Phosphorylation of the α-subunit of eukaryotic initiation factor 2 (eIF2α) is a conserved mechanism regulating protein synthesis in response to various stresses. A screening for negative factors in yeast salt stress tolerance has led to the identification of Gcn2p, the single yeast eIF2α kinase that is activated by amino acid starvation in the general amino acid control response. Mutation of other components of this regulatory circuit such as GCN1 and GCN3 also resulted in improved NaCl tolerance. The gcn2 phenotype was not accompanied by changes in sodium or potassium homeostasis. NaCl induced a Gcn2p-dependent phosphorylation of eIF2α and translational activation of Gcn4p, the transcription factor that mediates the general amino acid control response. Mutations that activate Gcn4p function, such as gcd7-201, cpc2, and deletion of the translational regulatory region of the GCN4 gene, also cause salt sensitivity. It can be postulated that sodium activation of the Gcn2p pathway has toxic effects on growth under NaCl stress and that this novel mechanism of sodium toxicity may be of general significance in eukaryotes.

Adaptation to changes in the extracellular environment is a critical event for cell survival. Single cell organisms such as the budding yeast *Saccharomyces cerevisiae* are able to adapt rapidly to extreme changes in extracellular salinity or nutrient availability and are consequently convenient model systems to study stress tolerances. Salt stress implies both exposures to osmotic stress and to specific cation toxicity (1). In yeast an increased extracellular osmolality causes the induction of a number of stress-protective genes of which the major outcome is the accumulation of glycerol and the restoration of turgor pressure (2). The high osmolarity glycerol mitogen-activated protein kinase pathway plays a dominant role in the signal transduction contributing to the adaptive response of yeast to high osmolarity or salinity (1–3). Additionally, the Ras-cAMP-protein kinase A pathway that responds more generally to stresses and nutrient availability also plays an important role in osmotic adaptation (4–6). Adaptation to salt stress also requires the modification of plasma or vacuolar membrane transport systems to exclude toxic ions from the cytosol. Both the high osmolarity glycerol pathway and the calcium-calcinurin pathway participate in the regulation of ion transporters during salt stress, especially of the Na⁺-pumping ATPase encoded by the ENA1 gene (1). Considerably less information exists about cellular targets of salt toxicity. Sodium and lithium inhibit Hal2p, a specific phosphatase acting on 3'phosphoadenosine 5'phosphate (PAP) (7, 8). PAP accumulation during salt stress inhibits sulfate assimilation and RNA processing, and this accounts for part of the salt toxicity. In addition lithium inhibits the essential ribonuclease MRP (9). Other targets of salt toxicity must exist, although previous mutational analyses have failed to identify them.

In the present work a genetic analysis has led to the identification of Gcn2p as a negative determinant of yeast salt tolerance. This protein kinase participates in one of the best characterized mechanisms of translational regulation in eukaryotes, which involves the phosphorylation of the α-subunit of eukaryotic initiation factor 2 (eIF2α) at serine 51 (10, 11). In yeast this regulatory pathway is activated when cells are subjected to amino acid or purine starvation and is called the general amino acid control (12). Yeast Gcn2 protein kinase regulates the translation of a single mRNA species, encoding Gcn4p, a transcriptional activator of genes involved in the synthesis of amino acids. This regulation is mediated by four short open reading frames (uORFs) in the leader of the GCN4 mRNA, which renders GCN4 expression hypersensitive to the levels of active eIF2 in the cell. In cells not limiting for amino acids and thus with abundant active eIF2, the uORFs block GCN4 translation. In cells deprived of an amino acid or purine, Gcn2p phosphorylation of eIF2α leads to reduced active eIF2 levels, which alleviates the inhibitory effects of the uORFs and allows increased GCN4 translation. Here we present a link between this regulatory pathway and the response to salinity stress. We demonstrate that salt stress induces the general amino acid control response and postulate that (over)activation of this pathway results in some toxic effect that inhibits growth under NaCl stress. The conserved nature of the Gcn2p pathway suggests that this mechanism of sodium toxicity may be of general significance in eukaryotes.

**EXPERIMENTAL PROCEDURES**

Plasmids and Strains

All plasmids used in this study are derived from YCP50 (p16), a low copy number plasmid marked with *URA3* and containing either the *GCN4* wild type allele (p164) (13), a *GCN4* mutant allele that contains only the first uORF (p235), or a *GCN4-lacZ* fusion including the *GCN4* 5' non-coding region with all four short uORFs (p180) (14). Yeast cells were transformed by the lithium acetate protocol according to Gietz et al. (15). Yeast strains H1725 (gcd7-201) and H1794 (gcd7-201 gcn2)

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were derived from the H1727 (Mata leu2-3,112 ura3-52) background and have been described previously (16). All other strains were derived from the W303-1A (Mata ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1) or W303-1B (Mata) background. Strains SKY697 and AG86 have been obtained by disrupting the ENA1–4 open reading frames and have been described (17). Disruption mutants, which suppressed the salt tolerance phenotype and the transposon insertion was verified. By crossing with the SKY697 strain (W303-1A ena1–4: HIS3) transformed with a mTn-lacZ/LEU2 mutagenized insertion library (21), disruption mutants that suppressed the salt sensitivity of this strain were isolated as colonies growing after 9 days in synthetic minimal medium plates (SD) supplemented with methionine (100 μg/ml) and 0.6 M NaCl. SD plates contain 2% Bacto-Agar (Difco), 2% glucose, 0.7% yeast nitrogen base without amino acids (Difco), and 2% glucose (18). A 171-bp hal2 disruption cassette (18) was used to generate the hal2 mutants, whereas cpc2, gcn1, gcn2, and gcn3 mutants were generated by replacing the respective open reading frames with a lox-P-kanMX-lox disruption cassette following the method described by Guldener et al. (19).

Culture Conditions and Analysis of Salt Tolerance

Standard methods for yeast culture and manipulation were used (20). In order to test for salt tolerance, the different strains were grown to saturation (48 h) in liquid-rich medium. Cultures were diluted 10-, 100-, and 1,000-fold, and volumes of about 3 ml were dropped with a stainless steel replicator (Sigma) on plates containing 2% Bacto-Agar (Difco) and rich medium with NaCl, KCl, or sorbitol as indicated. Rich medium (YPD) contained 1% yeast extract (Difco), 2% Bacto-peptone (Difco), and 2% glucose.

Construction, Isolation, and Genetic Analysis of Sed Mutants

Strain AG86 (W303-1B ena1–4::HIS3) was transformed with a mTn-lacZ/LEU2 mutagenized insertion library (21). Disruption mutants that suppressed the salt sensitivity of this strain were isolated as colonies growing after 9 days in synthetic minimal medium plates (SD) supplemented with methionine (100 μg/ml) and 0.6 M NaCl. SD plates contain 2% Bacto-Agar (Difco), 2% glucose, 0.7% yeast nitrogen base without amino acids (Difco), 50 mM succinic acid adjusted to pH 5.5 with Tris base, and adenine (30 μg/ml), tryptophan (80 μg/ml), and uracil (30 μg/ml) as required. Mutants with the strongest salt tolerance phenotype were isolated and designated sed mutants (for suppression of ena1–4 by disruption). By crossing with the SKY697 strain (W303-1A ena1–4::HIS3), sporulation, and tetrad dissection the linkage between the salt tolerance phenotype and the transposon insertion was verified. To identify the genomic site of transposon insertion in the most relevant mutants, a plasmid rescue method was followed (21).

Immunoblot Analysis

Immunodetection of eIF2α—Strains were grown in liquid YPD medium to mid-logarithmic phase and then shifted to fresh minimal SD medium without any amino acid supplements or YPD medium with NaCl (1.25 M) or KCl (1.25 M) as indicated. Protein extraction, SDS-polyacrylamide gel electrophoresis, Western blotting with the ECL Plus detection system (Amersham Pharmacia Biotech), using either a polyclonal antibody that specifically recognizes eIF2α phosphorylated at serine 51 or a polyclonal antibody that recognizes both phosphorylated and non-phosphorylated forms of eIF2α, were executed as described (22). The MacVector software version 2.5 was used to quantify the optical density of the eIF2α bands on scanned autoradiographs.

Immunodetection of Hal2p—Yeast strains were grown to mid-logarithmic phase in liquid YPD medium or YPD medium with KCl (1.25 M) or NaCl (1.25 M). Protein extraction, SDS-polyacrylamide gel electrophoresis, blotting, and immunoassay with a polyclonal antibody against Hal2p were performed as described (18).

β-Galactosidase Assay

Yeast cells were grown in liquid SD medium with all amino acids supplemented to mid-logarithmic phase and then shifted to fresh SD medium without any amino acid supplements or SD medium with all amino acid supplements and either KCl (1.25 M) or NaCl (1.25 M). In all cultures uracil was omitted to keep selection for the p180 plasmid. Preparation of cells and determination of the β-galactosidase activity was performed as described (23). β-Galactosidase activity was measured during a period of 10 h following shift of medium.

Amino Acid Uptake and Incorporation Experiments

Yeast cells were grown in liquid SD medium to mid-logarithmic phase, shifted to fresh SD medium or SD medium with either KCl (1.25 M) or NaCl (1.25 M), and incubated for an additional 2 h, corresponding to steady state intracellular amino acid concentrations (24). Subsequently, yeast cells were washed and incubated in 50 mM succinate/Tris buffer (pH 5.5), 2% glucose with 10 μM [3H]phenylalanine and 1 μM [14C]leucine and with either KCl (1.25 M), NaCl (1.25 M), or no extra salts added. Amino acid uptake was measured during 2 min of incubation by liquid scintillation counting as described (25).

Incorporation of Phenylalanine—[3H]Phenylalanine (10 μM) was added to the cultures after the 2-h incubation period, and cells were further incubated. Samples were taken after 0, 15, 30, 45, and 60 min, mixed with trichloroacetic acid to a final concentration of 10%, incubated on ice, and passed through 4.5-μm nitrocellulose filters. Filters were washed with 10% trichloroacetic acid and H₂O and were then assayed for [3H]phenylalanine activity by liquid scintillation counting as above.

Measurement of Intracellular Cation Accumulations

Cells grown in YPD medium to mid-logarithmic phase were supplemented with NaCl and incubated for an additional 120 min. The steady-state intracellular sodium and potassium concentrations were measured by atomic absorption spectrometry after centrifugation, washing, and extraction of the cells as described (24).

RESULTS

A Transposon Insertion into the GCN2 Locus Causes NaCl Tolerance—In order to identify novel determinants of salt tolerance in S. cerevisiae, we have designed a screening based on the isolation of recessive, loss-of-function mutations suppressing the salt sensitivity of ena1–4 disruptants in medium supplemented with methionine. To this end we used a transposon-tagging strategy in which the ena1–4 strain was transformed with a mTn-lacZ/LEU2 mutagenized insertion library (21). The ena1–4 strain was used to avoid the complex regulatory system of this major determinant of salt tolerance (1), whereas methionine supplementation bypasses the salt-sensitive Hal2p phosphatase (18). Disruption mutants, which suppressed the salt sensitivity of this strain, were isolated and designated sed mutants, for suppression of ena by disruption. In five out of seven tested sed mutants so far, the phenotype was linked to the transposon insertion. Identification of the insertion locus in the genome showed that these five sed mutants were allelic and that the insertion occurred 12 nucleotides upstream of the GCN2 open reading frame (sed1 mutant, Fig. 1A) on chromosome IV. To verify that this event resulted in a loss of GCN2 function, we constructed a gcna2 mutant by removing the complete GCN2 open reading frame. The sed1 and the gcna2 mutations suppress the Na⁺ sensitivity of the ena1–4 strain to the same extent, indicating that the transposon insertion in the proximal promoter region of GCN2 completely abolishes its activity (Fig. 1B).

Salt Tolerance of a gcna2 Strain Is Specific for Sodium and Is Not Due to Altered Cation Accumulation—GCN2 encodes a protein kinase involved in translational regulation during amino acid starvation, a regulatory pathway called the "general amino acid control response" (12). The connection of this pathway with salt tolerance could be mediated by the translation of some protein related to either osmotic regulation or ion homeostasis. Further analysis of the behavior of gcna2 mutants subjected to salinity or osmotic stress revealed that the salt tolerance phenotype was specific for Na⁺ ions (Fig. 2). No increased tolerance to toxic concentrations of KCl or sorbitol was observed. On the contrary, gcna2 mutants grew somewhat slower in the presence of high concentrations of K⁺ ions. The same phenotypes of the gcna2 mutation were observed in an ENA1–4 strain (Fig. 2).

Sodium tolerance may be due to reduced intracellular Na⁺ accumulation, and therefore we measured steady-state levels of intracellular sodium and potassium after a NaCl shock. As mentioned above, the enhanced salt tolerance by loss of GCN2 function is independent of the presence of ENA1, encoding the cation extrusion pump that acts as the major determinant of salt tolerance in S. cerevisiae. Although the influx of Na⁺ from yeast cells is mostly dependent on the Ena1p ATPase (26), activities of other transporters like the H⁺-antiporter Nha1p...
that the known systems mediating sodium homeostasis are not involved.

**Loss of Function of GCN1 and GCN3 Also Causes Sodium Tolerance**—It has been proposed that during amino acid starvation, activation of the general amino acid control response due to elF2α phosphorylation by Gcn2p is induced by uncharged tRNA (29–31). GCN2 function in vivo also requires GCN1 and GCN20, encoding two proteins that form a complex that associates with ribosomes, physically interacts with Gcn2p, and is proposed to mediate activation of Gcn2p protein kinase in response to elevated levels of uncharged tRNA (32, 33). To determine whether this complex is also responsible for the negative effect of GCN2 on salt tolerance, we constructed gcn1 mutant strains. Loss of function of GCN1 increased sodium tolerance in both ena1–4 and ena1–4 GCN2 cells to the same extent as loss of GCN2 function (Fig. 3).

A similar genetic analysis was carried out using cells disrupted for the GCN3 locus. During translation initiation elF2 delivers the initiator methionyl-tRNA (tRNA\(^{Met}\)) to 40 S ribosomal subunits in an elF2-GTP-Met-tRNA\(^{Met}\) ternary complex and, upon AUG recognition, is released as an inactive elF2-GDP binary complex. elF2 is recycled to the GTP-bound state by the guanine nucleotide exchange factor elF2B. Phosphorylation of elF2α by Gcn2p converts elF2 from a substrate to an inhibitor of elF2B, thereby blocking or lowering ternary complex formation. GCN3 encodes the α-subunit of elF2B and as such forms part of the regulatory subcomplex in elF2B that mediates inhibition of the guanine nucleotide exchange function by phosphorylated elF2α (34–36). As in the case of GCN1 and GCN2, loss of GCN3 function also increased sodium tolerance (Fig. 3). These results suggest that the initial participants of the general amino acid control response somehow negatively affect yeast salt tolerance.

**Sodium Activates Gcn2p-dependent Phosphorylation of elF2α at Serine 51**—It has been described that constitutive activation of the Gcn2p kinase in the GCN2c-517 dominant mutant allele results in growth inhibition (37). Therefore, it seemed plausible that the negative effect of Gcn2p during Na\(^{+}\) stress could be due to (over)activation of the kinase. This possibility was explored by determining whether saline stress enhances phosphorylation of elF2α, a known physiological substrate of the Gcn2p kinase. Experiments were carried out by immunoblot analysis following the method described by Yang et al. (22), using polyclonal antibodies that either specifically recognize elF2α phosphorylated at serine 51 or that recognize both phosphorylated and non-phosphorylated elF2α (Fig. 4). As expected, high extracellular NaCl concentrations increased elF2α phosphorylation. This phosphorylation was entirely Gcn2p dependent since in gcen2 cells there was no detectable phosphorylation of elF2α. The increase in phosphorylation was detectable 15 min after mid-logarithmic phase cells were shifted to medium containing NaCl and was visible for at least 24 h (data not shown). The highest ratio of phosphorylated to non-phosphorylated elF2α was observed after 1 h following the

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**Table I**

| [Na\(^{+}\)] | [K\(^{+}\)] |
|-------------|-------------|
| ENA1-4 GCN2 | 122.8 ± 5.3  | 72.0 ± 5.7  |
| env1-4 gen2 | 114.9 ± 1.7  | 73.2 ± 6.5  |
| ena1-4 GCN2 | 122.2 ± 4.2  | 131.1 ± 6.5 |
| ena1-4 gen2 | 117.4 ± 5.1  | 133.1 ± 6.2 |

*Results are the average (±S.D.) of two uptake experiments with three determinations each and are represented as nmol/mg of cells. The external concentrations of sodium were 1 and 0.5 M for ENA1-4 and env1-4 cells, respectively.*
shift to medium containing NaCl. This phenomenon is apparently due to the toxicity of the Na^{+} ions and not to an osmotic effect since equivalent concentrations (with respect to osmolality) of KCl (and sorbitol, data not shown) did not evoke increased phosphorylation of eIF2α (Fig. 4).

**Loss of GCN2 Function Does Not Improve Translation Under Salt Stress**—Although in yeast cells this is not very apparent, in mammalian cells eIF2α phosphorylation can lead to a severe down-regulation of the translation initiation rate and consequently of total protein synthesis (11). Therefore, we wanted to determine whether translation efficiency under salt stress is improved in gcn2 cells and whether this could explain the salt tolerance phenotype. To this end, we measured incorporation of radioactive labeled phenylalanine into proteins in GCN2 and gcn2 strains in the presence of high Na^{+} concentrations (Fig. 5A). As a control we performed the same measurements in medium with an equivalent concentration of K^{+} instead of normal medium since under these conditions the rate of amino acid uptake is similar to that in Na^{+}-supplemented medium (Fig. 5B). Also, during the period of the assay in both conditions cells suffer a similar temporary growth arrest (data not shown). Incorporation of phenylalanine was scored following a 2-h preincubation period in the presence of the salts to allow the cells to reach steady-state levels of intracellular cation concentrations. Under these conditions Na^{+} ions were found to inhibit translation substantially more than K^{+} ions. However, and most importantly, no significant differences in translation efficiency under salt stress conditions were observed between GCN2 and gcn2 cells (Fig. 5A), suggesting that the salt tolerance phenotype of gcn2 mutants cannot be attributed to a general improvement of translation initiation.

**Sodium Activates the Translational Control of GCN4 Expression**—In contrast with mammalian cells, in yeast the Gcn2p—transcriptional activator of genes encoding amino acid biosynthetic enzymes (12)—mediates sodium activation of GCN4 expression. In order to test the hypothesis that the negative effect of Gcn2p on salt tolerance could be attributed to GCN4 activation, we used three different genetic approaches. In the first approach, we checked the salt tolerance of the gcn7-201 mutant (16). GCD7 encodes the β-subunit of eIF2B that with Gcn3p and Gcd2p forms a regulatory subcomplex that mediates inhibition of eIF2B exchange function by phosphorylated eIF2α (34). The gcn7-201 mutation elevates GCN4 expression levels (16) and, as indicated in Fig. 7A, this results in decreased salt tolerance. Deletion of GCN2 in this mutant background improved salt tolerance.

In the second approach, we investigated the effect of loss of CPC2 function in the W303-1A background. CPC2 encodes a Gβ-like WD protein that is required for the inhibition of Gcn4p transcriptional activity in the absence of amino acid starvation (39). The cpc2 mutation leads to increased transcription of Gcn4p-dependent genes under non-starvation conditions without increasing GCN4 expression. Again we found a negative effect on salt tolerance, and this could also be counteracted by additionally deleting the GCN2 gene in the cpc2 mutant (Fig. 7B).

The third approach was designed to test directly whether enhanced GCN4 activity results in sodium sensitivity. In this experiment a mutant GCN4 allele with only the first uORF intact (thus lacking uORFs 2–4) was introduced in isogenic gcn2 and GCN2 strains. This mutant allele expresses GCN4 at a derepressed level, independent of GCN2 function, and turns on amino acid biosynthetic genes but does not affect translation (14). As shown in Fig. 7C, introduction of this mutant allele in a gcn2 strain again increased sodium sensitivity (compare p164 and p235). Here we show results for the ena1–4 strain, but identical results were also obtained in an ENA1–4 gcn4 strain (data not shown).

Testing the gcn2 phenotype in a gcn4 mutant is complicated by the salt sensitivity of the latter (40). Nevertheless, using a relatively low salt concentration (0.5 M NaCl), we could not detect salt tolerance conferred by the gcn2 mutation in the gcn4 background (data not shown). Altogether, these results demonstrate that elevated GCN4 activity causes sodium sensitivity.

**Implication of HAL2 in the GCN2-mediated Sodium Toxicity**—The activation of Gcn2p by sodium could be indirect, mediated by the known target of sodium toxicity in yeast Hal2p (8). HAL2/MET22 encodes a nucleotidase that dephosphorylates...
PAP, an intermediate of the sulfate assimilation pathway. This nucleotidase is inhibited by Na\(^+\) but not by K\(^+\) (7). PAP accumulation during salt stress inhibits sulfate assimilation into methionine and RNA processing (8, 9). The observation that Na\(^+\) but not K\(^+\) ions were able to induce Gcn2p-mediated eIF2\(\alpha\) phosphorylation led us to determine whether HAL2 is implicated in the GCN2-mediated sodium toxicity.

Three different experiments were performed. Since the

HAL2 gene was identified in a genetic screening as a gene that confers salt tolerance to yeast upon overexpression (18), the first experiment was designed to analyze whether loss of GCN2 function could benefit HAL2 expression levels. Comparison of Hal2p accumulation levels by immunoblot analysis in cells grown in the absence or presence of high concentrations of Na\(^+\) or K\(^+\) clearly showed that this was not the case (Fig. 8A). On the contrary, Hal2p levels were highest in GCN2 cells grown in the presence of NaCl. This latter observation is consistent with the idea that NaCl induces GNC4 expression and consequently also the expression of amino acid biosynthetic genes such as HAL2. In the second experiment we wanted to control whether we could still observe enhanced salt tolerance conferred by loss of GCN2 function in a hal2 background. The increased salt sensitivity of the hal2 mutant complicated this experiment, but nevertheless loss of GCN2 function was able to increase salt tolerance to some extent (Fig. 8B). Important, eIF2\(\alpha\) phosphorylation following the shift to medium with NaCl is still observed in a hal2 mutant strain and with similar kinetics as in the HAL2 strain (data not shown). This suggests that the presence of HAL2 is not a requisite for the activation of the Gcn2p kinase and that loss of HAL2 function does not abolish this activation.
FIG. 7. Activation of GCN4 function causes sodium sensitivity. Strain H1727 (GCN2 GCN7) and its derivatives H1725 (GCN2 gcn7-201) and H1794 (gcn2 gcn7-201) (A), strain W303-1A (GCN2 CPC2) and its derivatives AG254 (GCN2 cpc2) and AG255 (gcn2 cpc2) (B), and strain AG86 (ena1–4 GCN2) and its derivative AG209 (ena1–4 gcn2) transformed with plasmids p16[Ycp50], p164[Ycp50-GCN4], or p235[Ycp50–ORF1-only-GCN4] (C) were grown in liquid YPD medium to saturation, and serial dilutions were dropped on YPD plates with or without NaCl (1.25 M in A and B and 0.5 M in C). Growth was recorded after 2 days in the absence of stress or after 5 days in the presence of NaCl.

FIG. 8. Implication of HAL2 in the GCN2-mediated sodium toxicity. A, Hal2p accumulation in salt-stressed yeast cells depends on GCN2. Yeast strain W303-1A (GCN2) and its derivative AG207 (gcn2) were grown to mid-logarithmic phase in liquid-rich medium either in the absence of salts (YPD) or in the presence of KCl (1.25 M) or NaCl (1.25 M). Upper and lower panels, respectively, show the Ponceau-stained nitrocellulose blot and the immunoblot analysis with the polyclonal antibody recognizing Hal2p. As a control a protein extract of AG258 (W303-1A hal2) cells was added. Lane M contains marker proteins to which purified Hal2p was added. The molecular mass (in kDa) and the position of Hal2p (arrowhead) are indicated at the left and the right of the figure, respectively. B, salt tolerance of gcn2 mutants is independent of a functional HAL2 gene. Strain AG86 (ena1–4 HAL2 GCN2) and its derivatives AG267 (ena1–4 hal2 GCN2), AG268 (ena1–4 hal2 gcn2), and AG209 (ena1–4 HAL2 gcn2) were grown in liquid YPD medium to saturation, and serial dilutions were dropped on YPD plates with or without NaCl (0.2 M). Growth was recorded after 2 days in the absence of stress or after 5 days in the presence of NaCl.

DISCUSSION

High Salinity Induces the General Amino Acid Control Response—In the present study, a genetic screening for negative determinants of yeast salt tolerance has led to the following discoveries. (i) High salinity induces the Gcn2p-mediated phosphorylation of eIF2α and thereby activates the translational control of GCN4. (ii) This response negatively affects salt tolerance. The former observation again underscores the statement made by Yang et al. (22) that induction of Gen2p activity and GCN4 translational control occurs in response to a wider spectrum of nutrient deprivations than was previously thought. This phenomenon was originally found to occur when yeast cells are subjected to amino acid starvation (42). Later it was found that purine starvation (43) and, more recently, glucose limitation (22) could also induce the general amino acid control response. Alternate pathways activated by various stress situations that induce GCN4 mRNA translation without the requirement of a functional Gen2p kinase have also been described (44–46).

The mechanisms regulating this response under saline stress conditions seem to be conserved with those operating during amino acid limiting conditions because in both stress conditions the same factors seem to be involved in the activation of the response (e.g., Gen1p, Gen2p, eIF2α, eIF2B, and Gcn4p). However, as is also the case for the response to glucose limitation, there are some differences in the regulatory mechanisms in response to amino acid starvation and high salinity, the most important being the delay in time between activation of Gen2p and activation of Gen4p. In the case of starvation for amino acids and glucose both events are tightly linked in time and can be detected almost simultaneously. This delay might reflect the temporary growth arrest, which yeast cells suffer when transferred to media with high NaCl concentrations and which is necessary for cells to adapt to the new and severe growth conditions. During this period translation is drastically reduced as can be deduced from Fig. 5A and from the downshift in expression of genes encoding ribosomal proteins or other proteins involved in translation (47). This might affect de novo translation of GCN4 mRNA, whereas a signal transduction process with factors that do not need de novo synthesis, such as phosphorylation of eIF2α by the Gen2 protein kinase, could still proceed.

What Physiological Event Would Cause the Na⁺-Mediated Activation of the General Amino Acid Control Response?—Protein kinase Gen2p is a multidomain protein that contains a region homologous to histidyl-tRNA synthetases juxtaposed to the kinase catalytic moiety. This domain regulates Gen2p kinase function by monitoring the levels of uncharged tRNAs accumulating during amino acid limitations (29–31). Un-
charged tRNAs were shown to bind directly with the HisRS-related region, and mutations in this domain (gen2-m2) that block RNA interaction also abolish the kinase function in vivo. The fact that in contrast to the wild type GCN2 allele neither the gen2-m2 mutant allele nor the gen2-K628R allele, mutated in the kinase catalytic domain, could complement the salt tolerance phenotype caused by loss of GCN2 function (data not shown) seems to confirm that in response to saline stress this HisRS-related region also is responsible for the induction of Gen2p protein kinase activity.

It is reasonable to wonder whether the activation of Gen2p during salt stress is a consequence of amino acid limitations. Several authors (40, 41) have already reported on the strong inhibition of amino acid uptake by high concentrations of Na+. However, as shown in the present work, both high Na+ and K+ concentrations inhibit equally the rate of initial amino acid uptake. This suggests that the rapid activation of the amino acid control response by Na+, which does not occur with high concentrations of K+, cannot solely be due to amino acid starvation as a consequence of the decreased rate of amino acid uptake. Consistent with this view is the finding that toxic concentrations of Li+, which do not inhibit amino acid uptake (40), also induce eIF2α phosphorylation (data not shown).

Another hypothesis that could explain the rapid activation of the amino acid control response upon salt stress is that high intracellular Na+ concentrations somehow impede normal tRNA processing or synthesis, creating defective tRNAs that can activate Gen2p in the absence of amino acid starvation (30, 46, 48, 49). Various antecedents for toxicity of Na+ or Li+ cations to RNA processing are known. In the field of aminoacyl-tRNA synthetase recognition of tRNA, it has been reported that salts, such as sodium chloride, can inhibit nucleoprotein complex formation and/or enzymatic activity (50–54). PAP accumulation due to the salt-mediated inhibition of Hal2p inhibits the 5′ → 3′-exoribonucleases Xrn1p and Rat1p, and consequently 5′ processing of the 5.8 S rRNA and small nucleolar RNAs, degradation of pre-rRNA spacer fragments, and mRNA turnover are inhibited. Lithium also inhibits the activity of RNase MRP by a mechanism that is not mediated by PAP (9).

Interestingly, overexpression of the NME1 gene coding for the RNA component of the MRP ribonuclease, which causes improper 5.8 S ribosomal RNA maturation and which could thus affect ribosomal biogenesis and translation initiation, can stimulate GCN4 mRNA translation (45, 46).

Why Does the Activation of the General Amino Acid Control Response Negatively Affect Yeast Salt Tolerance?—Yeast salt tolerance seems to depend on three kinds of proteins as follows: cation transporters, regulators of these transporters, and cation toxicity targets (1). Loss of GCN2 function does not seem to up-regulate members of the first two categories as Na+ and K+ intracellular accumulation after a NaCl shock was not significantly altered by loss of GCN2 function. The only identified and probably most important in vivo target of lithium and sodium toxicity in yeast identified so far is the nucleotidease Hal2p (7, 8). Overexpression of Hal2p confers salt resistance in yeast (18). However, Hal2p accumulation levels were not increased by loss of GCN2 function, and both the salt tolerance conferred by the gen2 mutation and the Gen2p-mediated phosphorylation of eIF2α induced by sodium seemed to be independent of HAL2. Also, overactivation of the sulfur amino acid biosynthetic pathway and thus a potential increase in PAP accumulation cannot (solely) account for the observed phenotypes. For example, loss of function of MET16 could not enhance yeast salt tolerance (data not shown). Expression of MET16, encoding the reductase that converts 3′-phospho-5′-adenyllysulfate to sulfate, the biosynthetic step in which PAP is also generated as a by-product, is also regulated by Gen4p (55, 56). Apparently, salt tolerance caused by loss of GCN2 function cannot be simply explained by a single-gene model but instead may rely on a more general and complex effect on gene expression.

The most plausible possibility is that overexpression of many of the amino acid biosynthetic genes due to overactivation of the Gen2p-Gcn4p pathway is harmful for yeast growth during salt stress conditions and may create certain metabolic problems. Several observations support this hypothesis as follows. (i) In the gen2 strain induction of GCN4 expression by salt stress is almost completely abolished, and Gen4p stays more or less present at basal levels. (ii) Overexpressing GCN4 to derepressed levels without affecting translation initiation renders sodium sensitivity to gen2 strains. (iii) A cpc2 mutation that leads to high transcriptional activity of Gen4p without increasing GCN4 expression itself (39) negatively affects yeast salt tolerance.

However, elevated GCN4 expression may not be sufficient to fully account for the salt-sensitive phenotype, and a Gen2p-mediated decrease in translational efficiency, although modest, may also contribute. In yeast eIF2α phosphorylation does not lead to a severe down-regulation of total protein synthesis, but a transient decrease in the rate of translational initiation and amino acid incorporation following the removal of amino acids from the growth medium could be observed (57). Although in our amino acid incorporation assay we could not detect an effect of loss of GCN2 function on general translation efficiency under salt stress, some observations impede us to reject this possibility. First, loss of GCN2 function improved salt tolerance in the gcd7-201 background, although in the general amino acid control pathway GCD7 is epistatic to GCN2 (16); and second, overexpressing GCN4 to derepressed levels without affecting translation initiation dramatically reduces salt tolerance although not to the level of wild type yeast cells.

Consequently, it cannot be excluded that each of the above discussed aspects participates in some way, and therefore salt tolerance conferred by loss of GCN2 function may reflect the outcome of a sensitive balance between counteracting cellular processes. For example, it has been demonstrated recently that the presence of the GCN4 gene is vital for yeast growth under NaCl or KCl stress (40). We may hypothesize that there is a balance between the effects of two independent phenomena: the positive effect of the Gen2p-Gcn4p pathway on salt tolerance and the toxic overactivation by Na+ of this pathway. This leads to the apparent contradictory situation that the gcn4 mutation renders cells highly salt-sensitive, whereas the gen2 mutation confers NaCl tolerance. A final point is that, given the conservation of the Gen2p-eIF2α stress pathway in eukaryotes, the fact that sodium induction of this pathway mediates sodium toxicity may be of general significance.

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