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Global Changes in Protein Synthesis during Adaptation of the Yeast *Saccharomyces cerevisiae* to 0.7 M NaCl

ANDERS BLOMBERG*

Department of General and Marine Microbiology, Lundberg Laboratory, University of Göteborg, S-413 90 Göteborg, Sweden

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Exponentially growing *Saccharomyces cerevisiae* was challenged to increased salinity by transfer to 0.7 M NaCl medium, and changes in protein synthesis were examined during the 1st h of adaptation by use of two-dimensional gel electrophoresis coupled to computerized quantification. An impressive number of proteins displayed changes in the relative rate of synthesis, with most differences from nonstressed cells being found at between 20 and 40 min. During this period, 18 proteins exhibited more than eightfold increases in their rates of synthesis and were classified as highly NaCl responsive. Only two proteins were repressed to the same level. Most of these highly NaCl-responsive proteins seemed to constitute gene products not earlier reported to respond to dehydration. Applying a selection criterion to subsequent samples of a twofold change in the relative rate of synthesis, 14 different regulatory patterns were discerned. Most identified glycolytic enzymes exhibited a delayed response, and their rates of synthesis did not change until the middle phase of adaptation, with only a minor decrease in the rate of production. A slight salt-stimulated response was observed for some members of the HSP70 gene family. Overall, the data presented indicate complex intracellular signalling as well as involvement of diverse regulatory mechanisms during the period of adaptation to NaCl.

Water molecules possess a high permeability coefficient for passage over the cellular plasma membrane, a property that instigates a massive initial efflux of cellular water when cells are submerged into a more-saline (hypertonic) medium. This is a fast and purely osmotic phenomenon driven by the attainment of cellular water potential equilibrium with the surroundings, and the amount of water lost is a function of a number of cellular parameters as well as the stress magnitude (7). A prominent feature of the cellular response of yeast cells to an increase in the external salt concentration is the enhanced production and subsequent accumulation of the polyhydroxy-alcohol glycerol (6, 7, 10). A key enzyme in this production, at least for the yeast *Saccharomyces cerevisiae*, is glycerol-3-phosphate dehydrogenase (GPD), and cells have been shown to respond to the osmotic stress by an increased specific activity of this enzyme (6, 11). This activity increase reflects an enhanced number of protein molecules in the cell (3), and *GPD1* expression is osmotically regulated at the level of DNA transcription (1, 2, 14, 41).

The components of a signal transduction pathway that is activated by changes in the osmolarity of the extracellular environment and that has been shown to be involved in the glycerol response have been identified (9). This high-osmolarity glycerol response (HOG) pathway contains a mitogen-activated protein kinase called Hog1p and the mitogen-activated protein kinase kinase Pbs2p. In response to cellular dehydration, Hog1p is rapidly tyrosine phosphorylated, which seems to be a prerequisite for dehydration-induced transcriptional activation of the *GPD1* gene during adaptation to osmotic stress (1, 13a). Another gene under the control of the activity of the HOG pathway is *CTT1* (39).

In addition to glycerol accumulation, proper modulation of the intracellular salinity is of vital importance during NaCl stress. Na^+ homeostasis is achieved by the coordinate regulation of plasma-membrane-located influx and efflux systems. Under NaCl stress, the K^+ uptake system is converted to a high-affinity mode that results in higher K^+-Na^+ discrimination, which reduces the entrance of sodium ions (21). Na^+ efflux is mediated by the P-type ATPase encoded by *ENA1*, which is transcriptionally regulated by NaCl (15). These NaCl-induced cellular responses involved in ion homeostasis are under the control of a signalling path containing the Ca^{2+}-calmodulin-dependent protein phosphatase calcineurin (29).

Additional genes under NaCl control include *HSP26* and *HSP12* (41), both of which are also strongly regulated by heat treatment. Salinity-induced expression of heat shock genes may indicate heat shock factor-mediated osmotic signalling; however, this factor is apparently not involved in *GPD1* regulation (14, 40).

To further characterize this complex cellular response, a global system approach utilizing computerized two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) analysis was conducted in order to highlight qualitative and temporal changes in the synthesis of individual proteins during adaptation to NaCl-supplemented medium. Classification of responsive proteins into regulatory classes might reveal the amount of regulatory mechanisms and/or paths involved, as well as selecting interesting proteins for further studies.

The cellular processes taking place during adaptation to intermediate salt stress greatly increase the resistance of cells to a sudden exposure to high concentrations of NaCl (25). The tolerance determinant is not the intracellular accumulation of glycerol, since osmotically conditioned cells washed free of glycerol retain their capacity for colony formation on high-salt-concentration media (6). In addition, blockage of protein synthesis inhibits the acquisition of the tolerant state, indicating a protein-mediated phenotype. Furthermore, increased activity of the glycerol-producing enzyme GPD has been shown not to be the single tolerance determinant (8). Thus, the aim of this study was twofold: (i) to study the cellular response in terms of quantitative and temporal changes in the rate of synthesis of proteins during adaptation to osmotic stress and (ii) to identify

* Mailing address: Department of General and Marine Microbiology, Lundberg Laboratory, University of Göteborg, Medicinaregatan 9C, S-413 90 Göteborg, Sweden. Phone: 46 31 7732589. Fax: 46 31 7732599. Electronic mail address: Anders.Blomberg@gmm.gu.se.

proteins potentially responsible for the cellular state of tolerance to a sudden osmotic shock.

MATERIALS AND METHODS

Yeast strain and growth conditions. Strain Y41 (ATCC 38531) of *S. cerevisiae* was maintained short term on YPD agar (1% [wt/vol] yeast extract, 2% [wt/vol] peptone, 2% [wt/vol] glucose, and 2% [wt/vol] agar) or at −70°C in 20% (vol/vol) glycerol for long-term storage.

The defined medium that was used in all experiments contained 0.67% (wt/vol) yeast nitrogen base without amino acids (YNB) (Difco Laboratories, Detroit, Mich.) supplemented with 0.5% (wt/vol) glucose and, when indicated, with 0.7 M of NaCl. YNB (10× strength) was filter sterilized, while glucose and salt solutions were autoclaved separately. In all experiments, the flasks were incubated at 30°C on a rotary shaker (110 rpm). Primary cultures were grown for 24 to 48 h in 300-ml flasks containing 50 ml of medium and subsequently were used as an inoculum for 500 ml of medium, contained in 2.8-liter Fernbach flasks, to yield an initial concentration of about 2×10^4 CFU/ml. Adaptation experiments were performed with cells grown for about 15 h to an optical density at 610 nm (OD_{610}) of 0.50 (equivalent to 5×10^6 CFU/ml), as monitored with a spectrophotometer (model B; Beckman Instruments, Inc.). The exponentially growing culture was divided into 250-ml portions and centrifuged (4,000 × *g*, 5 min, 20°C). The pellets were suspended in 250 ml of prewarmed (30°C) YNB medium with 0.7 M NaCl. The cultures were incubated for 60 min at 30°C, which is referred to as the period of adaptation.

Warburg measurements to record CO_2 production were performed essentially as described earlier (8). The fraction of budding cells was estimated by snap-freezing samples in liquid nitrogen at intervals during the adaptation period, for later microscopic observation.

Radiolabelling. In order to measure the rate of incorporation during the adaptation period, 1 ml of the culture was mixed with 1.4 μCi of [^{35}S]methionine (1,270 Ci/mmol; Amersham Corp. product SJ1515) in microcentrifuge tubes and incubated at 30°C for 2 min. Trichloroacetic acid (0.5 ml, 10% [wt/vol]) was added, and the sample was placed on ice for at least 1 h. The precipitable material was collected by filtration through a 0.2-μm-pore-size Nucleopore filter and washed, and radioactivity was measured in a liquid scintillation counter.

For protein analysis by 2D-PAGE, 10-ml samples were withdrawn at three time points (*T*) during the osmotic adaptation: $T = 0$, $T = 20$, and $T = 40$ min. The control cells were taken during exponential growth in nonsaline medium. Samples were incubated in the presence of 15 μCi of [^{35}S]methionine per ml in 150-ml Erlenmeyer flasks for 20 min at 30°C on a rotary shaker. In no case was the added methionine depleted during the labelling period.

Sample preparation. Protein samples were prepared essentially as earlier described (16, 17). Briefly, 5×10^6 cells were pelleted in a microcentrifuge tube and disrupted by the addition of 100 μl of sample buffer containing 0.3% (wt/vol) sodium dodecyl sulfate (SDS; Biorad), 5% (vol/vol) β-mercaptoethanol (Biorad), and 50 mM Tris (Sigma) buffer, pH 8, plus 0.1 g of glass beads (0.5 mm in diameter), and the samples were subsequently vortexed four times for 30 s each, with cooling on ice in between. The tubes were then heated at 95°C for 15 min and placed on ice. Ten microliters of DNARNA:s solution containing DNase (Worthington product LSO 6330) plus RNase (Worthington product LSO 5679) was added, and the samples were incubated for 10 min on ice. The samples were then snap-frozen in liquid nitrogen, packed on dry ice, and shipped to Protein Databases, Inc., for 2D-PAGE and fluorography. The radioactivity levels and the protein contents of the 12 samples were recorded (17) to determine their specific activities ± standard deviations (SD) (67,188 ± 30,015 dpm/μg of protein) and protein concentrations ± SD (0.62 ± 0.13 μg of protein per μl). The samples were lyophilized and subsequently dissolved in isoelectric focusing buffer (9.5 M urea [Schwartz Mann product 821527], 2% Nonidet P-40 [Accurate Chemical], 100 mM dithiothreitol [Calbiochem], and 2% 3 to 10 pH ampholytes [LKB]) at 37°C for 30 min.

2D-PAGE. The 2D-PAGE was performed by the methods of Garrels (16, 17). Approximately 10 to 20 μl of the sample, containing about 250,000 to 400,000 dpm (300,559 ± 77,365 [SD]), and a maximum of 20 μg of protein were loaded onto a narrow-bore isoelectric focusing tube (0.8-mm diameter; ~ 20 cm long). The isoelectric focusing tube contained 2.9% acrylamide (Biorad), 2% Nonidet P-40, 9.5 M urea, and 2% 3 to 10 pH ampholytes (LKB). The proteins were focused for 19,000 V · h overnight. 2D-PAGE was carried out as follows. The SDS-equilibrated isoelectric focusing gel was mounted on the 2D gel (24 cm by 24 cm by 1 mm) with an acrylamide (Serva) concentration of 10%. Electrophoresis in the second dimension was performed at 60 W (constant wattage) for approximately 4 h, corresponding to the time for the bromphenol blue marker to just reach the bottom of the gel.

Image development by fluorography. The gels with their corresponding radioactive calibration strips containing known amounts of radioactive protein (17) were then processed for fluorography, and four exposures of each gel were made (exposure I, 2 to 3 days; exposure II, 3 to 4 days; exposure III, 5 to 7 days; and exposure IV, 14 to 24 days). The time of exposure was increased as the disintegration-per-minute counts of the protein loaded onto the first-dimensional gel decreased. Films were subsequently sent to me for evaluation and computerized analysis.

Scanning and computerized spot quantification. All analyses of films were performed by myself at the 2D facility at the Department of General and Marine Microbiology, University of Göteborg, Göteborg, Sweden. The four films per gel were processed by using the commercially available 2D-gel computer analysis program PDQuest version 4.0 (Protein and DNA ImageWare Systems, Inc.), based on the initial design and appropriate algorithms of the Quest program (18) and running under the Openwindows environment on a SUN sparc1 workstation with 1.5 gigabytes of internal memory and 16 megabytes of RAM. Gel films were scanned with a Howtech Scanmaster 3 white-light desktop scanner at a resolution of 200 by 200 μm (roughly 1,156 by 1,345 pixels) and subsequently saved on disk where one film scan used about 1 megabyte of memory space. Scanning was performed in the transparency mode, and the medium-illumination calibration strip was used to set the sensitivity of the scanner. The scanner was turned on at least 30 min before scanning, since 15 min was experimentally found to be the minimum time of prewarming to ensure a constant signal response; in addition, the lamp was prewarmed by at least five prescans. Calibration of the scanner signal (255-Gy levels) to ODs was performed with an OD step tablet 3 (0.05 to 3.05 OD) (catalog number 152 3422; Kodak).

The spot detection and quantification procedure was started by merging of the four film scans per gel into a composite synthetic gel image without any premerging processing of the raw scans. The merging is the slowest step of the whole spot detection and takes about 20 min for one gel (four scans). The synthetic image was then smoothed by an "average smooth" algorithm, before background subtraction proceeded. Vertical and horizontal streaks were removed by use of the rolling disk (disk radius, 60 pixels) prior to processing with the algorithm "Fourier smooth" (window size; $x = 4$ pixels, $y = 4$ pixels) which removes minor background noise. The background levels were averaged and subtracted before spot detection (medium sensitivity) was performed, during which the spots were fitted into Gaussian volumes. All these steps were automatically executed without any operator interference. An average of 1,355 ± 252 (SD) spots per gel were detected for the 12 gels, with no significant difference in the numbers of spots of the control and the samples for the three time points during osmotic adaptation recorded. By visual inspection of the four exposures of a gel, roughly 1,000 protein spots were identified for any single gel. A small number of spots (2 ± 1.5 [SD]) were recorded as saturated. The manual interventions on the computer-processed gel films were kept to a minimum to increase the objectivity of the data analysis. No combining or cancelling operations were performed, since they were not found to be necessary. In some of the gels, however, a number of the more-dominant spots had been allocated multiple spot centers, and these were edited to a more uniform number. Spots detected close to the background tend to be underestimated (18), and the quantity of an average of 121 ± 27 (SD) faint spots (OD of <0.2 over background) was corrected by making the *x* and *y* sigma values the same as those of the spots in the same *y*-coordinate region of the film.

Gel matching, spot identification, and statistical analysis. The 12 gels (one for each sample) were assembled into a matched set, i.e., a "matchset." This is the unit of the comparative analysis and has two components: the reference gel image and the individual sample gel images. The reference image was made from one of the gel images (gel 11874 at $T = 20$ during the osmotic adaptation to 0.7 M NaCl medium). The subsequent step in the data analysis is landmarking, a process during which individual spots are manually matched to the reference image. A total of 677 spots were manually landmarked in each gel, and any spot missed during the landmarking could be scored by the "partial match" function and subsequently matched. For a protein detected in a sample gel image and not in the reference image, the spot was manually added to the reference image so that its presence was recorded. Automatic matching of the rest of the spots identified no further spots exhibiting quantitative changes.

The M_r and pI values assigned to spots adhere closely to values in the 2D-PAGE protein database of *S. cerevisiae* at Cold Spring Harbor, N.Y. (25a). Some of the most abundant proteins resolved on the 2D gels were biochemically identified and linked to their corresponding genes by comparisons of their patterns with those in existing 2D-PAGE protein databases (19, 32), in which identifications are based mainly on sequence data. The identities of Pgk and Pyk were based on comigration with purified protein (25a), and their 2D locations fitted well with the theoretical positions of Pyk1p (p54.5/7.7) and Pgk1p (p44.7/7.9).

Individual quantifications of the proteins resolved were normalized to the amount of radioactivity applied to the gel. Systematic errors in quantification between gels were compensated for by log normalization (18). The statistical analyses were performed by utilizing the statistical function within the PDQuest software. Proteins displaying significant changes in synthesis between samples were selected by a Student *t* test on log-transformed values.

RESULTS

Overall cellular response to osmotic dehydration. Exponentially growing cultures of *S. cerevisiae* were transferred to 0.7 M NaCl medium. At the time of the osmotic challenge, the culture was proliferating in the basal medium with a generation time of 2.2 h and cells were in the respiro-fermentative state of metabolism, with the time for glucose exhaustion and entrance

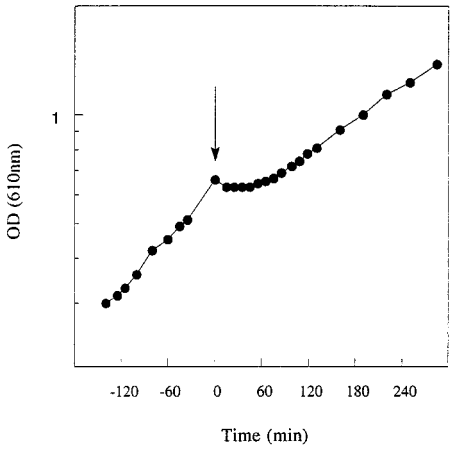

FIG. 1. Spectrophotometric recording at OD_{610} of the salinity-instigated growth response of *S. cerevisiae* Y41 upon transfer to medium containing 0.7 M NaCl. The arrow indicates the time of transfer ($T = 0$).

into the transition phase being preceded by 4 to 5 h (data not shown). The cellular dehydration brought about by the osmotic shock resulted in a rapid cessation of growth, and the time of adaptation before growth recommenced in the saline medium was found to be roughly 1 h (Fig. 1). Growth was slightly retarded in the saline medium, and the generation time for fully adapted cells during saline growth was estimated as 3.3 h. The proportion of budded cells in the exponentially growing culture was around 75% at the time of transfer from basal medium, and no significant change in the size of the budding fraction was observed during the initial hour of osmotic adaptation (data not shown). Exponentially growing cells in basal medium exhibited a fermentative capacity of 27 ± 2 (SD) μmol of CO_2 per mg (dry weight) per h, a rate consistent with earlier obtained results for cells with a high level of fermentative activity (8). A slight drop in the activity level to 20 ± 1 (SD) μmol of CO_2 per mg (dry weight) per h was recorded during the first 20 min, indicating a minor impact on fermentation during the initial period of adaptation. The CO_2 production displayed an increased rate at around 30 min, although it never returned to the preshift value. Some of the decline in gas-producing activity may be a consequence of the osmotically instigated increase in flux of carbon into glycerol (6).

Dehydration influence on the rate of total [^{35}S]methionine incorporation. In order to study global changes in protein synthesis during the 1-h osmotic adaptation, cells were to be labelled with [^{35}S]methionine during three consecutive 20-min periods (designated T = 0-20, T = 20-40, and T = 40-60). Fluctuations in incorporation during these labelling periods would influence the interpretation of the results, and cells in introductory experiments were thus labelled with [^{35}S]methionine for 2-min periods, either before transfer or at appropriate times during the adaptation (Fig. 2). The rate of incorporation initially decreased to 3% of its preshift value and then slowly recovered and peaked after 15 to 30 min of the adaptation period, although it never exceeded 30% of the rate of incorporation for exponentially growing cells in basal medium. From min 30 to 60, there were steady but minor declines in the rates of incorporation (Fig. 2). This result indicated that only during the first 20-min period (T = 0-20) is some incorporation-change-implemented bias put on proteins synthesized late (10 to 20 min) in the period.

2D-PAGE analyses of osmotically controlled synthetic changes. Cultures were labelled with [^{35}S]methionine for 20-min periods, either before transfer during exponential growth in nonsaline medium or at $T = 0$, $T = 20$, and $T = 40$ min after the osmotic challenge. Cells were mechanically disrupted in SDS and β-mercaptoethanol solution, and extracts were nuclease and heat treated prior to the 2D separation. Gels were subjected to fluorography at four different time intervals, after which the four exposures were scanned and merged by the image analysis software. Growth, labelling, and gel running were performed in three independent experiments, and the subsequent data analysis was accordingly based on comparisons of the sample triplicates. The central portion of a typical 2D gel of *S. cerevisiae* Y41 growing exponentially in nonsaline medium can be seen in Fig. 3A. The computer analysis allocated 1,355 ± 252 (SD) protein spots in each gel, and by visual inspection roughly 1,000 individual proteins could be identified when all four fluorographic exposures were inspected. It has been concluded from kinetic analysis of RNA-cDNA hybridization that the yeast genome expresses roughly 4,000 genes under normal growth conditions (22). Thus, about 25% of the proteins of the cell were detected in the 2D gels. This number agrees well with the portion of the total disintegrations per minute of protein loaded that was resolved in quantifiable spots, since on average 25% of that loaded onto the first-dimension gel tubes were detected as valid spots in the 2D pattern. The remaining disintegration per minute values encompass the background level subtracted, those below the level of detection, or those of proteins with pIs or M_rs outside the indicated range. The proteins separated span an apparent pI range of about pH 4.5 to 7.5 and an apparent M_r range of 20,000 to 150,000.

A global comparison of the numbers of statistically significant changes in the relative rates of synthesis of individual proteins among the three time points during the period of adaptation was performed. This analysis revealed that the T = 20-40 sample displayed the greatest number of changes, with 202 of the resolved proteins (roughly 20% of the total) exhibiting a significant alteration in synthesis relative to the control. During the initial 20 min of adaptation, 95 significant changes were scored. The middle phase (T = 20-40) was also the most

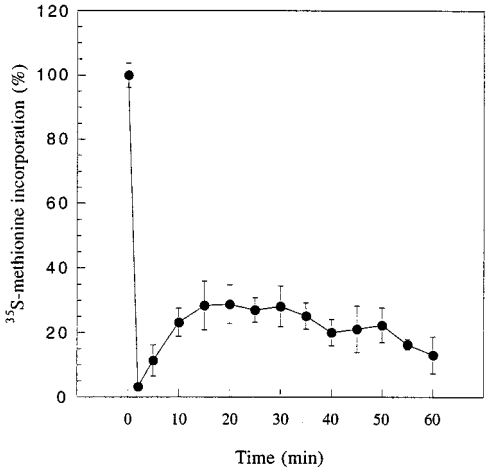

FIG. 2. Total rate of [^{35}S]methionine incorporation during osmotic adaptation of *S. cerevisiae*, to 0.7 M NaCl medium, measured as trichloroacetic acid-precipitable material from cells labelled with [^{35}S]methionine during 2-min periods. The rate of incorporation during exponential growth in basal medium (0 M NaCl), immediately prior to transfer into 0.7 M NaCl, was set to 100% (absolute value of 5×10^{-2} dpm/min/10^7 cells). The values represent averages ± SD (error bars) of duplicate experiments.

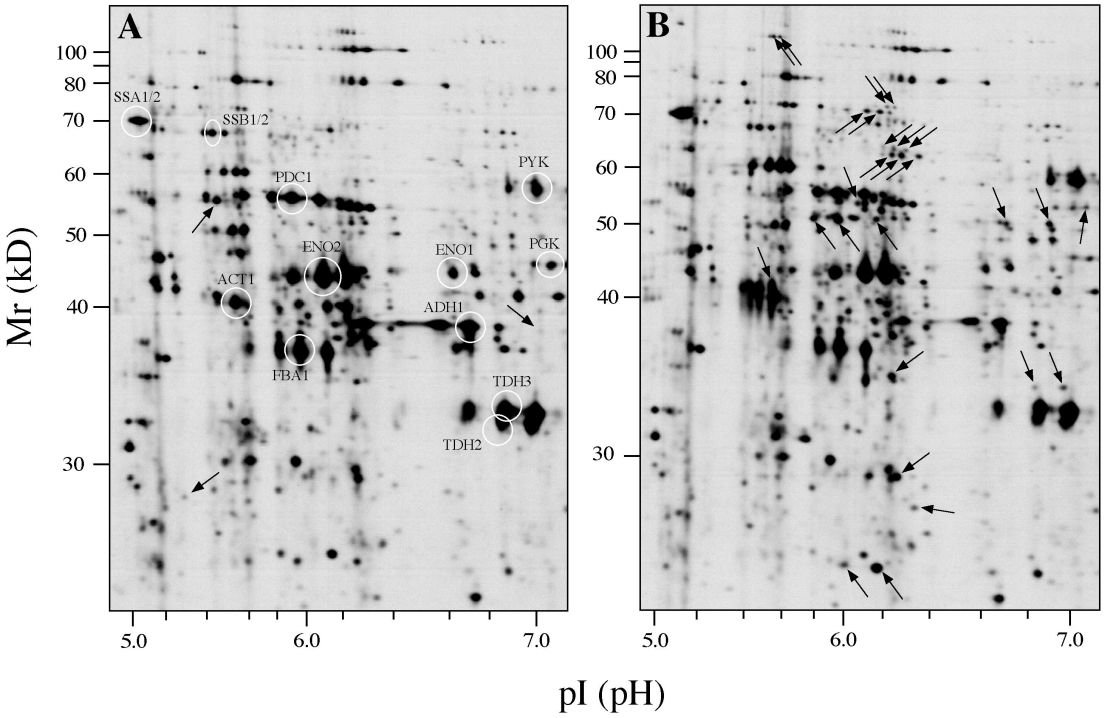

FIG. 3. Fluorograms of 2D-PAGE-separated [^{35}S]methionine-labelled total protein extract of *S. cerevisiae*. (A and B) Five-day exposures of the central regions of gels loaded with 400,000 dpm. (A) Exponentially growing cells in basal medium (0 M NaCl). Proteins which displayed at least a sixfold decrease in the relative synthetic rate during T = 20-40 of the adaptation compared with the preshift rate (arrows) and proteins with known identities (circles) are indicated. The following gene and/or protein designations have been used: ACT1, actin; ADH1, alcohol dehydrogenase; ENO1 and ENO2, enolase; FBA1, fructose-bisphosphate dehydrogenase; PDC1, pyruvate decarboxylase; PGK, phosphoglycerate kinase; PYK, pyruvate kinase; SSA1/2 and SSB1/2, HSP70 homologs; TDH2 and TDH3, glyceraldehyde-3-phosphate dehydrogenase. (B) Cells labelled during min 20 to 40 of the adaptation to 0.7 M NaCl medium. Proteins with at least an eightfold-increased relative rate of synthesis compared with the preshift rate are indicated (arrows).

different on the basis of a twofold-change criterion, with 143 proteins changing by at least this factor. For all samples, almost equal numbers of proteins increased and decreased their relative rates of synthesis by twofold (68 and 75 changes, respectively, during the middle phase). From the exponential growth phase to T = 0-20, 30 and 31 proteins showed increases and decreases, respectively, of more than twofold; from T = 0-20 to T = 20-40, increases and decreases numbered 74 and 64, respectively. From the exponential growth phase to T = 40-60, 61 and 57 proteins showed increases and decreases, respectively, while from T = 20-40 to T = 40-60, the increases and decreases numbered 36 and 42, respectively.

The twofold-responsive proteins during T = 20-40 were further classified according to their magnitude in change of synthesis. The numbers of proteins in the different fold change categories were distributed over a wide range, with the most frequent response being a twofold decrease, observed for 60 proteins (Fig. 4). Regarding greater changes in the relative rate of synthesis, however, the predominant mode of regulation was induction: 24 protein spots increased their synthesis by more than 10-fold, while only 1 protein displayed a diminished rate of the same magnitude.

The data related to the global comparison deserve some additional remarks regarding the normalization of the synthetic rate. A consequence of the roughly fourfold decrease in the absolute rate of total protein synthesis during the period of adaptation (Fig. 2) is that all proteins exhibiting a less-than-fourfold increase in the relative rate of synthesis will diminish their absolute rate of synthesis. Thus, even during the phase with the fewest significant changes in the relative rates of synthesis (T = 0-20), there were an overwhelming number of proteins that by the absolute-rate criterion would not have been classified as constant but as repressed. In a global study of the influence of a temperature shift from 23 to 37°C on protein synthesis, it was found that among the approximately 500 proteins examined, more than 300 peptides were transiently repressed (31). These responses concerned changes in the absolute rate of synthesis, and many of the repressed proteins followed the general change in synthesis of total protein; i.e., a vast proportion of them, in terms of relative rates, would have been designated constant. It should be stressed that neither criterion is right or wrong; however, in my view changes in the relative rate of synthesis better reflect an active cellular "decision" in terms of specific changes in gene expression during the cell's adaptation to its new environment, changes which are not influenced by an increase or decrease in the overall activity of the translational machinery.

Highly NaCl-responsive proteins. Proteins that increased their relative rate of synthesis to at least eightfold-higher levels during the middle phase of the adaptation period are indicated in Fig. 3B and listed in Table 1. These highly responsive proteins were distributed over the whole range of pIs and M_rs studied and were not restricted to any particular size or charge fraction. It was also apparent that some proteins tentatively identified as having charge modifications, with similar M_rs and the same osmotic response but with different pIs, existed. This phenomenon was most apparent for the proteins with M_rs greater than 60,000. It was clear, however, that charge modification in general was not an NaCl-induced phenomenon, and charge trains were observed for many proteins even in the

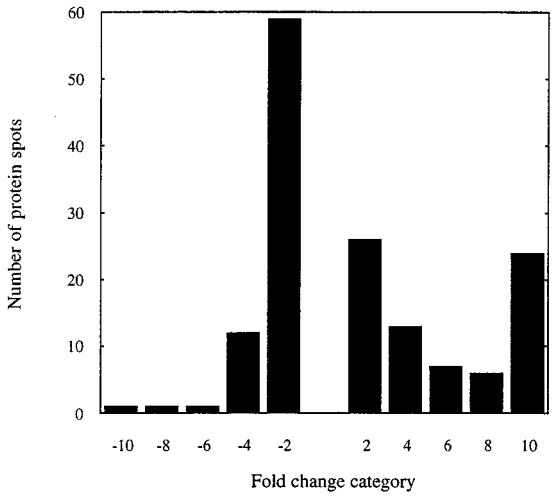

FIG. 4. Fold change frequency plot of the 143 proteins that displayed a statistically significant, by log Student *t*-test criteria, and more-than-twofold change in relative rate of synthesis at T = 20-40 of the adaptation period compared with the control (growth in basal medium). Fold change categories represent positive values [*x*-fold and greater increased relative rate of synthesis but less than $(x + 2)$-fold] and negative values [*x*-fold and less but greater than $(x - 2)$-fold (e.g., a value of 2 means a fold change factor equal to or greater than 2 but less than 4)]. The exceptions are fold change values of 10 and −10, which indicate 10-fold and greater change (increase) and −10-fold and less change (decrease), respectively.

absence of osmotic stress. When charge modifications were taken into account, the 30 highly responsive spots tentatively represented 18 core proteins (Table 1).

During exponential growth in basal medium, roughly half of these responsive proteins were synthesized at rates which were below the level of detection (roughly 5 ppm) and thus at this level of resolution were classified as novel proteins (Table 1). One exception was p25.1/6.2 (sample spot protein [ssp] 5103) which under nonsaline conditions was produced at a relative rate of 80 ppm. During the adaptation, this protein was synthesized at a rate of about 1,300 ppm; thus, during the adaptation period it was one of the 50 most frequently synthesized proteins in the cell. Most other eightfold-induced proteins peaked at 100 to 300 ppm, thus being more moderately expressed than many nonresponsive proteins. The only highly NaCl-responsive proteins tentatively identified in these gels are Hsp104p (p106.0/5.5) (19) and Gpd1p (p41.2/5.5) (33). In addition, the *CTT1* gene product has a theoretical M_r of 64,000 and a pI of 6.6, which are close to those of the NaCl-responsive proteins p62.9/6.2 and p61.4/6.3.

The one protein whose synthetic rate decreased more than 10-fold during the adaptation period was p25/5.2 (ssp 2206), which was moderately abundant in exponentially growing cells in nonsaline medium (Fig. 3A). The relative synthetic rates of two additional proteins decreased by at least a factor of 6, and all three highly salt-repressed proteins are indicated in Fig. 3A. One of them, p53.7/5.5 (ssp 2621), was somewhat dominant during normal growth and exhibited a preshift rate of 645 ppm.

Identification of regulatory classes. In order to identify proteins that were coregulated and potentially controlled at the molecular level by the same regulatory mechanism(s), responsive proteins were classified into regulatory classes with similar synthetic profiles. Firstly, proteins were grouped according to the phase of their first response of greater-than-twofold magnitude as early (E) (T = 0-20), middle (M) (T = 20-40), and late (L) (T = 40-60). Secondly, induction (I) or repression (R) in the relative rate of synthesis as the first response during the adaptation period was scored. Thirdly, classification was based on whether the response was transient (T) or sustained (S) throughout the 1-h period (by a twofold threshold criterion). The regulatory patterns obtained for responsive proteins are depicted in Fig. 5, and it is evident that 14 of the 26 possible patterns are represented, considering all combinations of increased (*In*), decreased (*De*), and sustained (*Su*) responses. These regulatory classes comprised 80 proteins, i.e., about 10% of the proteins resolved.

Early-phase proteins. Eight regulatory classes contained proteins for which the first salt-induced change in synthesis occurred during the initial 20 min of the adaptation to salt. The largest regulatory group of these early responsive proteins comprised eight proteins, designated EIT_{InInDe}, which all displayed a transient response and a peak rate of synthesis in the middle phase. The members of this class were also among the highly responsive proteins, all being salt induced more than 10-fold during the middle phase. None of the proteins in this EIT_{InInDe} group, despite their transient response, returned to their control values during the course of the experiment. Two representatives of this regulatory class are depicted in Fig. 6.

An interesting but small regulatory class is EIT_{InDeSu}, which contained only one member, p53.9/5.4 (ssp 2622). This protein was expressed at about 50 ppm during exponential growth in

TABLE 1. Proteins exhibiting at-least-eightfold-increased rates of synthesis at T = 20-40 during adaptation to 0.7 M NaCl compared with control rate (exponential growth without NaCl)

Proteins	M_r[a]	pI	ssp no.[b]	Status[c]	Rate of synthesis (ppm)[d]
p106.0/5.5	106.0	5.5	3805		305
	105.8	5.6	3809		279
p72.3/6.2	72.5	6.2	5709		80
	72.2	6.2	5717		69
p71.1/6.2	71.3	6.1	4723		160
	71.1	6.2	5705		206
p62.9/6.2	63.3	6.2	5708	*	42
	62.9	6.2	5716	*	101
	62.5	6.3	5726	*	95
p61.4/6.3	61.6	6.2	5714	*	128
	61.4	6.3	5722	*	224
	61.3	6.3	6702	*	204
p52.5/6.1	52.5	6.1	4612		417
p52.3/>7	52.3	>7	8611	*	175
	52.2	>7	8620		166
p50.2/5.9	50.2	5.8	3619	*	263
	50.2	5.9	4609		827
p50.2/6.2	50.2	6.2	5604		267
p49.8/6.9	49.8	6.9	7621	*	298
	49.8	6.7	7607	*	229
p44.7/<5	44.7	<5	514		197
p41.2/5.5	41.2	5.5	3502		1,094
p34.4/6.2	34.4	6.2	5307	*	314
p33.8/7.0	33.8	6.8	7311		111
	33.8	7.0	8301		222
p29.2/6.2	29.2	6.2	5217		740
p27.8/6.3	27.8	6.3	6205	*	193
p25.1/6.2	25.2	6.0	4110	*	302
	25.1	6.2	5103		1,273
p22.2/<5	22.2	<5	0009	*	62

[a] M_r is given in thousands.
[b] Sample spot protein number, a unique number automatically assigned to each spot by the PDQuest software.
[c] *, proteins not detected in the control (novel proteins).
[d] Rates were determined for 0.7 M NaCl at T = 20.

A

Regulatory class	Number of spots	Average kinetic profile	Proteins in class (pMr/pI)	Max synthetic rate (ppm)
EIT_{InInDe}	13		p72.5/6.2	79
			p61.4/6.3	223
			p52.9/5.9	465
			p52.3/>7	175
			p50.2/5.8	263
			p44.7/<5	197
			p33.8/7.0	222
			p25.1/6.2	1272
EIT_{InDeIn}	1		p43.8/>7	103
EIT_{InDeSu}	1		p53.9/5.4	337
EIS_{InSuSu}	3		p172.2/<5	100
			p43.4/>7	66
			p39.3/5.5	321
MIT_{SuInDe}	11		p71.1/6.2	206
			p59.1/5.6	3329
			p53.4/6.1	452
			p50.2/5.9	827
			p47.6/5.4	148
			p42.0/6.4	287
			p35.9/>7	105
MIS_{SuInSu}	5		p72.6/5.6	353
			p38.8/5.6	137
			p22.8/5.5	49
LI_{SuSuIn}	3		p93.5/<5	58
			p67.3/7.0	129
			p26.8/<5	150

B

Regulatory class	Number of spots	Average kinetic profile	Proteins in class (pMr/pI)	Max synthetic rate (ppm)
ERT_{DeDeIn}	1		p28.6/5.3	103
ERT_{DeInSu}	4		p71.2/5.4	64
			p68.3/6.7	69
			p31.6/5.9	91
			p30.7/6.9	89
ERT_{DeSuIn}	1		p36.4/6.7	521
ERS_{DeSuSu}	6		p57.5/6.3	106
			p46.1/5.6	538
			p36.8/6.3	672
			p35.9/<5	59
			p35.3/5.5	150
MRT_{SuDeIn}	4		p61.6/6.7	111
			p39.5/6.2	1569
			p36.2/7.0	272
			p29.6/<5	291
MRS_{SuDeSu}	13		p54.9/5.4	303
			p49.4/5.5	388
			p45.1/>7	827
			p42.0/5.3	730
			p41.0/>7	884
			p39.9/6.0	571
			p29.6/6.5	79
			p29.0/6.2	704
			p25.4/6.2	346
			p24.0/6.0	81
			p23.9/6.3	281
			p23.2/5.2	284
LR_{SuSuDe}	1		p25.0/6.7	317

FIG. 5. Regulatory classes identified during 1-h adaptation of *S. cerevisiae* to 0.7 M NaCl. Proteins were classified into different regulatory groups according to (i) their first responsive phase during osmotic adaptation (early [E], T = 0-20; middle [M], T = 20-40; and late [L], T = 40-60), (ii) increase or decrease as the first response in the relative rate of synthesis (induction [I] and repression [R]), and (iii) their subsequent response (sustained [S] and transient [T]). (A) Proteins whose first response was induction; (B) proteins whose first response was repression. M_r is indicated in thousands. Max, maximum. Profile graphs are as described in the legend to Fig. 7.

nonsaline medium, but the relative rate of synthesis was raised transiently to roughly 400 ppm during the early phase of adaptation. During the period from min 20 to 60, it was expressed at or below the level of detection (Fig. 6). The only protein that displayed an early induced oscillatory response and was thus classified EIT_{InDeIn}, p43.8/>7 (ssp 8506), was a minor protein with a maximal level of expression in any phase of about 100 ppm.

Of the proteins that were repressed during the initial phase of adaptation, the five proteins that exhibited sustained low levels of expression throughout the 60 min were classified ERS_{DeSuSu}. Two representatives of this class with control values of about 600 ppm were p46.1/5.6 (ssp 3506) (indicated in Fig. 6) and p36.8/6.3 (ssp 5424). Two of the early repressed proteins displayed transient responses and returned to relative synthetic rates similar to those determined under the nonsaline growth conditions in the middle phase of adaptation (class ERT_{ReInSu}). The most pronounced example was p30.7/6.9 (ssp 7217), which exhibited about a fourfold-decreased rate and displayed expression rates of roughly 100 ppm in the preshift and later phases. A single protein p28.6/5.3 (ssp 2206) displayed a high level of repression (at least 10-fold) and was also the only representative of the regulatory class ERT_{DeDeIn}. This protein was moderately expressed in the control cells, with a relative synthetic rate of about 100 ppm.

Middle-phase proteins. The relative rates of synthesis of members in the four regulatory classes were not affected until 20 min subsequent to osmotic dehydration. The most dominant of the members in the class MIT_{SuInDe} was p59.1/5.6 (ssp 3607). The protein was synthesized at a rate of roughly 700 ppm in the control cells, and that rate was sustained until the middle phase of adaptation where it was increased by about fourfold and was subsequently repressed to almost preshift synthetic values for the last 20 min (Fig. 6). A total of three proteins whose rates of synthesis were induced during the middle phase and that sustained the increased rate throughout the course of the experiment belonged to the MIS_{SuInSu} class. The representatives of this group displayed rather minor changes. The largest regulatory class encountered included 13 members and contained proteins that were repressed during the middle phase and subsequently displayed sustained synthesis. The quantitative response of these proteins was rather minor, with changes in the level of expression in the range of two- to fourfold. Some of the more predominant representatives were p25.4/6.2 (ssp 5109) and p29.0/6.2 (ssp 5215). The biochemical identities of these two proteins are unknown, but one other protein from this regulatory class with an even higher relative rate of synthesis was identified from the 2D gel protein database at Cold Spring Harbor Laboratories (25a). This spot was found to represent phosphoglycerate kinase (Pgk, p45.1/>7 [ssp 8510]) and exhibited about twofold repression. Some proteins were transiently repressed during the middle phase and thus belonged to the regulatory group MRT_{SuDeIn}. The four members could be further classified according to their responsive patterns: (i) spots (ssp 1203 and 5409) which at min 40 to 60 resumed preshift rates and (ii) spots (ssp 5409

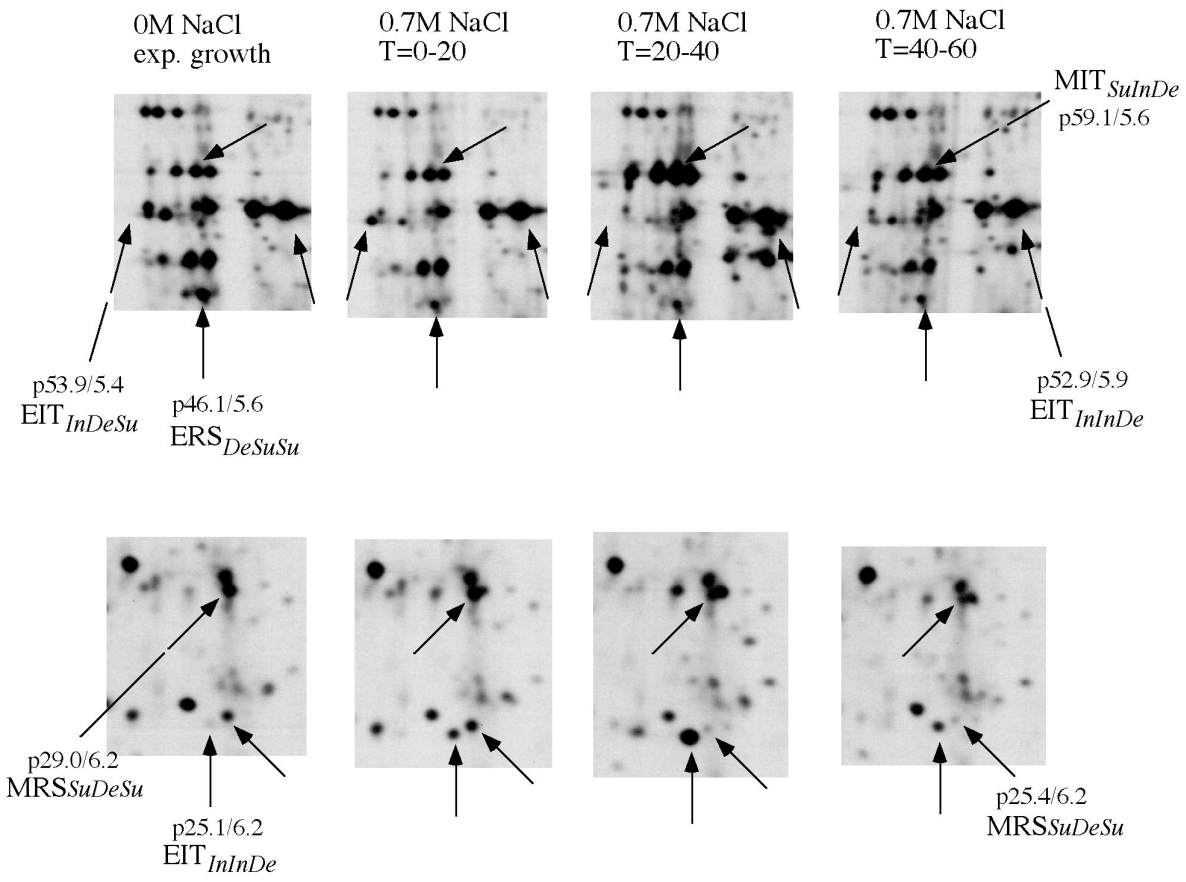

FIG. 6. Exemplified regulatory classes for some responsive proteins in two portions of the gels. The upper panel encompasses M_rs of 45,000 to 70,000 and pIs of 5.3 to 6.0, while the lower panel represents M_rs of 24,000 to 31,000 and pIs of 5.8 to 6.4. exp., exponential. Specific protein spots are indicated by the arrows.

and 8403) which during the late phase still displayed rates lower than those of the control.

Late-phase proteins. A small number of proteins were classified as having a late response. The proteins that belonged to the regulatory group LI_{SuSuIn} were all expressed at low levels (10 to 50 ppm) during nonsaline steady-state growth which increased to a maximum of about 150 ppm. There was only one representative of the group LR_{SuSuRe}, and that was the moderately abundant protein p25.0/6.7 (ssp 7103).

Regulatory patterns of identified proteins. A number of abundant proteins resolved on the 2D gels were biochemically identified from existing 2D-PAGE protein databases (19, 32). These were identified as glycolytic, ethanol-producing, stress, or structural proteins, and their changes in synthesis are depicted in Fig. 7. The glycolytic enzymes displayed levels of expression that during the late phase of adaptation decreased to about 50% of the preshift values. Even though only one of these proteins, Pgk, was classified in the regulatory group MRS_{SuDeSu} (described above), almost all the other glycolytic enzymes exhibited regulatory patterns that were similar to that of Pgk but less pronounced. One clear exception was pyruvate kinase, Pyk, whose synthesis increased almost twofold during the initial 20 min of adaptation, although it subsequently adhered to the repressed pattern of the other proteins. The pyruvate produced in glycolysis is further processed to ethanol by Pdc1p and Adh1p, and the rates of synthesis of both these fermentative enzymes were diminished in a similar fashion to Pgk1p. Two heat shock proteins that belong to the HSP70 family were identified. A stimulatory effect on Ssa1/2p production was observed during the adaptation to NaCl; however, the response level increased by only slightly more than twofold. Ssa1p is strongly induced by heat treatment (42). The other HSP70 member identified is a heat stroke protein (Ssb1/2p) whose rate of synthesis diminishes dramatically during heat shock (42). This protein exhibited an almost constant relative rate of synthesis during the 60-min adaptation, only exhibiting a minor transient decrease at T = 20-40. The only structural protein identified was actin, which during the osmotic adaptation exhibited almost constant levels of expression.

DISCUSSION

The impressive number of proteins that displayed changes in their relative rates of synthesis during the adaptation to NaCl conveys the idea that *S. cerevisiae* is an organism with a highly salt-stress-responsive gene expression. The great number of synthetic changes was striking considering the relatively mild osmotic shock applied; growth recovered within roughly 1 h to an only slightly retarded rate. This result should be considered in relation to the fact that the maximum concentration of NaCl tolerated for growth of this strain of *S. cerevisiae* is roughly 2 M, which requires cells to adapt for several days before sluggish proliferation starts (data not shown). A twofold change in the synthetic rate during the adaptation period was observed for about 150 of the resolved protein spots. It should be realized, however, that even if 2D-PAGE is a powerful tool for

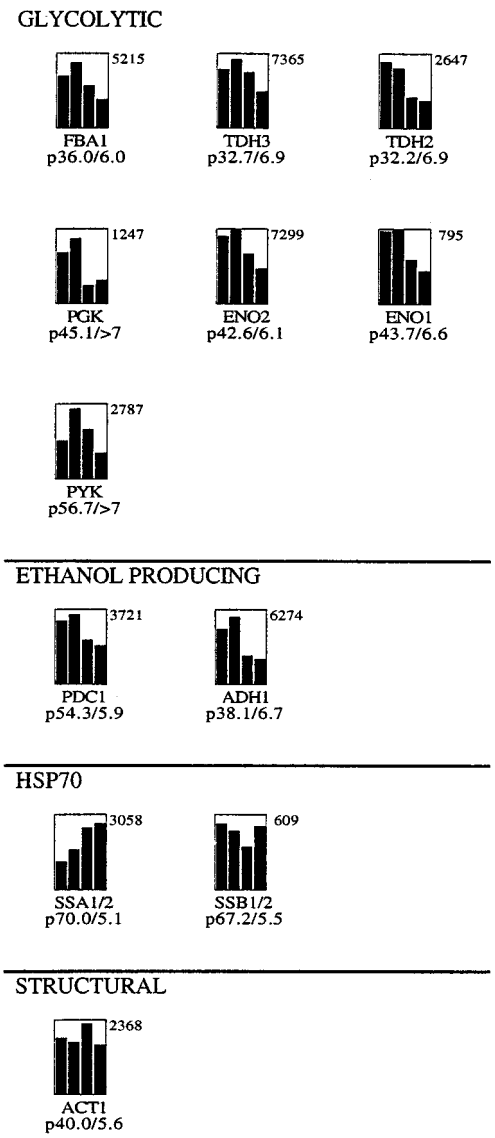

FIG. 7. Histograms displaying the response for some proteins identified in Fig. 3A. The number in the upper righthand corner of each graph represents the parts-per-million value for the highest bar. The leftmost bar in each depicts the control value obtained during exponential growth without salt, and the other bars represent from left to right $T = 0$, $T = 20$, and $T = 40$ min during the adaptation to NaCl.

whole-cell analysis, the technique visualizes only a portion of all cellular proteins. So, what is the estimate of the total amount of salt-responsive genes? Roughly 25% of the proteins of the cell were detected in the 2D gels. Some of these were clearly the result of charge modifications of the same core protein, and from the data for the 18 highly-salt-responsive proteins, it can be predicted that about half of the scored 2D spots in these gels represent pI variants. In addition, some of the responsive proteins may constitute M_r variants as a result of posttranslational modifications of the same primary product. Thus, it is estimated that *S. cerevisiae* contains roughly 200 genes and/or proteins which respond to salt stress.

The great number of synthetic changes also implies that the salt-implemented osmotic dehydration instigated impact on many cellular activities. This assumption is strengthened by the fact that genes involved in diverse cellular functions have been reported to be regulated by the osmotic properties of the medium: *HAL1*, which is involved in Na^+-K^+ selectivity of the cell (20); *ENA1*, encoding a P-type ATPase participating in the efflux of sodium ions (15); *GPD1*, which is involved in glycerol production (1, 14, 41); and *TPS2*, which encodes one of the subunits of the trehalose synthase complex (20a). Both the maintenance of a proper intracellular Na^+ balance and the accumulation of the compatible solute glycerol are key features of the osmoregulatory response in yeast cells (7). The disaccharide trehalose has been invoked as an agent in severe dehydration resistance by proposed membrane-stabilizing properties (13). Thus, regulation of genes involved in these processes is likely to be salinity controlled.

However, even proteins participating in cellular activities of less obvious involvement in adaptation to osmotic stress have been reported to be strongly regulated: *CTT1*, the catalase T gene which plays a protective role during oxidative stress (28); *HSP26*, *HSP12* (41), and *HSP104* (39), which are heat shock regulated; *DDR2*, a DNA damage-inducible gene (39); and *GAC1*, a tentative protein phosphatase (39). Most of the genes reported to be under salinity control display high levels of induction and are thus presumably among the highly responsive proteins presented in Table 1. The only three which at present can be tentatively identified within this group are Hsp104p, Gpd1p, and Ctt1p. Some of the other gene products responding to osmotic stress will presumably not be resolved on these 2D-PAGE gels, since they have either too basic theoretical pI values (like Tps2p [p102.8/7.9] and Hal1p [p33.0/9.2]) or too small M_r values (as for Hsp12p [p11.7/5.1]) or do not contain methionine and thus cannot be labelled (like Hsp26p [p23.7/5.22]). For the remaining two gene products, Gac1p (p88.6/5.49) and Ena1p (p120.4/5.32), there was no corresponding salt-responding protein in the vicinity of their presumptive 2D locations. Thus, for 14 of the highly regulated proteins there is not even a tentative identification, which indicates a vast potential in further identification and/or cloning of salinity-controlled genes.

The multitude of revealed regulatory classes indicated complex intracellular signalling in response to salt stress. Proteins that belong to the same regulatory class may be coregulated by the same regulatory mechanism(s). Although this type of grouping is by no means proof of any kind of coregulation, it can serve as a useful selective tool for interesting proteins to be enriched and sequenced so that the corresponding genes subsequently can be cloned for further molecular and genetic characterization. The initial osmotic signal is believed to be transmitted through a mitogen-activated protein kinase cascade by Hog1p- and Pbs2p-mediated phosphorylation (9). This signal has been shown to generate a rapid response, and the Pbs2p-dependent tyrosine phosphorylation of Hog1p is maximal 1 min after the osmotic challenge. It has been shown that the *CTT1* gene is salt regulated by a *HOG1*-dependent mechanism via an upstream activating sequence named STRE in the *CTT1* promoter (39). The position in the 2D map of Ctt1p is not at present known, but because of its reported great magnitude in salt-stimulated synthetic increase and early response, it is likely that the *CTT1* protein is included in the regulatory class EIT_{InInDe}. An additional gene under Hog1p control is *GPD1* (1). The synthetic profile of the tentatively identified Gpd1p largely mimicked the response of the members of the class EIT_{InInDe}, even though it was not included in this set because of too great a variance in one sample point. Thus, one can hypothesize that many if not most of the members of the EIT_{InInDe} class constitute a *PBS2-HOG1*-dependent gene family. Preliminary results of the synthetic profiles during adaptation to osmotic stress of a *pbs2*Δ mutant supports this assumption (unpublished results).

Roughly equal numbers of proteins displayed increases or decreases in the synthetic rate as the initial response. In theory, only one signalling path could differentially and sequentially affect the expression of a number of genes; however, it is tempting to envisage a number of parallel signalling paths. This line of thought is partly supported by the reported impact on salt-dependent *ENA1* expression by the Ca^{2+}-dependent protein phosphatase calcineurin (29) and by the importance of protein kinase C, Pkc1p, for the osmotic integrity of the yeast cell (34). These paths in conjunction with the *HOG1*-mediated signal may trigger most of the observed regulatory effects.

The phosphorylation of Hog1p and thus its presumptive activity are transient during osmotic adaptation (9), resulting in a transient synthetic profile for those genes under *HOG1* control. This profile is indeed found for a number of both induced and repressed regulatory classes. It is unclear if these transient changes reflect induction of proteins needed only during a short time of the cellular adaptation or if the general strategy of acclimation to a changed environment involves an overresponsive increase in synthetic rates of proteins necessary for fully adapted cells during resumed growth. The transient profile may thus reflect a feedback mechanism that responds to a sufficient amount of the desired product.

The synthesis mechanisms of many of the proteins were clearly nonresponsive during the initial 20 min of adaptation but displayed significant changes during the later phases. It is presently unknown if this temporal response is due to delayed signals strictly dependent on the initial osmotic dehydration. Unfortunately, the salt-dependent kinetics of signalling through the calcineurin or PKC1 paths is presently not known. However, the delayed response may also be a consequence of the metabolic changes taking place during the initial 20 min, in both the protein setup and the intracellular metabolite and ion pools. One of the proteins of MRS_{SuDeSu} was identified as phosphoglycerate kinase, and the glycolytic and ethanol-producing enzymes identified (Fig. 7) displayed rather similar patterns of synthesis even though they were not strictly included in that regulatory class. This simultaneous decrease in the relative synthesis levels of these central metabolic enzymes may indicate general adjustments to the new metabolic state. In addition, these changes may be prerequisites for increased glycerol production, since overexpression of Gpd1p did not enhance the formation of glycerol for glucose-grown cells (1). However, the control of Gpd1p on glycerol production was apparent when cells were derepressed, either during galactose growth (1) or when glucose was added to fully derepressed cells growing on ethanol (24). Most of the enzymes indicated in Fig. 7 that displayed decreased relative rates of synthesis are normally produced at reduced levels under derepressed conditions (5). The implication is that the decreased expression of these genes during adaptation to osmotic stress could instigate Gpd1p flux control over the glycerol production. A high coefficient of control of the GPD reaction over glycerol production has been reported (6). The theoretical basis for such control analysis is a search for flux changes instigated by changes in the activity and/or amount of one component of the system, with all the other components kept at constant levels. The present data clearly indicated that control analysis of cells adapting to salt is strongly hampered by massive changes in the overall protein content. Furthermore, the high reported control coefficient of 0.63 for the GPD reaction was probably overestimated, and it is to be expected that more enzymes have significant controlling power over the flux into glycerol.

The regulation of *PGK1* has earlier been shown to be under heat shock control (35). Even the synthesis of Eno1p is enhanced during heat treatment, and it was the first heat-responding protein to be identified in yeast cells (23). During the adaptation to salt, these proteins displayed decreased rates of synthesis, indicating a cellular response distinct from heat acclimation. The distinction between heat shock and osmotic shock is further substantiated by the differential regulation of the salt-controlled *GPD1* gene, since no impact of heat on GPD activity (40) or *GPD1* transcription (14) was found. However, some kind of overlap or cross talk between the heat and osmotic stimulons is apparent from the reported *HSP26* and *HSP12* salt-dependent induction (41). The tentative cross talk was also evident from the salt response of the HSP70 homologs *SSA1/2* and *SSB1/2* (Fig. 7); their salt response was consistent with their regulation during heat treatment, although the heat response is more pronounced (30). On the other hand, one of the heat shock proteins not induced during the adaptation to salt was Hsp83p, for which the 2D position is known and no increased synthesis was found (data not shown). The lack of Hsp83p induction is interesting because of the reported absolute involvement of the heat shock upstream element in the heat-induced expression of this gene (27). This involvement indicates that the salt-induced synthesis of some of the heat shock proteins was not solely a heat shock upstream element-dependent phenomenon and that some other regulatory mechanism acting during salt adaptation must be implemented for these genes. It is well documented that heat stress is not the sole inducing condition for many of the HSP genes, since their synthesis is highly upregulated during nutritional limitation (4).

Single proteins have been shown to confer tolerance to a stressful environment. Synthesis of Hsp104p appears to be required to acquire the state of thermotolerance (37), and in a genetic background without *HSP104* expression, the *SSA1* gene is linked to heat stress resistance (38). Synthesis of Ctt1p seems to partly but not fully account for the increased resistance to high NaCl concentrations acquired by short-time conditioning at low-level salinities (39). Since protein synthesis is a prerequisite for acquiring the state of osmotolerance (6), any of the protein changes occurring during the period of osmotic conditioning is a potential candidate for osmotolerance determinance. However, since both Hsp104p and Ctt1p were expressed during adaptation to 0.7 M NaCl, these genes, together with genes involved in glycerol accumulation and sodium efflux, appear good candidates for use in studies unravelling the mechanisms involved in osmotolerance.

A cell cycle-dependent relation to cellular heat resistance has been proposed, and the majority of cells remain unbudded during the acquisition of thermotolerance (36). It was evident from the constant proportion of budding cells throughout the period of adaptation to salt that cells were frozen at their actual point in the cell cycle at the time of dehydration. Thus, the acquired osmotolerance of the culture is not just a reflection of a gradual selective stop of the main fraction of the population in one part of the cell cycle during the osmotic conditioning. In contrast to the acquired resistance to heat shock, the acquired osmotolerance thus seemed to be a cell cycle-independent phenomenon.

The fairly constant overall catabolic activity of the dehydrated cells, which was indicated by the almost unaffected rate of CO_2 production, contrasted sharply with the profound impact on the rate of biomass production and the total rate of protein synthesis. The reason for these diminished rates of growth and translation was thus possibly not a decreased supply of ATP. More likely, they reflect dehydration-induced disturbances of general proliferation controls and are part of programmed and controlled cellular adaptation to the new

environment. Increased external osmolarity causes a rapid loss of actin filament cables, structures which are believed to be prerequisites for asymmetrical bud growth of *S. cerevisiae* (12). Actin filament organization may act as an osmosensor responding to changes in ion content or turgor pressure and rapidly mediating this response to general control points for growth. On the other hand, a tentative osmosensor gene which encodes a protein with presumptive integral membrane-spanning regions was recently isolated (26). This gene product, Sln1p, was characterized as a histidine kinase that is part of a two-component system, with its osmotic signal transmitted via the *PBS2-HOG1* signal transduction path. However, a cellular response of the complexity displayed in this 2D study may be triggered not by one single sensor but as a result of a multitude of responding signalling paths.

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