ (Fig. 5), was therefore calculated for each of the more than 3-fold responsive proteins between 0 and 1.4 M NaCl, and this $Q$ value was subsequently used to group these responders into three classes depending on between which salinities the main expression change occurred. A value for $Q$ falling between 0.4 and 2.3 was somewhat arbitrarily chosen as an indication of a protein expression regulation more or less linearly with salt concentration, a response that we will hereafter refer to as halometric. Proteins displaying their major change already at 0.7 M NaCl, corresponding to a $Q$ value below 0.4, were characterized as having a low-salt response while a major change at the highest salinity, indicated by a $Q$ above 2.3, was termed high-salt response. A number of proteins, especially among the increasing, have a very low ppm value in the uninduced state, and quantifications of spots close to background values tend to be overestimated or uncertain. For this reason, no $Q$ value was calculated for spots with an uninduced level of below 100 ppm, and these were therefore grouped together as unclassified responders (Table II).

Since the selection into classes described above will naturally yield border straddlers, we have also indicated the proteins that fulfill a more stringent classification by an asterisk in Table II. By these more stringent criteria, a high-salt response should have a $Q$ of $\pm$ 0.5, a low-salt response should have a $Q$ value below 0.1, and a high-salt response should have a $Q$ value of more than 10.

High-salt Responding Proteins—This class of proteins constitute the largest group, both for increasing and decreasing proteins, comprising 29 of 45 classified proteins. 17 of the 29 halometric proteins were found to decrease, most by 3–4-fold, and about half of the proteins in this class have been identified (Table II). Glutamate dehydrogenase (NADP$^+$), encoded by the $GDH1$ gene, was identified as spot p53.3/5.5 as the most repressed protein present, and it is likely that the slightly less regulated p53.5/5.4 is a charge variation, satellite spot, of the same protein, thus explaining the similar regulation. The same would apply to the pair of spots p39.8/6.3 and p40.0/6.4, which were also among the most repressed enzymes, the first of which has been identified as the product of the alcohol dehydrogenase 1 ($ADH1$) gene. The spot corresponding to Enol2p, p46.8/5.8, was repressed approximately 2-fold, which on account of the very high level at which this protein is synthesized (Table III) makes it the most repressed enzyme seen as absolute change in number of protein molecules/cell. Pyruvate decarboxylase (Pdc1p) and the glycolytic enzyme fructose bisphosphate aldolase (Fba1p) were both repressed by salt, although the latter only about 2.2-fold.

Only 3 of 22 halometrically increasing proteins have been identified as yet (Table II), and all of these are induced to a rather low degree, a maximum of 4-fold. Gpd1p (p46.1/5.4), encoding sn-glycerol-3-phosphate dehydrogenase (NADH) and Gpp2p, encoding one of the recently identified glycerol-3-phosphatases, have previously been shown to be induced by growth in salt, and their positions were also known (8, 22). The third identified halometrically responsive protein, p59.5/5.2, was shown to correspond to the open reading frame SC9745.2 (YML070w) (Table I) and to be induced by approximately the same -fold change as Gpd1p and Gpp2p. Most of the halometrically increasing proteins have to date not been obtained in sufficient quantities to allow N-terminal sequencing, and the function and gene linkage of these, therefore, remain unknown.

High-salt Responding Proteins—This is the smallest class with only five members (Table II). None of the three high salt increasing proteins have been linked to a corresponding gene, since they are synthesized at low levels, below 750 ppm, which makes the identification of them difficult. The most dominant of the two high salt decreasing proteins, p85.7/6.1, was identified as methionine synthase (Met6p). It is probable that the other member is a charge variety of Met6p, since it is of the same-fold change as Gpd1p and Gpp2p. Most of the halometrically increasing proteins have to date not been obtained in sufficient quantities to allow N-terminal sequencing, and the function and gene linkage of these, therefore, remain unknown.

Low-salt Responding Response—This group is made up of four increasing proteins and seven decreasing proteins. The most dominant of the increasing proteins, p48.9/6.3, was identified as enolase I, which is the product of the ENO1 gene. As is the case for other dominant spots such as Gdh1p and Adh1p,
Identification of Enzymes in the Dihydroxyacetone Pathway—The halometrically induced protein p59.5/5.2, which was identified as the product of SC9745_2 (YML070w) in 1.4 M NaCl, is together with YFL053w, one of two yeast homologs of the recently purified and characterized dihydroxyacetone kinase (DhaK) from Citrobacter freundii (29). The two yeast genes are 46% identical, and both are approximately 37% identical (56% similar) to the C. freundii enzyme. We will therefore henceforth use the names DAK1 (dihydroxyacetone kinase) and DAK2, respectively, for the SC9745_2 and YFL053w genes. An aligning of the three genes shows that most regions that are conserved between Dak1p and Dak2p are also conserved in the C. freundii DhaK protein (Fig. 6). The longest conserved regions are located N-terminally, with the C terminus also containing several short regions of identity. In the middle part of the Dak1p sequence (approximate position 330–400) is a stretch of amino acids that shows a very low degree of conservation between the two yeast genes and that is largely missing in the sequence of the bacterial protein. The predicted positions on two-dimensional gels are 62.1 kDa/pI 5.28 and 62.1 kDa/pI 5.69 for Dak1p and Dak2p, respectively, which fits well with the position of Dak1p on two-dimensional gels (p59.5/5.2), while no spot corresponding to Dak2p has yet been identified.

To further substantiate the putative identity of Dak1p, the activity of dihydroxyacetone kinase was measured in crude extract by the method previously utilized for Schizosaccharomyces pombe (25) and Z. rouxii (20). For cells from 0 M NaCl, the activity was 0.9 units/mg of protein while the corresponding value for 1.4 M NaCl grown cells was 3-fold higher, or 22.9 ± 0.4 units/mg of protein. This is in good agreement with the 3.6-fold increase of protein amount seen on the two-dimensional gels (Table II).

The existence of the dihydroxyacetone kinase genes also implies the presence of the enzyme GLD in S. cerevisiae. However, we were not able to measure any such activity, despite several attempts by different methods (see “Experimental Procedures”). The GLD activity has also previously been reported as very low in D. hansenii (21). No corresponding gene has to our knowledge been cloned and sequenced in any eukaryote, and regions conserved between the four known bacterial glyceraldehyde dehydrogenase genes do not share homology with any unidentified open reading frame (FUN gene) in the yeast genome sequencing project. There is, however, glycerol dehydrogenase activity purified and partially characterized from A. niger, which was found to be mediated by a 38-kDa protein (30). Such NADP⁺-dependent glycerol dehydrogenase activity from A. niger is commercially available. Protein from a batch of this enzyme was run on SDS-PAGE, which yielded two major bands of approximately 37 and 34 kDa (Fig. 7A). These were digested with trypsin as described previously (22), and a number of
peptides were N-terminally sequenced. The 37-kDa protein was found to share significant homology with four yeast FUN genes putatively identified as members of the aldoreductase/ketoreductase family. The two genes showing the best homology to the peptides generated from the *A. niger* enzyme, *GCY1* (31) and *YPR1*, are furthermore 65% identical to each other over the whole sequence (Fig. 8B). The other two genes with significant homology to the *A. niger* enzyme, *YDL124w* and *YBR149w* (*YBZ9*), share between 30 and 40% identity with the other two putative GLD genes. Not two-dimensional spot corresponding to any of the putative GLD genes has been identified, and both Gcy1p (p35.1/8.0; theoretical values) and Ypr1p (p34.8/6.9; theoretical values) have predicted pI outside the range of our gels. From Northern analysis it is, however, evident that *YBR149w* (*YBZ9*) is almost constitutively expressed, while *GCY1* is strongly up-regulated and *YPR1* is slightly induced under salt stress conditions (Fig. 8). No signal from *YDL124w* was detected, suggesting that this gene is silent under the experimental conditions. All four genes have a codon bias of 0.2–0.35, which is a property they share with both *DAK1* and *DAK2*, probably indicating similar, moderate, levels of expression.

We obtained two peptide sequences from the protein of 34 kDa (Fig. 7A). One of these, IQFGGDEVVK, is 100% identical to amino acids 224–233 in malate dehydrogenase 1, *MDH1*, from *S. cerevisiae*. A second peptide, IHXVGPNVEYEQGLIXXALDGLK, had homology to amino acids 338–362 in mitochondrial malate dehydrogenase from the algae *Chlamydomonas reinhardtii* (GenBank™ accession number U40212). A homology search of the two peptides directly to these two malate dehydrogenase genes indicated the same regions as conserved, and these regions of homology were furthermore in both cases preceded by a lysine or arginine, constituting potential trypsin-cutting sites. Similar homologous regions are also found in malate dehydrogenase from *Cucumis sativus* and *Citrullus vulgaris* (data not shown).

**Fig. 6.** Alignment of *DAK1* (SC79425) gene, encoding a putative dihydroxyacetone kinase, to DhaK from *C. freundii*. Boldface letters indicate identity between *DAK1* and *DAK2* genes, a line indicates identity between DhaK and *DAK1*, and an asterisk points to amino acid residues found in all three genes.
difficult. It did, however, seem to be expressed at a roughly constant level by visual inspection of the very weak signal (data not shown).

Strain Y41 Is Much Less Responsive than SKQ2n on Protein Level—In a previous study we showed that *S. cerevisiae*, strain Y41, displayed very few changes in protein expression in response to 0.7M NaCl in the medium, the only identified increasing protein being Gpd1p (19). A noteworthy difference between that study and this work is that the glucose concentration in the present case was 2% (0.5% in Ref. 19), a fact that might affect the salt-instigated protein pattern. We therefore ran two-dimensional gels of salt-grown cultures of Y41 from medium with 2% (w/v) glucose including medium with 1.4M NaCl to enable a more strict comparison with strain SKQ2n. It was found that the trend from the earlier work persisted under these conditions and also that, even at 1.4M NaCl, very little change in protein expression was encountered in strain Y41. This is exemplified in a close up of a region from a two-dimensional gel of cells from 1.4M NaCl, where it can be seen that the protein pattern from Y41 is very similar to that seen for the control cells of SKQ2n at 0M NaCl with Hxk2p constitutively high and with only slight induction of Dak1p and no induction of protein p53.1/5.7 (Fig. 4).

DISCUSSION

73 Proteins Change Their Expression More than 3-fold in 1.4M NaCl—We have found 73 proteins to be regulated during exponential growth in minimal glucose medium with 1.4M NaCl...
sodium chloride. Of these salt-responsive proteins, 30 showed decreased and 43 showed increased expression by a factor of at least 3-fold compared with the situation in basal medium. At higher magnitudes of expression changes, induction was almost exclusively the mode of regulation encountered. Furthermore, most of the repressed proteins were clearly expressed also during osmotic stress, while many of the increasing proteins were induced from a nonstress level close to or below the background of detection. The implication of this is that during growth under osmotic stress there was mainly a need for induction and the addition of new metabolic pathways rather than the repression and silencing of existing ones.

A high proportion of the proteins exhibiting decreased expression have been identified. A striking feature is the dominance of enzymes involved in the synthesis of amino acids, such as Ilv5p, Lys9p, Met6p, and Sam1p, and proteins implicated as having a role in translation or protein folding such as Ssb1p asIlv5p, Lys9p, Met6p, and Sam1p, and proteins implicated as having a role in translation or protein folding such as Ssb1p. Among the other identified down-regulated proteins was Adh1p, the repression of which is probably a consequence of the demand for NADH in salt-instigated synthesis of glycerol. The down-regulation of Adh1p will presumably favor an increased flux of carbon from acetaldehyde to acetate instead of to ethanol, thus generating NADH instead of consuming it. This salt-induced flux to acetate (3) is probably mediated by a cytosolic aldehyde dehydrogenase previously shown to be induced by osmotic stress (15).

Several putative aldehyde dehydrogenases have been identified in the yeast genome sequencing project, among them Ald6p (YPL061W), which was in the present study classified as a low salt decreasing protein. The metabolic significance of Ald6p, as well as that of the other aldehyde dehydrogenase genes, can at present only be speculated upon. However, the fact that two aldehyde dehydrogenases display opposite regulation of expression, one increasing and one decreasing, might suggest differences in the substrate affinity of the two forms or different interactions with other proteins.

Dak1p Increases in Parallel with Gpd1p, Suggesting a Novel Pathway for Glycerol Turnover under Osmotic Stress—The increased production of glycerol in response to osmotic stress has been shown to involve the enhanced expression of one isogene of glycerol-3-phosphate dehydrogenase (NAD\(^+\)), GPD1 (4), and one isogene of glycerol 3-phosphatase, GPP2 (8), both of which are identified also in the present work as halometrically induced enzymes. During growth on glycerol as carbon and energy source, the reverse pathway is utilized, involving glycerol kinase, GUT1 (34), and a mitochondrial glycerol-3-phosphate dehydrogenase, GUT2 (35). An alternative pathway for glycerol catabolism, involving glycerol dehydrogenase (NAD\(^+\)) and dihydroxyacetone kinase, exists in various organisms and has been shown to be utilized by S. pombe (25).

Four putative NADP\(^+\)-dependent GLD genes were identified in this study in a homology search of the S. cerevisiae genome, using peptides generated from A. niger glycerol dehydrogenase (NAD\(^+\)), which has been purified and shown to have an \(M_w\) of 38 kDa (30). A pair of homologous genes (65% identical) were the most likely candidates for yeast GLD genes, and they were shown via Northern analysis to have different responses to salt stress. GCY1 belongs in the class of strongly halometric responders, while YPR1 was only slightly induced by salt. A third GLD homolog, YBZ9 (YBR149w), showed also a slight salt induction, while no transcripts could be detected under any growth condition utilizing probes for the fourth GLD homolog, YDL124w.

The sequences of these putative NADP\(^+\)-dependent GLD enzymes in yeast displayed no apparent sequence homology to the bacterial NAD\(^+\)-dependent counterparts. Will this fact disqualify the tentative GLD functionality of these open reading frames? A GLD (NAD\(^+\)) has been purified from S. pombe as an octamer of 47-kDa subunits (36). The GLD (NAD\(^+\)) from S. pombe and that of C. freundii (DhaD) (29) exhibit very low \(K_m\) values for the cytosolic NADP\(^+\) from S. pombe and that of C. freundii (DhaD) (29) exhibit very low \(K_m\) values for glycerol (approximately 1 mM), compared with the reported \(K_m\) values of almost 1 M for the A. niger GLD (NAD\(^+\)) (30). This suggests that the GLD enzymes requiring NAD\(^+\) and NADP\(^+\) constitute different protein families, from which the former are likely to be utilized for glycerol catabolism. Whether there are at least two evolutionary families of GLD (NAD\(^+\)) is uncertain, since no sequence is available for any of these enzymes. However, a partially purified GLD (NAD\(^+\)) enzyme from S. pombe seems to be different from the A. niger counterpart, and purification yielded a protein of native size 57 kDa, consisting of two subunits of 25 and 30 kDa (37).

We furthermore report that Dak1p (SC9745_2), a S. cerevisiae homolog of DhaK from C. freundii, was induced approximately 4-fold during growth under salt stress. The fact that the expression of both a putative glycerol dehydrogenase and a dihydroxyacetone kinase are increased during salin growth provides tentative evidence that glycerol is metabolized via this pathway during salin growth, perhaps providing a metabolic overflow path involved in the fine tuning or sensing of intra-
Protein Expression of S. cerevisiae in 1.4 M NaCl

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TABLE IV
A site from the ENO1 upstream repressor sequence found in the four salt-induced genes GPD1, GPP2, GCY1, and DAK1

| Gene | Consensus |
|------|-----------|
| ENO1 | TATGCCCTCT |
| GPD1 | TATGCCCTCT |
| GPP2 | TATGCCCTCT |
| GCY1 | TATGCCCTCT |
| DAK1 | CTCTTGCCTC |

Position is given relative to translation start ATG, with the first A as +1.

Although Dak1p and Gpd1p are present in roughly equimolar amounts induction by salt stress was comparable with these species. In addition to its GPD activity and to a lesser extent dihydroxyacetone kinase activity reported previously (3). Acetate metabolism, which includes the glycerol facilitator Fps1p (38), has been suggested for Dunaliella salina (39).

We have measured the activity of dihydroxyacetone kinase at levels in parity with those found for D. hansenii (21) and Z. rouxii (20) and have furthermore shown that the degree of induction by salt stress was comparable with these species. Although Dak1p and Gpd1p are present in roughly equimolar amounts in S. cerevisiae, the measured activity of dihydroxyacetone kinase seems to be approximately 10% of the GPD activity reported previously (3). D. hansenii also induced both GPD activity and to a lesser extent dihydroxyacetone kinase activity during growth in 1.4 M NaCl (21). In addition to its tentative role in being a glycerol pool regulator, the path via dihydroxyacetone, in conjuction with GPD and GPP enzymes, could provide the cell with an enzymatic cycle functioning as a transhydrogenase converting NADH to NADPH at the expense of one ATP. No transhydrogenase enzyme from yeast has yet been described, although the corresponding activity has been reported to be mainly cytosolic in S. cerevisiae (40).

Different Regulation of Isogenes—A striking observation is that most of the salt-responsive proteins have isogenes that are differentially expressed during salt stress (Fig. 9). Some gene pairs display an opposite regulation by salt stress; examples include GLK1/HXK2, ENO1/ENO2, and ALD5/ALD6. The other main pattern is for one isogene to be regulated while the other gene shows roughly constant expression, which was found for the increasing GPD1, GPP2, GCY1, and DAK1. It is possible that the function of the two isoforms is to mediate differential interactions with proteins, e.g. Eno2p, the main form of enolase expressed during nonstressed growth in glucose medium, might perhaps be part of a glycolytic complex associated with Pdc1p, Adh1p, and Ald6p, while Eno1p might similarly be part of an alternative, stress-induced glycolytic protein complex, associated with Ald5p and perhaps also with the salt-induced enzymes involved in glycerol metabolism.

Enolase I Is Induced by Salt Stress—The enolase genes, ENO1 and ENO2, were found to display opposite regulation under salt stress conditions, and a similar pattern of regulation of the enolase genes has also earlier been reported for cells entering the stationary phase (41, 42). The promotor of ENO1 contains at least two upstream activating sequences, and there is also an upstream repressor sequence (43). It is probable that one of the upstream activating sequence elements is responsible for the increase of Eno1p during stationary phase (42). The upstream repressor sequence element is located between nucleotides −181 and −143 from the start of transcription, and deletions within this region will make Eno1p expression glucose-inducible to the same level as Eno2p (43). It has furthermore been demonstrated that this upstream repressor sequence is functional when inserted into minimal promoter constructs, but not in the reverse direction (42). The 38-base pair upstream repressor sequence was compared with the promoters of GPD1, GPP2, GCY1, and DAK1, all of which were found to show similar regulation as ENO1 (this study), and a consensus sequence 5′-TATGCCCTCT-3′, centered at −163 in the ENO1 promotor, was found in all four salt-induced genes (Table IV).

Enolase I was the first heat shock protein, Hsp48, to be identified (44), and it was also shown to be induced upon entry into G₀ arrest (45), which also makes cells constitutively more resistant to heat shock. A heat shock-resistant mutant, hsr1 (46), later located to the adenylate cyclase gene, CYR1 (47), constitutively synthesized four proteins, one of them Eno1p. Since elevated synthesis of Eno1p was the only overlap between the different heat shock-tolerant physiological states of yeast, it was suggested that increased expression of the ENO1 gene is responsible for the stress-tolerant phenotype (46).

Proteins Required for Growth and Acquisition of Stress Tolerance—Gpd1p has previously been reported to be induced by salt in several studies (4, 7, 9, 19), thus indicating the importance of this gene in allowing growth under hyposomatic stress. A decrease in the activities of glyceroldehyde-3-phosphate dehydrogenase and enolase, as well as a decrease in the level of Sam1p was also suggested to be of importance during growth in saline medium (19). However, most of the other salt-dependent protein responses found in strain SKQ2n seem to be dispensable during growth under hyposomatic stress, since they were not seen for strain Y41 (19), even at high salinities (this study). It could be speculated that the transient protein response to salt stress shown for Y41 (18) and the very
minor changes in protein synthesis seen during growth in 0.7 M NaCl medium (19) are responsible for, or reflect, the low tolerance to osmotic shock seen for this strain (48). Enolp is only found to be induced by salt in the more halotolerant strain SKQ2n, which provides further evidence that this protein itself might determine stress tolerance, as suggested previously (46). It could also be that the levels of Enolp and many other genes affected by salinity are regulated by some signaling pathway that is involved in determining stress tolerance. A good candidate is the Ras-cAMP path, which has been implicated in the regulation of several stress-induced genes such as SSA3 (49), CTT1 (50), and HSP12 (14). The level of cAMP, and thus presumably the activity of the cAMP-dependent protein kinase A, has indeed been reported to be lowered during saline growth (48). Eno1p is only affected by salinity in the more halotolerant strain (48). Eno1p is only found to be induced by salt in the more halotolerant strain (48).

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REFERENCES

1. Blomberg, A., and Adler, L. (1992) *Adv. Microb. Physiol.* 33, 145–212
2. Ölz, R., Larsson, K., Adler, L., and Gustafsson, L. (1993) *J. Bacteriol.* 175, 2205–2213
3. Blomberg, A., and Adler, L. (1989) *J. Bacteriol.* 171, 1087–1092
4. Larsson, K., Ansell, R., Eriksson, P., and Adler, L. (1993) *Mol. Microbiol.* 10, 1101–1111
5. Eriksson, P., André, L., Ansell, R., Blomberg, A., and Adler, L. (1995) *Mol. Microbiol.* 15, 95–107
6. Garrels, J. I., Hutchings, B., Kobayashi, R., Latter, G. I., Schwender, B., Volpe, T., Warner, J. R. and McLaughlin, C. S. (1994) *Electrophoresis* 15, 1–21
7. Albertyn, J., Holmgren, S., and Prior, B. A. (1994) *Curr. Genet.* 25, 12–18
8. Norbeck, J., Pahlman, A.-K., Akhtar, N., Blomberg, A., and Adler, L. (1996) *J. Biol. Chem.* 271, 13875–13881
9. Hirayama, T., Maeda, T., Saito, H., and Shinozaki, K. (1995) *Mol. Gen. Genet.* 249, 127–138
10. Brewster, J. L., De Valoir, T., Dwyer, N. D., Winter, E., and Gustin, M. C. (1993) *Science* 259, 1760–1763
11. Maeda, T., Wurgler-Murphy, S. M., and Saito, H. (1994) *Nature* 369, 242–245
12. Maeda, T., Takekawa, M., and Saito, H. (1995) *Science* 269, 554–558
13. Schüller, C., Brewster, J. L., Alexander, M. R., Gustin, M. C., and Ruis, H. (1994) *EMBO J.* 13, 4382–4389
14. Varela, J. C. B., Prachelt, U. M., Meeacock, P. A., Planta, R. J., and Mager, W. H. (1995) *Mol. Cell. Biol.* 15, 6232–6245
15. Miralles, V. J., and Serrano, R. (1995) *Mol. Microbiol.* 17, 653–662
16. Márquez, J. A., and Serrano, R. (1996) *FEBS Lett.* 382, 89–92
17. Hirata, D., Harada, S., Namba, H., and Miyakawa, T. (1995) *Mol. Gen. Genet.* 249, 257–264
18. Blomberg, A. (1995) *J. Bacteriol.* 177, 3563–3572
19. Norbeck, J., and Blomberg, A. (1996) *FEBS Microbiol. Lett.* 137, 1–8
20. Van ZyI, P. J., Prior, B. A., and Kilian, S. G. (1991) *Appl. Microbiol. Biotechnol.* 36, 369–374
21. Adler, L., Blomberg, A., and Nilsson, A. (1985) *J. Bacteriol.* 162, 300–306
22. Norbeck, J., and Blomberg, A. (1995) *Electrophoresis* 16, 149–156
23. Blomberg, A., Blomberg, L., Norbeck, J., Farnham, S., Larsen, P., Larsen, M., Roepstorff, P., Degand, H., Boutry, M., Posch, A., and Garg, A. (1995) *Electrophoresis* 16, 1935–1945
24. Garrels, J. I. (1983) *Methods Enzymol.* 100, 411–423
25. Gancedo, C., Llebell, A., Ribas, J.-C., and Luchi, F. (1986) *Eur. J. Biochem.* 159, 171–174
26. Fuge, K. E., Braun, E. L., and Werner-Washburne, M. (1994) *J. Bacteriol.* 176, 5802–5813
27. Bataille, N., Régnacqo, M., and Boucherie, H. (1991) *Yeast* 7, 367–378
28. Demianova, M., Formosa, T. G., and Ellis, S. R. (1996) *J. Biol. Chem.* 271, 11383–11391
29. Daniel, R., Steuritz, K., and Gottschalk, G. (1995) *J. Bacteriol.* 177, 4392–4401
30. Schuurink, R., Busink, R., Hommers, D. H. A., Witteveen, C. F. R., and Visser, J. (1990) *J. Gen. Microbiol.* 136, 1043–1050
31. Oechsner, U., Magdolen, V., and Bandlew, W. (1988) *FEBS Lett.* 253, 123–128
32. Nelson, R. J., Ziegenhoff, T., Nicolet, C., Werner-Washburne, M., and Craig, E. (1992) *Cell* 71, 97–105
33. Hu, Y., Cooper, T. G., and Kihliah, G. B. (1995) *Mol. Cell. Biol.* 15, 52–57
34. Pavlik, P., Simon, M., Schuster, T., and Ruis, H. (1993) *Curr. Genet.* 24, 21–25
35. Ronnow, B., and Kieland-Brandt, M. C. (1993) *Yeast* 9, 1121–1130
36. Marshall, J. H., May, J. W., and Sloan, J. (1985) *J. Gen. Microbiol.* 131, 1581–1588
37. Marshall, J. H., Kong, Y.-C., Sloan, J., and May, J. W. (1989) *J. Gen. Microbiol.* 135, 697–701
38. Luyten, K., Albertyn, J., Fourie-Skibbe, W., Prior, B., Ramos, J., Thevelein, J. M., and Hofmann, S. (1995) *EMBO J.* 14, 1360–1371
39. Lerner, H. R., Sussman, J., and Avren, M. (1980) *Biochim. Biophys. Acts.* 615, 1–9
40. Evans, T. C., Mackler, B., and Grace, R. (1985) *Arch. Biochem. Biophys.* 243, 492–503
41. McAlister, L., and Holland, M. J. (1983) *J. Biol. Chem.* 257, 7181–7188
42. Carmen, A. A., Brandile, P. K., See Park, C., and Holland, M. J. (1995) *Yeast* 11, 1031–1043
43. Cohen, R., Yoki, T., Holland, J. P., Pepper, A. E., and Holland, M. J. (1987) *Mol. Cell. Biol.* 7, 2573–2571
44. Iida, H., and Yahara, I. (1985) *Nature* 315, 688–690
45. Iida, H., and Yahara, I. (1984) *J. Cell. Biol.* 99, 199–207
46. Iida, H., and Yahara, I. (1984) *J. Cell. Biol.* 99, 1441–1450
47. Iida, H. (1988) *Mol. Cell. Biol.* 8, 5555–5560
48. Blomberg, A. (1996) *Yeast*, in press
49. Beerst, W. R., and Craig, E. A. (1993) *EMBO J.* 12, 2543–2553
50. Marchler, G., Schuller, C., Adam, G., and Ruis, H. (1993) *EMBO J.* 12, 1997–2003