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Cloning and characterization of genes required for osmoregulation in the yeast *Saccharomyces cerevisiae*

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Rice University, 1994
RICE UNIVERSITY

CLONING AND CHARACTERIZATION OF GENES REQUIRED FOR OSMOREGULATION IN THE YEAST SACCHAROMYCES CEREVISIAE

by

Jay Brewster

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE DOCTOR OF PHILOSOPHY

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ABSTRACT

CLONING AND CHARACTERIZATION OF GENES REQUIRED
FOR OSMOREGULATION IN THE YEAST SACCHAROMYCES CEREVISIAE

by

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Salt stress induces multiple physiological responses in unicellular organisms. Responses include both immediate effects upon membrane permeability to select cytoplasmic solutes and changes in gene expression. This study has sought to isolate genes required for osmoregulation in the yeast, Saccharomyces cerevisiae. Eighteen mutants, with an inability to grow under osmotically stressed conditions (Osm°), and deficient in salt induced glycerol accumulation were isolated. Each of the mutants fell into one of four complementation groups hog1 through hog4 (High Osmolarity Glycerol response). Three genes, HOG1, HOG2, and HOG4, that rescued growth of representative mutants under osmotically stressed conditions were cloned and disruption alleles generated. Disruption mutants of each gene displayed Osm° growth and depressed glycerol accumulation following salt stress. Sequence analysis of HOG1 revealed striking sequence homology with mitogen-activated protein (MAP) kinases, including 50% identity with FUS3, a MAP kinase in the yeast mating response pathway. Immunoblots of normal and osmotically stressed cells detected an inducible tyrosine phosphorylation of HOG1, an event shown to activate homologous kinases. Site-directed mutagenesis of
**HOG1** on residues required for activation in related MAP kinases resulted in a loss of complementing activity on high salt plates and a lack of tyrosine phosphorylation of Hog1p when Tyr^{176} was mutated to Phe. **HOG4** was identified to be a previously sequenced gene, **PBS2**, with strong sequence homology to MAPK kinases, kinases upstream of MAP kinases and thought to be responsible for the activating phosphorylation events. Hog1p was not tyrosine phosphorylated in a pbs2 mutant consistent with **PBS2** being the kinase responsible for Hog1p phosphorylation. Osmotic stress was shown to result in aberrant terminal morphology (elongate, multi-budded, multi-nucleate cells) in both hog1 and pbs2 null mutants. Further characterization of the mutants using time lapse microscopy revealed that salt stress induces bud abandonment and aberrant bud site selection. **HOG1** and **PBS2** therefore are two genes involved in carrying an osmostress induced signal to stimulate adaptive cellular responses and are required for normal bud site selection following salt stress.
Acknowledgements:

The following study would not have been possible without the help of Dr. Michael Gustin, Dr. Edward Winter, Dr. Tamsen deValoir, Noelle Dwyer, Margie MacIntosh and Doriann Allen.

I am deeply grateful for the expertise, patient guidance, and friendship Mike has shared with me over the last several years. The relationship we've had has instilled in me an understanding of the progression in research from an idea on paper to the generation of real data. Mike is always ready to lend an ear to a demoralized graduate student following another failed experiment, and to offer his suggestions without criticism. He also has repeatedly made himself available whenever an experiment needs an extra hand, no matter what time of day (or night) the help is needed. I thank Mike for the professional guidance he has offered, but more importantly for his friendship, which has made my years at Rice truly enjoyable.

Dr. Edward Winter was also critical to the development of this project. Ed unselfishly shared his expertise in immunoblots, performing the first blots which identified tyrosine phosphorylation of Hog1p, and carefully taught the technique to us through several lengthy phone conversations. Dr. Tamsen deValoir helped in the subcloning of the HOG1 and HOG2 clones, and performed Northern analyses on all three genes as well as a variety of other experiments. Tamsen brought an expertise in molecular biology which was greatly appreciated. Noelle Dwyer, Margie MacIntosh and Doriann Allen helped with the cloning and genetic analyses early in this study. I am grateful for their very competent and committed effort as well as their friendship while working on the project.

I would also like to thank the members of my thesis committee; Dr. Charlie Stewart, Dr. Michael Stern, Dr. James Campbell, Dr. Jackie Shanks, and Dr. Michael Gustin. I appreciate the time each has spent in overseeing my research project at Rice, including the advice they have offered which has benefitted the development of this work.

This document is dedicated to my wife Stephanie. Throughout the peaks and valleys of graduate school, she has been unfailing in her encouragement and a constant source of strength.

To my parents, who have always encouraged my interests in science and nature, I offer my deepest gratitude. I will always be indebted to them for the guidance and support they've offered over the years.
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ABBREVIATIONS

ATP       adenosine triphosphate
BCIP      5-bromo-4-chloro-3-indolyl-phosphate
BSA       bovine serum albumin
DAPI      4,6-diamidino-2-phenylindole hydrochloride
DCPIP     2,6-dichlorophenolindophenol
GPD       glycerol-3-phosphate dehydrogenase
IPTG      isopropyl-β-thiogalactopyranoside
MAP kinase mitogen-activated protein kinase
MAPK kinase mitogen-activated protein kinase kinase
NAD       nicotinamide adenine dinucleotide
NBT       nitro blue tetrazolium
Osm\textsuperscript{R}  osmoresistant
Osm\textsuperscript{S}  osmosensitive
PBS       phosphate buffered saline
PMSF      phenylmethylsulfonyl fluoride
Chapter 1:

Introduction to Osmoregulation

Microorganisms comprise simple yet prolific cellular systems with staggering growth rates. However, environmental conditions limit these growth rates. Adaptive responses to changing temperatures, introduction of antibiotics or heavy metals, and depletion/introduction of nutrients have been characterized in a variety of microbial systems, offering important insight into the control of cell growth (reviews: Parkinson, 1993, Lindquist and Craig, 1988, also McAlister and Finkelstein, 1980, Entian et al, 1982, Celenza, 1986). Cell growth in higher organisms is generally controlled by different stimuli. However, similar genes have been shown to regulate cell cycle progression in both systems (reviews: Naeve et al, 1991 Nurse, 1990, Lewin, 1990). Characterization of growth control in simple systems therefore benefits an understanding of both microorganisms and more complex cellular systems.

The budding yeast, *Saccharomyces cerevisiae*, employs extensive regulatory mechanisms which control the expression of genes necessary for growth. Specific nitrogen sources in the growth media induces the expression of genes necessary for their utilization and represses the expression of those employed in the metabolism of poorer nitrogen sources (Cooper, 1982, Wiame et al, 1985). Similar mechanisms have been identified in the metabolism of carbon sources. Yeasts can utilize a wide array of sugars to generate energy for growth (for review, Barnett, 1976), but employ complex
regulatory mechanisms to exhaust supplies of the "favored" carbon source before metabolizing others. The availability of glucose in the growth media results in repression of the genes involved in metabolism of galactose, maltose, sucrose, glycerol, and ethanol (for review: M. Johnston and M. Carlson, 1992). As glucose levels are depleted, repression is relaxed and cellular metabolism converts to another carbon source. Thus, as conditions outside the cell vary, regulation of gene expression inside the cell is optimized for efficient growth.

A prominent threat to single celled organisms is dehydration. As water evaporates from a microenvironment, solute concentrations increase, resulting in cellular dehydration as cytoplasmic water is lost. In order to grow, the cells must detect these external fluctuations in osmolarity and make the necessary physiological adjustments. Under extreme conditions the osmolarity rises above concentrations permissive for growth and the cells must arrest growth completely. The focus of this thesis is to employ the budding yeast *Saccharomyces cerevisiae* to characterize cellular mechanisms by which cells "sense" or "feel" dehydration and signal for adaptive responses.

Growing cells demand an inward flux of water in order to gain volume. Under normal growth conditions, higher cytoplasmic solute concentrations result in a water influx as water flows down its concentration gradient (for review, Brown et al, 1986). This influx is limited only by physical resistance from the cell membrane or wall which cannot expand beyond certain dimensions without rupture. Pressure exerted on the cell
membrane and wall by the osmotic gradient is termed turgor pressure. Turgor is needed by the cell to maintain pressure on the cell membrane and keep the cell "inflated", this pressure is also needed to maintain normal cellular morphology as cells grow and divide. The magnitude of turgor exerted on the cell membrane is dependent upon the concentration gradient across the membrane. If the external osmolarity is raised to equal or exceed the internal osmolarity, turgor is lost or reversed and growth is inhibited. An understanding of cellular responses to osmotic stress and how these responses are stimulated should offer guidance in the production of drought\saline resistant plants.

Osmoregulation in *Escherichia coli*:

The first studies to isolate genes involved in osmoregulation were performed in *Escherichia coli*. Concentrations of NaCl above 800 mM inhibit the growth of most strains of bacteria unless the media is supplemented with members of a class of small, extremely water soluble molecules termed osmoprotectants. Both plants and bacteria were found to accumulate similar osmoprotectants when growing under solute rich conditions (for review; Le Rudulier et al, 1984). The role these molecules play in enabling cells to grow under stressed conditions and the reason for conservation of such molecules in diverse organisms has been critically evaluated. As discussed earlier, high external solute concentrations dehydrate the cell if the internal osmolarity is surpassed. The accumulation of an osmoprotectant, through elevated synthesis or transport, can offset high external osmolarity and restore normal turgor if the solute can be retained in
The cell. The limiting factors regarding osmoprotectants and the probable reasons for conservation are 1) accumulation of the solute to high levels must not be toxic to normal cellular functions, and 2) production of the solute should be energetically feasible. Osmoprotectants used in bacteria include glycine betaine, betaine aldehyde, proline, proline betaine, and dimethyl glycine. In higher plants, either glycine betaine or proline accumulate proportionally with increasing NaCl in the growth media. Each of these are described as "compatible" solutes, identifying their ability to accumulate to very high levels without inhibiting normal cellular functions.

The actual mechanism of sensing external osmolarity has not yet been determined, but a membrane associated protein has been cloned in E. coli and shown to trigger downstream events affecting expression of osmotically regulated genes (for review Mizuno and Mizushima, 1990). The outer membrane of bacterial cells contains a few classes of predominant proteins (10^5-10^6 molecules/cell); one of these is a class of small pore-forming molecules termed the porins. These proteins have been shown to function primarily as non-selective diffusion channels for small hydrophilic molecules (Sugawara et al, 1992), but also as phage receptors (Datta et al, 1977), mediators in F-factor conjugation (van Alphen et al, 1977), stabilizers of bacterial shape (Sonntag et al, 1978), and important to the transport of amino acids (Manning et al, 1977). The best understood of these functions is that of pore-formation and solute diffusion. Two major porins, OmpC and OmpF, are thought to play a vital role in growth adaptations to extracellular osmolarity. Under normal conditions OmpF is preferentially expressed and OmpC is
found in the membrane at much lower levels. An increase in the osmolarity of the growth media results in increased \textit{OmpC} expression and decreased levels of \textit{OmpF}. The absolute concentration of both proteins combined is similar under either condition. The physiological relevance of differential porin expression appears to be related to the size of molecules each protein allows through the membrane. \textit{OmpF} forms a larger hole than \textit{OmpC} (Parkinson, 1993). Under osmotically stressed conditions where compatible solutes are being accumulated, membrane impermeability of those solutes may depend upon higher levels of \textit{OmpC} expression.

A two-component regulatory system, similar to other previously identified bacterial systems involved in chemotaxis, nitrogen metabolism, plant infection, and phosphate metabolism (Stock, 1988), was found to mediate the osmotically controlled expression of porins. The membrane-associated cytoplasmic protein, \textit{EnvZ}, has been shown to have both kinase and phosphatase activities appearing to detect changes in external osmolarity and alter its phosphorylation/dephosphorylation of the second protein, \textit{OmpR}. \textit{OmpR} is a transcription factor with sequence-specific DNA binding activity towards the promoters of both \textit{ompF} and \textit{ompC}. Though not yet completely understood, the transfer or removal of phosphate groups onto or from \textit{OmpR} is thought to result in the differential expression of the porins.

\textbf{Osmoregulation in Yeast and Algae:}

A great deal of work has been done in the characterization of osmoprotectant
production in various salt-resistant microorganisms. The natural environments of the yeasts *Debaryomyces hansenii* and *Zygosaccharomyces rouxii* include sea water and brines which contain high levels of salts (Norkrans, 1966, Onishi, 1963). A third yeast, *Saccharomyces cerevisiae*, is not as osmotolerant but displays similar physiological responses to salt stress. The unicellular algae *Dunaliella* also grows well in extremely saline environments, and the physiology of that growth has been studied (Brown and Borowitzka, 1979). Characterization of internal solutes within each of these species revealed that the production and accumulation of glycerol was dependent upon external osmolarity (Andre et al, 1988, Yagi, 1988, Brown, 1978). Despite the similarities in glycerol production, other physiological characteristics varied and were summarized into three categories by Brown and Borowitzka (1979); 1) The compatible solute is produced at a constant rate and the percentage retained within the cell is varied depending upon external osmolarity. The cell membrane appears to control permeability of the solute through unknown mechanisms (*Z. rouxii*). 2) The rate of synthesis of the compatible solute is varied depending upon external osmolarity and the percentage retained within the cell remains constant (*S. cerevisiae*). More recent information has shown *S. cerevisiae* to have some control over membrane permeability to glycerol (see below), but even in high salt a great deal leaks out of the cell. 3) The rate of synthesis of the osmoregulator is controlled by external osmolarity as above, but all of the synthesized solute is retained in the cell (*Dunaliella*). Decreases in external osmolarity result in the conversion or metabolism of the solute. The degree of osmoresistance of each of these species has been linked to the method of glycerol accumulation as described above. The
yeast *S. cerevisiae* is the least osmotolerant of the examples given and employs the most wasteful method of osmoregulation described. Glycerol is made at elevated levels to allow accumulation and restoration of turgor in high osmolarity media, but a significant amount of the glycerol leaks from the cell. In extremely high osmolarity, the wastefulness of this method becomes limiting as cells lose too much "energy" in the form of leaking glycerol. The highly efficient system displayed in *Dunaliella* depends upon the membrane impermeability to glycerol.

Characterization of physiological responses to osmotic stress in eukaryotic systems is most complete in *S. cerevisiae*. As described above, an increase in external osmolarity results in the synthesis and accumulation of glycerol up to molar levels (Figure 1-1, from Reed et al, 1987). Trehalose has also been shown to accumulate, but at much lower concentrations, and is thought to aid in the stabilization of membranes (Crowe et al, 1984, Crowe et al, 1988, Wiemken, 1990). Blomberg and Adler (1989) found glycerol permeability of the yeast cell membrane to be variable. Cells grown in high osmolarity media were washed in various solutions of lower osmolarity, revealing glycerol to be lost from the cell to the wash solution in a manner dependant upon its osmolarity. Transfer of cells to higher osmolarity results in a decrease in the membrane permeability to glycerol. Experiments testing the viability of yeast cells growing exponentially in culture which were suddenly exposed to 1.4M NaCl in the media tested the requirement for protein synthesis in osmoregulation (Blomberg and Adler, 1989). Only .02% of the cells were viable following a shift to 1.4M NaCl; however, if a preincubation in .7M NaCl
Figure 1-1: Levels of intracellular glycerol in exponentially growing *Saccharomyces cerevisiae*. A wild type yeast strain was exposed to increasing concentrations of NaCl in the growth media and the accumulation of intracellular glycerol determined using $^{13}$C-nuclear magnetic resonance spectroscopy. The intracellular osmolarity resulting from glycerol accumulation was calculated from published osmolarities. Values generated were then used to determine the percentage of external osmotic increases due to NaCl which were counterbalanced by internal glycerol. Data from Reed et al (1987).
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<td>0.86</td>
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Reed et al, 1987
preceded the shift, 100% of the cells survived. Cycloheximide treated cells displayed .2% viability despite preincubation in .7M NaCl. These experiments identified a requirement for protein synthesis in cellular adaptive responses to osmotically stressed conditions. Andre et al (1991) revealed that osmotic stress induces expression of glycerol-3-phosphate dehydrogenase (GPD), the key enzyme in glycerol synthesis. Two-dimensional analysis of metabolically labelled cells revealed the induction of nine proteins following salt stress (Varela et al, 1992). Overall protein synthesis and amino acid uptake from the medium have been shown to be sharply reduced following osmotic stress. The above data seem to point towards an immediate signalling of osmotic stress, resulting in changes in cellular physiology (glycerol permeability) and in gene expression.

Stretch-Activated Channels:

The most prominent candidates for eukaryotic osmotic "sensors" are stretch-activated or mechanosensitive (MS) ion channels which are associated with the cell membrane and activated by increased tension on the cell membrane. The application of a small amount of pressure (5-15 mmHg) into isolated E. coli (Martinac et al, 1987) or S. cerevisiae (Gustin et al, 1988) cells has been shown to result in anion and cation flow. Similar stretch-activated and stretch-inactivated channels have been detected in animal cells (for review Chamberlin and Strange, 1989, Morris, 1990). The physiological relevance of MS channels is not yet clear though two prominent possibilities involve a role in osmoregulation (Gustin et al, 1988). Ion flow immediately following salt stress could function as an osmotic stabilizer, to help minimize dehydration before solutes could
be accumulated. A similar system has been proposed in *E. coli*, where potassium transporter expression is controlled as a function of external osmolarity and is believed to play a role in osmoregulation (Laimins, 1981). A second proposed role for the MS channel is in the generation of a cytoplasmic signal for cellular adaptations to fluctuations in osmolarity. The MS channel is the only known calcium transporter in yeast, and calcium is known to play a role in signalling in other eukaryotic systems. Accumulation of calcium in the cytoplasm could trigger a cascade of osmoregulatory events including changes in gene expression and influences upon growth. The presence of the MS channel in a variety of systems identifies it as important for general cellular function and highlights the importance of its isolation.

**Signalling in Yeast:**

Of interest to the work in osmoregulation is the process of getting a signal to the nucleus from the cell membrane. The mechanism in *S. cerevisiae* by which haploid cells detect and mate with cells of the opposite mating type has been well-characterized and is an excellent example of intercellular signalling (for review Reed, 1991, Marsh et al, 1991). Haploid yeast cells produce a small, mating type-specific, peptide pheromone and contain a membrane-associated receptor which detects pheromone from cells of the opposite mating type. The receptor is composed of three subunits, which bind peptide pheromone and initiate a cascade of kinase-mediated phosphorylation events. One final substrate for the cascade is the transcription factor *STE12* which stimulates mating-specific gene expression (Dolan et al, 1989, Errede and Ammerer, 1989). Another target
Figure 1-2: Signal transduction pathways in *S. cerevisiae* and *S. pombe*. Components of the protein kinase C, and mating response signalling pathways reveal similar ordering and activation of homologous kinases. Shaded boxes identify kinases with highly conserved residues. The boxes include MAPKK kinases (*BCK1/SSP31, STE11*, and *byr2*), MAPK kinases (*MKK1/MKK2, STE7, and byr1*), and MAP kinases (*MPK1, FUS3, KSS1*, and *spk1*). Figure adapted from Irie et al (1993).
is *FARI* which, activated by phosphorylation, associates with the cell cycle machinery to inactivate it and arrest growth (Peter et al., 1993). A similar mating response signalling cascade has been identified in the fission yeast *Schizosaccharomyces pombe* (for review, Errede and Levin, 1993). Following activation by mating pheromone *byr1*, *byr2*, and *spk1*, three protein kinases with homology to kinases in the *S. cerevisiae* pathway, are thought to carry the kinase mediated signal to the nucleus. Genetic evidence as well as sequence homologies to other components of other signal transduction cascades indicates the displayed ordering.

A second signalling pathway was recently identified in yeast involving protein kinase C (*PKCl*). In mammalian systems, this kinase is thought to mediate a variety of external signals affecting secretion, exocytosis, ion conductance, smooth muscle contraction, gene expression and cell proliferation (for review Nishizuka, 1988). Deletion of yeast *PKCl* results in a lethal cell lysis defect and is due to incomplete cell wall synthesis (Levin et al., 1990). Four suppressors of this lethality have been isolated and sequentially ordered using genetics (Irie et al., 1993). As shown in Figure 1-2, both the mating response pathway and the protein kinase C pathway display similar kinases in the "kinase cassette". They are ordered similarly and include a MAPKK kinase (*STE11, BCK1, byr2*), which act upon a MAP activator or MAPK kinase (*STE7, MKK1, MKK2, byr1*), followed by the MAP kinase (*FUS3, KSS1, MPK1, spk1*) which is activated by phosphorylation on tyrosine and threonine (Lee et al., 1993, Zhou et al., 1993). Yeast has proven to be a powerful system for the isolation of additional components of these
pathways and for the functional and genetic analysis of the genes involved. Signal transduction pathways containing MAP kinases, displaying similar activation and ordering have been detected in several other systems (for review Thomas, 1992).

**Goals of project:**

The following research was undertaken to isolate and characterize genes involved in osmoregulation in *S. cerevisiae*. Mutants sensitive to high osmolarity were isolated and found to represent mutations in four genes, three of which have been cloned. Characterization of those genes has led to the discovery of an osmotically activated signal transduction pathway containing kinases similar to those found in the mating response and protein kinase C pathways of *S. cerevisiae*. Downstream influences of this pathway beyond solute accumulation, affecting cell morphology and budding were unexpected and indicate important influences of the pathway upon cell growth. The following results provide some exciting insights into how cells detect and respond to osmotic stress but much more experimentation is needed to complete the story.
Chapter 2:

The Isolation of Genes Required for Osmoregulation

In order to isolate genes involved in the *S. cerevisiae* osmoregulatory response, a classical mutant screening approach was undertaken. Random mutations can be introduced into genomic DNA through exposure to strong alkylating agents. Cells are then screened for those with the mutant growth characteristics of interest. Recent efforts at the isolation of genes conferring osmoreistance in yeast have been undertaken. Cloning from high copy libraries, transformants are screened for a plasmid dependent growth advantage on media containing salt concentrations which are normally prohibitive to growth (>1M NaCl). These strategies have yielded genes specifically conferring resistance to NaCl, but with no influence upon growth in other solutes (Gaxiola et al, 1992). Another technique was developed in *Debaromyces hansenii* to isolate mutants with an abnormal glycerol response (Morales et al, 1990). In cultures grown under salt-stressed conditions, mutants with low intracellular glycerol were separated from wild-type cells using a Percoll-sorbitol gradient. Glycerol accumulation results in an increase in cell density and enables the separation of cells as a function of their density. This screen yielded several osmotically sensitive mutants which are currently being further characterized (Adler et al, unpublished results). Despite the similarities in premise, these mutant screens did not yield clones overlapping with those described below.
Mutagenesis of wild-type yeast:

Random mutagenesis of wild-type cells was accomplished using ethyl methanesulfonate to a cell viability count of 30%. Mutagenized cells were plated out onto rich media for growth of single colonies. Primary screening involved isolation of colonies showing little or no growth when printed from rich media (YEPD) to YEPD + 0.9M NaCl. To ensure the osmosensitivity (Osm8) was resulting from general osmotic stress, and not NaCl specifically, isolated mutants were rescreened on rich media containing high concentrations of other solutes (sorbitol, KCl, glucose). "Petite" mutants (loss of mitochondrial function) and translational suppressors (Singh, 1977), both of which have been shown to result in osmotically sensitive cells were eliminated from the mutant pool as described in Materials and Methods. The decision to eliminate petites and translational suppressors was based on a desire to remove mutants carrying non-specific growth defects. The mutagenesis was repeated several times and of 10,000 colonies, 95 (1%) were found to be generally Osm8 and retained for further analysis.

Assay for intracellular glycerol:

Within the first few hours following osmotic stress, the budding yeast, S. cerevisiae, osmoregulates through the production of glycerol (Brown, 1978). In order to screen the isolated mutants, an enzymatic assay was developed to measure intracellular glycerol under normal and osmotically stressed conditions. Several salt concentrations were first evaluated to determine the optimal glycerol accumulation response one hour after stress induction. Figure 2-1 displays glycerol accumulation in wild-type cells after
Figure 2-1: Assay of intracellular glycerol following exposure to NaCl. The wild-type yeast strain, MGY159A, was grown up in YEPD to log phase (1 x 10^7 cells/ml). Cultures were then divided into aliquots and each aliquot stressed with from 0.0 to 0.9M NaCl for 1 hour at 30°C. Intracellular glycerol was determined as described in Materials and Methods. A peak of glycerol accumulation was observed in cells exposed to 0.4-0.6M NaCl (duplicate experiments displayed).
1 hour in YEPD containing from 0.0 - 0.9M NaCl. A peak of accumulation was observed in cells stressed with 0.4 - 0.6M NaCl. Two possible explanations for why the maximal glycerol accumulation did not coincide with the maximal salt concentration are available from existing data. Extremes in salt concentrations are toxic to a percentage of the cell population, especially log phase cells (Mackenzie et al, 1986). Viable cells within the population will osmoregulate and accumulate glycerol but the number of responding cells will be overestimated. The concentration of glycerol in each cell will therefore be underestimated in assayed extracts. Osmotic stress has also been shown to dehydrate cells significantly, depending upon the gradient across the cell membrane, and higher salinities may delay solute accumulation (Morris et al, 1986). In screening the isolated mutants, an osmotic stress of 0.4M NaCl was chosen to get the maximum difference between stressed and unstressed cells within one hour.

To identify mutants that were unable to respond to osmotic stress, intracellular glycerol was measured for each of the 95 isolates in YEPD and YEPD + 0.4M NaCl. Intracellular glycerol lower than 60% that of wild-type cells was considered abnormal and the mutant retained for further analysis. As shown in Fig. 2-2 most of the mutants (~70%) displayed wild-type levels of glycerol under both conditions and were therefore eliminated. However, eighteen of the mutants did display an abnormally low glycerol response and were retained for further characterization.
Figure 2-2: Glycerol response in osmosensitive mutants. Each member of the Osm⁶ mutant pool (95 total) was assayed for accumulation of intracellular glycerol under normal and osmotically stressed conditions (assay described in Materials and Methods). Isotonic media was YEPD (252 mOs.), while hypertonic media in the assay was YEPD + 0.4M NaCl (1003 mOs.). Each mutant was assayed at least one time, standard deviation calculated for mutants assayed three or more times. Growth on hypertonic media was determined by printing cells to YEPD containing 0.9M NaCl, 1.6M sorbitol.
# Glycerol Response in Osmosensitive Mutants

<table>
<thead>
<tr>
<th>Strain</th>
<th>Growth on Hypertonic Media</th>
<th>Glycerol&lt;sub&gt;in&lt;/sub&gt; (nmol/10&lt;sup&gt;7&lt;/sup&gt; cells)</th>
<th>Glycerol&lt;sub&gt;isotonic&lt;/sub&gt;</th>
<th>Glycerol&lt;sub&gt;hypertonic&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>yes</td>
<td>40 ± 10</td>
<td>188 ± 48</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Aberrant glycerol response mutants</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NDY2</td>
<td>no</td>
<td>28 ± 10</td>
<td>103 ± 33</td>
<td></td>
</tr>
<tr>
<td>NDY12</td>
<td>no</td>
<td>30 ± 15</td>
<td>90 ± 18</td>
<td></td>
</tr>
<tr>
<td>DAC14</td>
<td>no</td>
<td>25 ± 10</td>
<td>115 ± 40</td>
<td></td>
</tr>
<tr>
<td>JBY1</td>
<td>no</td>
<td>23 ± 10</td>
<td>90 ± 35</td>
<td></td>
</tr>
<tr>
<td>(plus 14 additional mutants)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Normal glycerol response mutants</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NDY40</td>
<td>no</td>
<td>28 ± 15</td>
<td>198 ± 5</td>
<td></td>
</tr>
<tr>
<td>(plus 75 additional mutants)</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
Figure 2-3: Tetrad analysis of *hog1-l* backcross. A representative tetrad from a *hog1-l* X MGY159B backcross displaying cosegregation of depressed osmotically induced glycerol accumulation and Osm$^8$ (determined by evaluation growth on YEPD + 0.9M NaCl plates and indicated as + or -). Accumulation of intracellular glycerol was following a 1 hour incubation in YEPD or YEPD + 0.4M NaCl as described in Materials and Methods.
Genetic Analysis:

To establish whether the Osm\textsuperscript{S} phenotype was dominant or recessive, each mutant was crossed by a wild-type strain and the diploid tested for growth on high osmolarity medium. Osm\textsuperscript{S} was determined to be recessive in all eighteen mutants. To identify if the phenotype was the result of a single mutation, the diploid strains were sporulated under nitrogen starvation conditions. The resulting tetrads were dissected, and the haploid cells printed to high osmolarity media. Each of the mutants produced 2 Osm\textsuperscript{S} and 2 Osm\textsuperscript{R} (wt) haploids in each complete tetrad indicating a mutation in a single gene was causing the Osm\textsuperscript{S} phenotype. Tetrads were also assayed for glycerol accumulation, revealing cosegregation of Osm\textsuperscript{S} on plates with depressed levels of intracellular glycerol in the assay (Fig. 2-3). In each of the mutants, the two phenotypes were found to be genetically linked, and thus likely the result of mutation in the same gene.

Complementation testing was undertaken to determine the number of genes represented in the isolated mutants. When working with recessive mutants one can test if two phenotypically similar isolates are mutations in the same gene by examining the phenotype of heterozygous diploids. In this case, two Osm\textsuperscript{S} haploids are mated together and the diploid tested for growth on high osmolarity media. If the diploid is Osm\textsuperscript{S} the mutants are likely alleles of the same gene, if Osm\textsuperscript{R}, two separate genes are represented. Exceptions to this rule include complementing mutations in the same gene (different parts of the same protein have different functions), non-complementing mutations in different genes (haploid sufficiency; loss of one functional copy of a gene in a diploid has a
phenotype when paired with a mutated form of a second gene). A complementation group is not an absolute group, but most often contains mutant alleles at a single locus (cited exceptions are uncommon). This procedure was performed for each of the twenty mutants and resulted in identification of four complementation groups. As displayed in Table 1, the groupings were named hog1, hog2, hog3, and hog4 (High Osmolarity Glycerol response).

**TABLE 1: COMPLEMENTATION GROUPS**

<table>
<thead>
<tr>
<th>hog1</th>
<th>hog2</th>
<th>hog3</th>
<th>hog4</th>
</tr>
</thead>
<tbody>
<tr>
<td>NDY2 (1)</td>
<td>NDY12 (1)</td>
<td>DAC14 (1)</td>
<td>JBY1 (1)</td>
</tr>
<tr>
<td>DAC16 (2)</td>
<td>NDY17 (2)</td>
<td>DAE11 (2)</td>
<td>NDY1 (2)</td>
</tr>
<tr>
<td>DAD3 (3)</td>
<td>NDY33 (3)</td>
<td>DAC1 (3)</td>
<td>NDY11 (3)</td>
</tr>
<tr>
<td></td>
<td>NDY35 (4)</td>
<td></td>
<td>NDY26 (4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NDY36 (5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>DAD4 (6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>DAE9 (7)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>DAE10 (8)</td>
</tr>
</tbody>
</table>

To decrease the number of background mutations, representative alleles of each complementation group were backcrossed by the parental wild-type strain MGY159A at least three times. Tetrads from each outcross were analyzed for growth under osmotically stressed conditions and shown to display segregation of 2 Osm^S:2 Osm^R in all complete tetrads. For each mutant, at least 5 tetrads from the final outcross were assayed for high osmolarity-induced glycerol accumulation. Cosegregation of depressed intracellular glycerol and Osm^S was seen in all cases. Heterozygous diploids carrying hog3 displayed
a dominant sporulation defect removing the opportunity for backcrosses of available alleles. *hog3* was therefore not further evaluated within the scope of this study.

**Growth Curves:**

To evaluate cell growth under normal and osmotically stressed conditions, comparative growth curves were performed on representative backcrossed alleles from each complementation group and the parental wild-type strain. Cultures were grown in YEPD or YEPD + 0.5 M NaCl and the cell density measured every 2 hours for 48 hours. As shown in Figure 2-4a, all mutants grew similar to wild-type in YEPD. *hog2-1* reached log phase several hours later than the other strains but displayed a similar maximal growth rate. Following the shift to high salt media, wild-type cells grew normally while each of the mutants displayed only minimal growth. These results establish that the growth defect of the three *hog* mutants does not affect growth rates in rich media and almost eliminates growth under osmotically stressed conditions.

**Cloning of *HOG1*, *HOG2* and *HOG4:***

The cloning of *HOG1*, *HOG2*, and *HOG4* was accomplished using representative alleles of each complementation group. NDY2 (*hog1-1 ura3-52*), NDY12 (*hog2-1 ura3-52*), and JBY1 (*hog4-1 ura3-52*) were each transformed with a yeast genomic, single copy plasmid library (Rose, 1987). The whole cell transformation protocol (Ito, 1983) was found to work effectively with *hog1-1* and was thus used to clone the genomic locus of *HOG1*. *hog2-1* and *hog4-1* each gave only low numbers of transformants using the
Figure 2-4: Growth of wild-type, hog1-1, hog2-1 and hog4-1 under osmotically stressed conditions. Cells were grown to log phase in YEPD, diluted into YEPD + 0.5M NaCl, and shaken vigorously at 30°C. Cell density was determined every 2 hours for 48 hours by measurement of $A_{600}$. The wild-type strain MGY159A grew normally under both conditions while each hog mutant grew only minimally. No significant growth was observed after 36 hours. Strains were MGY159A (wild-type), NDY2 (hog1), NDY12 (hog2), and JBY1 (hog4).
whole cell procedure and therefore the spheroplast method of Beggs (1978) was employed. All Ura\(^+\) colonies (~10\(^5\) for each mutant) were screened for rescue of the Osm\(^8\) phenotype through analysis of growth on media containing 0.9M NaCl. Several Osm\(^R\) colonies, carrying putative complementing DNA, were isolated for each of the mutants and retained for further analysis.

**Confirmation of Clones:**

Confirmation of Ura\(^+\)Osm\(^R\) colonies as carrying *bona fide* complementary plasmid DNA was accomplished in a stepwise manner. Mitotic coininstability, which evaluates segregation of Ura\(^+\) and Osm\(^R\) phenotypes was first employed. By growing transformants on YEPD for several days, selection for plasmid maintenance is relaxed. Analysis of these cells by streaking out for single colonies on YEPD, and printing to uracil depleted media reveals 10-50\% of the colonies were Ura\(^-\) and have lost the plasmid. If the plasmid carries complementing DNA, analysis of individual colonies will reveal that every Ura\(^-\) colony is also Osm\(^8\). Reversion of the original mutation will result in all Osm\(^R\) colonies regardless of plasmid (Ura) segregation. Putative clones for each of the complementation groups displayed cosegregation of Ura\(^+\)Osm\(^R\) and Ura\(^-\)Osm\(^8\) phenotypes.

Plasmid DNA was isolated from yeast colonies that had passed mitotic coininstability analysis and transformed into the bacterial strain HB101 according to the method of Hoffman and Winston (1987). Following amplification in bacteria, each
putative clone was retransformed into the original yeast mutant to determine if the plasmid again rescued the $Osm^6$ phenotype. Plasmids which conferred $Osm^6$ were retained and a restriction map of the genomic DNA was determined for the major restriction enzymes. Multiple clones for the same mutant were evaluated to look for overlapping insert regions which could speed the subcloning process.

A 9.6 kb fragment of genomic DNA was isolated and shown to complement both the $Osm^6$ phenotype and the reduced glycerol response of $hog1-1$. The restriction map of the cloned fragment is shown in Figure 2-5A. To locate the complementing gene, restriction fragments were cloned into the centromere plasmid pRS316 (Sikorski and Hieter, 1989), and individually tested for complementation of the $Osm^6$ phenotype of $hog1-1$. Nested deletions were also used to remove DNA from the right-hand-side of the clone as drawn. The smallest subclone to carry complementing activity was a 2.1 kb $Eco$ RI-Sau 3A fragment. Results from Northern blot analysis indicated the transcript from $HOGL$ was ~1.4 kb size (not shown). This information combined with the inability of a slightly smaller 1.9 kb nested deletion fragment to rescue the $Osm^6$ indicated the 2.1 kb subclone to be very close to the borders of the $HOGL$ coding region.

Four putative $HOG2$ clones were isolated which resulted in complete phenotypic complementation. Restriction analysis revealed each of these clones to contain similar 11 kb inserts of genomic DNA. As shown in Figure 2-5B subclones were constructed in pRS316, transformed into $hog2-1$ cells, and tested for complementation of the salt
Figure 2-5: Restriction maps and subclones of complementing genomic DNA. Different fragments of genomic DNA were found to complement the Osm$^S$ phenotype of each hog complementation group. + indicates full complementation of the Osm$^S$ phenotype of the hog mutant; ±, partial complementation; -, no complementation. Smaller restriction fragments of each original clone were introduced into pRS316, transformed into a hog mutant, and the transformants tested for growth on plates containing YEPD + 0.9 M NaCl. Abbreviations of restriction enzymes include; B, Bam HI; Bg, Bgl II; C, Cla I; H, Hind III; Hp, Hpa I; K, Kpn I; N, Nhe I; P, Pst I; R, Eco RI; Rv, Eco RV; S, Sal I; Sc, Sac I; Sp, Spe I; Su, Sau 3A; and X, Xba I.
HOGL

Su  P  SR  PR  C  R  K  Hp  Sc  R  ClpP  Hr  K  H  Rbg  K  Hp  Su

HG2

Su  PvK  P  R  Sp  PvSc  Rv  C  SH  SpPv  H  X  Pv  Bg  P  Pv  Su

HOGL4

Su  C  Sp  C  H  Rv  H  Rv  Sc  Rv  X  KR  A  PCSc  Su

900 Na
Growth

+ 
+ 
+ 
+ 
- 
+ 
+ 

HOG2

Su  Bg

HOG4

Su  Bg

1 kb
sensitivity phenotype. The smallest positive subclone was the 2.8 kb Bgl II-Sal I fragment. Fragments missing 400 bases off the Sal I end or 500 bases off the Bgl II end failed to complement the mutation. Information from northern analysis indicated the transcript to be ~3 kb in size (not shown). The combination of the above data indicate that HOG2 is contained on the Bgl II-Sal I fragment.

Four putative HOG4 clones were generated, with only one being transferred successfully into HB101 for further analysis. The plasmid was found to contain a small 6.5 kb insert of genomic DNA with complementing capacity. A combination of subcloning and nested deletions yielded a 3.4 kb Kpn I-Spe I segment of complementing DNA as shown in Figure 2-5C. Deletion of 600 bases from the Spe I end results in a loss of complementing capacity.

Genetic linkage of cloned fragment and point mutation:

Integrative confirmation of the genetic linkage between the cloned DNA and the locus of the original point mutation was performed for both HOG1 and HOG4. Each gene was subcloned into the integrative vector pRS306 (Sikorski and Hieter, 1989). The plasmids were restriction digested a single time in the middle of the inserted gene to yield a linear fragment of DNA carrying the URA3 in the middle and half the gene on each end. In yeast this linear fragment of DNA will integrate into the chromosome at the locus of the genomic DNA sequences (recombination guided by homology between fragment and chromosome). HOG1 and HOG4 integrative transformants now carried a
marker at the locus of the cloned gene.

The purpose of generating these strains is to map the genetic distance from the cloned DNA (now marked with \textit{URA3}) to the point mutation generating the Osm$^5$ phenotype. The method relies upon recombination or crossing over of chromosomes during meiosis as diploid cells sporulate. Transformants are mated, the diploid cells sporulated, and the resulting tetrads (four haploid cells/tetrad) evaluated by analysis of auxotrophic markers. Tight genetic linkage between the integrated marker and the related point mutation indicates the cloned fragment is the gene or is in close proximity to the gene. Mating the strain containing the integrated \textit{URA3} by either wild-type Ura$^+$ or the related Ura$^-$ \textit{hog} strain yielded results confirming genetic linkage in both cases (see Materials and Methods). Figure 2-6 displays segregation from the \textit{HOG4} integration and mating. Mating the integration carrying strain by a Ura$^+$ wild-type yielded all Osm$^R$ tetrads and non 4:0 Ura segregation, confirming that the marker was not inserted at the locus of the \textit{URA3} gene. Mating the integration by the Ura$^-$ \textit{hog} strain resulted in 2 Osm$^R$Ura$^+$:2 Osm$^5$Ura$^-$ segregation in all complete tetrads. These results indicate that the cloned \textit{hog1} and \textit{hog4} complementing DNA represent genes that are tightly linked to the original \textit{hog1-1} and \textit{hog4-1} mutations and are likely \textit{HOG1} and \textit{HOG4} themselves rather than unlinked suppressor genes. Similar experiments were later carried out on the \textit{HOG2} clone resulting in similar tight linkage between \textit{hog2-1} and the cloned DNA (Nye et al, 1993).
Figure 2-6: Identification of genetic linkage between hog4-1 and the cloned fragment of genomic DNA. Tetrad analysis of marker segregation following integration of a plasmid containing HOG4 and URA3 at the locus of the hog4-1 mutation (transformation into JBY2). The marked strain was then mated by two strains JBY1 (hog4-1 ura3-52) and MGY159A (HOG4 URA3), the diploids sporulated and the subsequent tetrads analyzed for marker segregation. Results identify the cloned HOG4 DNA to be genetically linked to the original hog4-1 mutation (details in text). Media was YEPD, YEPD + 0.9M NaCl, and uracil drop out (uracil depleted minimal media).
**Chromoblot analysis:**

The chromosome on which each of the three genes are located was determined using the chromoblotting technique described by Chu et al (1986). Yeast chromosomes were separated electrophoretically using a pulsed field system, stained with ethidium bromide, photographed, and transferred to nylon membrane. The membrane was then probed with $^{32}\text{P}$-radiolabelled fragments of each of the cloned genes. The washed filter was exposed to autoradiographic film overnight at $-70^{\circ}\text{C}$. The three genes were found to be located on three different chromosomes. As shown in Figure 2-7, $HOG1$ is located on chromosome XII, $HOG2$ on chromosome IV, and $HOG4$ on chromosome X.

**Chromosomal locus of $HOG1$ detected using cosmid "dot" blots:**

Determination of the chromosomal location of cloned genes can be accomplished using genetic linkage mapping (Mortimer et al, 1989), or probing of grid filters which have recently been developed and contain 96% of the yeast genome on lambda and cosmid clones (Link and Olson, 1991). Linkage mapping is very time consuming and problematic, therefore the filter grid system was employed. Radiolabelled probes were generated from $HOG1$ DNA and used to probe each of the three filter grids. Hybridization was detected on two out of one thousand clones on the grids. Evaluation of the clones detected revealed they were overlapping clones from chromosome XII, located near the $GAL2$ locus. $HOG2$ sequence data revealed close proximity of the gene to a previously cloned gene, $PPH2$, located on chromosome IV (Nye et al, 1993). $HOG4$
was determined to be a previously cloned gene (see below) which had been localized to chromosome X by chromoblotting, and between *INO1* and *ARG3* by genetic linkage analysis (Boguslawski et al, 1987). Cosmid "dot" blots were therefore not performed for either gene.

**Generation of Disruptions/Deletions:**

To better characterize the functions of the three genes, null mutants of each were generated by replacing the chromosomal gene with an in vitro mutated gene. In *S. cerevisiae* cloned genes can be used to direct a recombination event that will disrupt or delete the gene at its chromosomal locus (Figure 2-8). Markers are used to disrupt or replace the coding sequence, the construction is then cut out of the plasmid to yield a linear fragment with sequences encoding genomic DNA on each end and carrying a genetic marker (Rothstein, 1991). The fragment is used to transform wild-type yeast by selecting for the inserted marker. The fragment is not stable in the nucleus without integrating into the chromosomal DNA, an event directed by homologous recombination between the ends of the construction with the matching wild-type DNA.

Disruption/deletion mutations of *HOG1*, *HOG2*, and *HOG4* were constructed in a wild-type strain by replacing large fragments of each gene with an auxotrophic marker. A *TRP1* (1 kb) marker was inserted into the *Bgl I-Sal I* sites of *HOG1* (Fig 2-9A), replacing 400 bp of the open reading frame, and eliminating ~35% of the gene (sequence data - see below). *HOG2* was disrupted by inserting *URA3* (1.8 kb) into the
Figure 2-7: Chromoblots of \textit{HOG1}, \textit{HOG2}, and \textit{HOG4}. Yeast chromosomes were isolated from MGY159A, separated electrophoretically, and transferred to nylon membrane. $^{32}$P-labelled probes were used to probe strips of the membrane containing 1-2 lanes of yeast chromosomes. $^{32}$P-labelled probes were generated from fragments of the cloned \textit{HOG1} or \textit{HOG4} DNA isolated from agarose gel slices. The \textit{HOG2} probe was generated from random $^{32}$P-labelling primed off of an entire plasmid (\textit{HOG2} subclone on YCp50), which contains two other yeast sequences, \textit{CEN/ARS} (400 bp), and \textit{URA3} (1500 bp). This resulted in low level detection of several chromosomes (\textit{CEN/ARS} fragments), and strong hybridization to chromosome V (\textit{URA3}). Following autoradiography, the distance of identified bands from the gel origin was measured and compared with a photograph taken of the agarose gel. \textit{HOG1} was shown to be located on chromosome XII, \textit{HOG2} on chromosome IV, and \textit{HOG4} on chromosome X. In each case the chromosomal locus of the cloned fragments were confirmed using other genetic techniques (see text).
Figure 2-8: One step gene disruption by homologous recombination. Figure adapted from Rothstein (1991). An auxotrophic marker (HIS3) is introduced into the gene of interest (GENEZ) replacing a significant section of the coding region. The construction is then restriction digested on each side of the marker and used to transform a wild-type (his3GENEZ) yeast strain. The genomic sequences guide a recombination event that results in replacement of the chromosomal locus of the gene of interest with the construction (now HIS3geneZΔ). Transformants are isolated for on selective media (histidine depleted media) and the recombination event confirmed through Southern blot analysis.
GENE Z

GENE Z

GENE Z

Rothstein, 1991
Figure 2-9: Construction of disruption mutations in the HOG genes. Disruptions of each gene were constructed by replacing internal fragments of the complementing DNA with the marker shown. A) The 0.4 kb Bal I-Sal I fragment (sites marked v in Figure 2-12), containing part of the consensus kinase domain of HOG1 (Figure 2-13), was replaced with the TRPI marker to create hog1-Δ1. B) HOG2 was disrupted by the replacement of the 0.5 kb Eco RI-Sal I fragment with URA3, creating hog2-Δ1. C) HOG4 was disrupted by the replacement of the 2.9 kb Hind III-Hind III fragment with URA3, creating hog4-Δ1. The construction was digested with restriction enzymes which cut in the genomic DNA on each side of the marker and used to transform the wild-type diploid YPH501. Trp+ or Ura+ transformants were sporulated and subsequent tetrads analyzed. In each case, tetrads showed a 2:2 cosegregation of the Trp+ or Ura+ phenotype with Osm8.
Eco RI-Sal I sites, replacing 500 bp near the N-terminus and deleting ~20% of the
coding sequence (Fig 2-9B). A larger disruption, deleting 50% of HOG2 was later
generated and shown to be phenotypically indistinguishable (Nye et al, 1993). A near
complete deletion of HOG4 was generated (Fig 2-9C), replacing a 1.6 kb Hind III-Hind
III fragment containing 95% of the coding sequence with URA3 (1.2 kb). Detailed
descriptions of each of the plasmid constructions is included in the Materials and Methods
section. Each plasmid was restriction digested to yield a linear fragment of DNA with
 genomic sequences bordering each end of the selected marker. The fragment was then
introduced into a wild-type diploid strain YPH501, selecting for transformants by
detection of the auxotrophic marker. Transformants were sporulated on nitrogen depleted
media, tetrads dissected (12/transformant), and the subsequent haploids analyzed for both
 marker segregation and osmotic sensitivity. Disruption constructions yielded 2:2
segregation of the respective marker and cosegregating Osm⁸ phenotypes. Successful
integration of the disruption construction results in a predictable change in the restriction
site pattern over that region. Probes were generated with fragments of each clone and
used to probe southern blots of the transformants. All three of the gene disruptions were
confirmed by this analysis (data not shown).

**Determination of Intracellular Glycerol in Disruption Alleles:**

The three disruption mutants were analyzed phenotypically to identify the
osmoregulatory deficiencies upon elimination of the genes. Total cell extracts were
prepared from cells grown in YEPD and YEPD + 0.4M NaCl. As shown in Figure 2-
10, the wild-type strain YPH98, a haploid derived from YPH501 (see Table 4), was assayed for glycerol and shown to accumulate 8-times more glycerol following an hour in high salt media. hog1Δ and hog4Δ each displayed depressed glycerol accumulation under osmotically stressed conditions. In rich media, both showed normal basal levels of glycerol but following salt stress showed only a 3-fold increase in glycerol accumulation. hog2Δ displayed slightly higher levels of glycerol following salt stress, but still significantly less than wild-type cells. Mutants carrying both hog1Δ and hog4Δ disruption alleles displayed glycerol accumulation results similar to either disruption alone. However, combining hog2Δ with either hog1Δ or hog4Δ resulted in dramatically lower glycerol accumulation than any of the single mutants.

Osmotic-sensitive growth:

Analysis of cell growth under various stresses was also analyzed for each of the disruption alleles. Strains were grown in YEPD to log phase, and the cell density of each culture equalized by spectrophotometric measurement (A600) and dilution into YEPD. Equal aliquots of the cultures were then spotted onto each plate, and the plates incubated at 30°C for 36 hours before being photographed (Fig. 2-11). Cell growth can be evaluated visually with normal growth (see 0.1M NaCl) generating round white patches of cells. The results correlated with the glycerol assays (lower glycerol accumulation corresponding with greater osmotic sensitivity). Observation of the single mutants reveals hog1Δ and hog4Δ to be slightly more osmotically sensitive than hog2Δ. Double mutants including hog2Δ are much more sensitive than any of the single mutants or the
Figure 2-10: Disruption alleles display depressed glycerol accumulation. Deletion mutations in the *HOG* genes cause a reduction in osmotic-stress induced glycerol accumulation. Intracellular glycerol levels following a 1 hr incubation of cells in either YEPD or YEPD + 0.4 M NaCl. Glycerol determined as described in Methods. Strains used were: YPH499, JBY10 (hog1Δ), JBY20 (hog2Δ), JBY40 (hog4Δ), JBY512 (hog1Δhog2Δ), JBY514 (hog1Δhog4Δ), and JBY524 (hog2Δhog4Δ).
Figure 2-11: Disruption alleles are osmosensitive. Deletion mutations in the HOG genes cause an osmotic-sensitive growth defect. Double mutants containing hog1Δ or hog4Δ in combination with hog2Δ display an enhanced Osm⁸ phenotype. Growth of wild-type (YPH499) and hogΔ mutants after spotting 2 x 10⁷ cells of each on YEPD, YEPD + 0.4 M NaCl, or YEPD + 0.9 M NaCl. The plates were photographed after 36 hours at 30 °C. Strains are identical to those listed in legend to Figure 2-10.
hog1Δhog4Δ double mutant. Comparisons of the hog1Δhog4Δ double mutant with either hog1Δ or hog4Δ reveal each of the strains to phenotypically identical. However, double mutants of hog2Δ with either hog1Δ or hog4Δ results in amplification of associated phenotypes. These data indicate that HOG1 and HOG4 are acting through a similar mechanism whose action is eliminated following the deletion of either gene. HOG2 likely has a role in addition to that played by HOG1 and HOG4 in the regulation of cellular responses to osmolarity.

Sequence Determination of the HOGS:

A 2.5 kb Bam HI- Cla I fragment of genomic DNA carrying hog1-1 complementing activity was sequenced on both strands and is displayed in Fig 2-12. Within this DNA, an open reading frame of 1.2 kb was detected that has the potential to encode a protein 416 amino acids in size (47 kDa protein). Homology searches through GenBank showed HOG1 to display striking sequence similarities (~50% identity) to FUS3 and KSS1, two genes encoding protein kinases involved in the mating response pathway (Elion, 1990). The NH2 half of HOG1 has each of the amino acid residues conserved in known protein kinases (Hanks et al, 1988). The central portion of the gene encodes residues found in a subclass of important proteins termed mitogen-activated protein kinases (MAP kinases) which are described in Chapter III. Figure 2-13 displays sequence homology shared between HOG1, FUS3, KSS1, and the rat MAP kinase ERK1. Sequence comparisons generally reveal MAP kinases to show ~40% identity to each other and ~30% identity to other protein kinases that contain residues necessary for ATP
binding and catalysis.

Sequence analysis and further characterization of HOG2 was performed (Nye et al, 1993), and HOG2 was found to be a previously identified gene TPS2, encoding a subunit of the enzyme trehalose synthase (de Virgillo et al, 1993). Further phenotypic characterization of the gene revealed HOG2 to most likely be involved in general stress responses. What role HOG2 plays in glycerol synthesis following salt stress is not yet clear. In this study, subsequent experiments involving HOG2 will primarily employ the disruption allele as a control.

HOG4 was also determined to be a previously identified gene, PBS2 (Brewster et al, 1993). Restriction maps of the two genes were identical and both clones were localized to chromosome X. Subsequent transformation of hog4-1 cells with a single copy plasmid containing PBS2, obtained from G. Boguslawski, resulted in complementation of all associated phenotypes. Partial sequence analysis of HOG4 revealed 100% sequence identity with PBS2, confirming that the two genes were indeed identical. PBS2 was originally cloned as a gene conferring resistance to the antibiotic polymyxin B when overexpressed in wild-type yeast cells (Boguslawski, 1987). Polymyxin B is thought to kill sensitive cells by disruption of the cell membrane (lower doses affect membrane permeability of some drugs). How overexpression of PBS2 affects polymyxin B sensitivity is not known. Within the sequenced region, a 2100 bp open reading frame was identified which translates into a putative 78 kDa protein.
Sequence comparisons revealed PBS2 to display strong homology to protein kinases (Table 2), most notably STE7, wis1 and byrl. Both STE7 and byrl kinases appear to regulate MAP kinases located in the S. cerevisiae and S. pombe mating response pathways (Zhou et al, 1993, Toda et al, 1991). wis1 is a second S. pombe protein kinase with strong homology to PBS2, and is thought to play a role in signalling nutrient levels in the growth media (Warbrick and Fantés, 1991). Two recently isolated kinases in the protein kinase C pathway, MKKI and MKK2, show 38% identity to PBS2 over a 298 amino acid region (Irie et al, 1993, see Figure 1-2). These proteins are grouped as MAPK kinases, or MAP activators which are thought to play a role in the activation of MAP kinases by direct phosphorylation (Anderson et al, 1990). The substrate for STE7 has been shown to be FUS3 (Cairns et al, 1992), a MAP kinase with strong homology to HOG1.

The sequence data point to the exciting prospect that a kinase mediated signal may be the result of salt stress and that the proteins involved may carry strong homologies to the mating response pathway. As described in the introduction, the mating response pathway is fairly complex. Identified thus far are at least 20 genes including a seven-transmembrane segment membrane receptor, multiple kinases, and downstream targets affecting mating competence, cell cycle arrest, cell morphology, and recovery from mating (for review: Sprague and Thorner, 1992). Osmotic stress may induce a pathway of similar complexity with multiple downstream targets effecting cell physiology and a "core" MAP kinase cassette involving several protein kinases.
Figure 2-12: Sequence determination of \textit{HOG1}. Nucleotide and predicted amino acid sequence of \textit{HOG1}. The \textit{Bal} I and \textit{Sal} I sites marked with \( \triangledown \) border the fragment replaced in the construction of \textit{hog1} \( \Delta \).
Figure 2-13: The HOG1 protein shows homology to FUS3, KSS1, and ERK1.

Conserved subdomains present in all protein kinases indicated by Roman numerals (Hanks 1988). FUS3, KSS1, and ERK1 are aligned as shown in Boulton et al. (1991). HOG1 was visually aligned, and gaps (represented by dashes) introduced to improve alignment. Identities and conservative substitutions (Dayhoff et al. 1978) are boxed, and residues conserved by all four sequences are labelled. The sequences shown are: HOG1, FUS3 (Elion et al. 1990), KSS1 (Courchesne et al. 1989), and ERK1 (Boulton et al. 1990).
Table 2: The *HOG4*/PBS2 protein encodes a putative MAPK kinase. Amino acid sequence identity over the kinase domain of *HOG4*/PBS2 to *STE7*, *wis1*, and *byr1* determined from published sequences (Warbrick and Fantes, 1991).
### Table 3: STRAIN LIST

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Materials and Methods: II

Media:

Yeast strains used in this study are listed in Table 3, diploid wild-type and hog-strains used in Chapters 3 and 4 are congenic to the listed haploid strains (JBY13/JBY13 = JBY13D). YEPD media (non-selective rich media) contained 1% yeast extract, 2% bactopeptone, 2% dextrose, and 20 μg/ml adenine sulfate. Osmotic sensitivity was tested on YEPD agar plates containing 0.9 M NaCl, 1.2M KCl, or 1.8M sorbitol. YEPG and YEPE plates are as YEPD except the 3% glycerol or 2% ethanol, respectively, were supplied as the carbon source in place of dextrose. Transformants were selected on synthetic complete (SC) media lacking uracil or tryptophan. Other media was prepared as described by Rose et al (1990). Transformation of yeast was performed using either the LiCl (Ito et al. 1983) or the spheroplast (Beggs 1978) procedure. Yeast genomic DNA for Southern hybridizations was isolated as described in Rose et al. (1990).

E. coli strains HB101 or SURE™ (Stratagene) were used for plasmid amplification. Standard techniques for plasmid DNA isolation from E. coli, restriction analysis, DNA ligations, etc. were as described (Sambrook et al, 1989). ³²P-labelled DNA probes for Southern analysis and chromoblots were generated by the random hexamer priming method (Feinberg and Vogelstein, 1983).

Mutagenesis and Initial Mutant Screens:

The following procedure was used to isolate mutants defective for growth in high
osmolarity media (Osm$^5$) and for high osmolarity-induced accumulation of glycerol. Two-day-old yeast cultures in YEPD were mutagenized with ethyl methanesulfonate (EMS) (Lindegren et al., 1965) to 30% survival and spread onto YEPD plates. Strains used for mutagenesis were MGY159A, MGY159B, YPH98, and YPH102. Colonies grown from mutagenized cells were tested by replica-plating for growth on NaCl and sorbitol plates. Mutants which failed to grow on both plates were deemed Osm$^5$. Before assaying Osm$^5$ strains for high osmolarity-induced glycerol accumulation, we eliminated Osm$^5$ mutants with a defect in mitochondrial function (petites) and where possible those with defects in the fidelity of protein synthesis (translational suppressors). Petite mutants were identified by their failure to grow on ethanol (YEPE) and glycerol (YPEG), two non-fermentable carbon sources. Putative translational suppressors were identified in the YPH98/YPH102 background by suppression of the lys2-801$^{amber}$ or ade2-101$^{ochre}$ mutations.

The remaining 95 Osm$^5$ mutants were assayed for high osmolarity-induced glycerol accumulation (see below), the "glycerol response". The 20 Osm$^5$ strains with a reduced glycerol response were termed the hog mutants. Each hog mutant was mated with the parental wild-type strain, prototrophic diploids isolated on minimal media, and the osmotic sensitivity of the diploids tested on NaCl plates. All heterozygous diploids grew as well as an Osm$^6$ wild-type homozygous diploid on NaCl plates, showing that each hog mutant was recessive. Each of the hog mutants were mated with each other and the resulting diploids tested for growth on NaCl plates. Two mutants were assigned to
the same complementation group if the diploid strain derived from the mating of the two mutants was Osm⁸. All 18 hog mutants fell into one of four complementation groups: hog1, hog2, hog3, and hog4. Representative mutants from the hog1, hog2, and hog4 complementation groups were backcrossed to MGY159B at least three times. The Osm⁸ phenotype segregated 2 Osm⁸:2 Osm⁸ in all complete tetrads. For each mutant, at least 5 complete tetrads from the final backcross were assayed for high osmolarity-induced glycerol accumulation. In each tetrad, the two Osm⁸ progeny had lower glycerol accumulation than the two Osm⁸ progeny. In crosses of hog1 with hog2, hog1 with hog4, and hog2 with hog4, approximately 1 out of 6 tetrads in each case had a non-parental ditype (2 Osm⁸:2 Osm⁸). This ratio is that expected for unlinked mutations.

Measurement of Intracellular Glycerol:

The culturing of cells and the preparation of cell extracts for measurement of intracellular glycerol followed the procedures used by Blomberg and Adler (1989). A log-phase yeast culture containing 2 x 10⁷ cells in 2 ml was diluted with 8 ml of either YEPD or YEPD plus 500 mM NaCl (final concentration of NaCl = 400 mM). After a 30°C incubation for 1 hr, cells were collected by centrifugation, and then resuspended in an isotonic saline solution (135 mM NaCl at 242 mOsm was isosmotic with YEPD, 600 mM NaCl at 1003 mOsm was isosmotic with YEPD plus 0.4 M NaCl, all determined by osmometer). Cells were washed 1X by centrifugation in the isotonic NaCl solution. The final pellet was resuspended in 500 µl of boiling distilled H₂O and boiled in a tightly-capped tube for 15 minutes. An aliquot of the boiled sample was transferred
to a microfuge tube and frozen at -20°C. Immediately prior to assay, samples were allowed to thaw at room temperature and centrifuged and 13,000 rpm for 2 min to remove insoluble debris.

The quantitation of glycerol was performed using a two step colorimetric assay that was a modification of that described by Frings and Pardue (1966). The assay mixture in a 1 ml cuvette contained 0.83 U of glycerol dehydrogenase (Sigma G3512), 680 µM nicotinamide adenine dinucleotide (NAD$^+$), 0.22 U of diaphorase (Sigma D2381), 20 µM 2,6-dichlorophenolindophenol (DCPIP) and 27.5 mM potassium phosphate (pH 7.0). Reaction was started by adding sample and the rate of absorbance change at 600 nm measured. In this assay, the glycerol dehydrogenase catalyzes the oxidation of glycerol and the reduction of NAD$^+$ in a coupled reaction. The NADH formed by this first reaction is oxidized by diaphorase in a reaction coupled to the reduction of DCPIP. The oxidized form of this dye is blue, the reduced form is colorless.

\[
\text{glycerol} + \text{NAD}^+ \xrightarrow{\text{glycerol dehydrogenase}} \text{dihydroxyacetone} + \text{NADH} + \text{H}^+
\]

\[
\text{NADH} + \text{H}^+ + \text{dye}_{(\text{ox.})} \xrightarrow{\text{diaphorase}} \text{NAD}^+ + \text{dye}_{(\text{red.})}
\]

The rate of dye reduction and thus the rate of glycerol oxidation was measured by determining the decrease in absorbance at 600 nm over time. The initial rate of dye
reduction was a linear function of glycerol concentration in the range between 25-250 μM glycerol. Rates were determined for each sample and concentrations of glycerol calculated using a standard curve.

Linkage analysis of *HOG1* and *HOG4*:

Phenotypic complementation of a recessive mutation by a fragment of genomic DNA (in a single copy plasmid), generally indicates one of two possibilities. Either the genomic clone contains the wild-type copy of the mutant gene or it contains a gene from another locus which when present at more than one copy (1 chromosomal + 1 plasmid copy) suppresses the mutant phenotype. To distinguish between these two possibilities, we took advantage of the high frequency of homologous recombination to test whether an integrated copy of complementing DNA is genetically linked to the mutation (Rose and Broach, 1991).

To test whether the 5.5 kb *Hind III-Bgl II* fragment of complementing genomic DNA contains the wild-type *HOG1* allele, the fragment was ligated into the *Bam HI-Hind III* sites of an integrating yeast vector, pRS306 (*URA3*, Sikorski and Hieter, 1989). The new plasmid was linearized by cutting at the Hpa I site of the genomic DNA and used to transform a *hog1-1 ura3-52* (NDY2) strain. Ura+ OsmR transformants now had *URA3* tightly linked to an extra copy of the complementing fragment at its chromosomal locus. If the cloned DNA represents the gene itself, the integration event also links the
wild-type copy of the gene \((HOG1 \ URA3)\) to the original allele \((hog1-I)\). This allows for genetic analysis of linkage between \(hog1-I\) (Osm\(^8\)) and the cloned gene (Osm\(^R\)), or between \(hog1-I\) and \(URA3\). Two transformants were crossed with the wild-type strain MGY159A (Ura\(^+\)Osm\(^R\)), the diploid cells sporulated by printing to nitrogen depleted media, and the resulting tetrads analyzed. In the 23 complete tetrads from these two crosses, only one haploid progeny was Osm\(^8\), indicating tight linkage of the complementing DNA to \(hog1-I\). In the 8 complete tetrads from a genetic cross between one transformant and NDY2, only the parental ditype \((2:2\ Ura^+Osm^R:Ura^-Osm^8)\) was observed. The tight linkage between \(HOG1\) and \(URA3\) genes are consistent with integration of the plasmid by homologous recombination at the \(hog1-I\) locus.

To test whether the 5.5 kb \(Sal\ I-Spe\ I\) fragment of complementing genomic DNA genomic clone contains the wild-type \(HOG4\) allele, the fragment was subcloned into the \(Sal\ I-Spe\ I\) sites of pRS306. The new plasmid was linearized by cutting at the unique \(Eco\ RI\) site of the insert and used to transform a \(hog4-1\ ura3-52\) strain (JBY1) to Ura\(^+\) Osm\(^R\). Two transformants were crossed with the wild-type strain MGY159A (Ura\(^+\)Osm\(^R\)). In 12 complete tetrads from these two crosses, there were no Osm\(^8\) progeny, indicating tight linkage of the complementing gene to \(hog4-I\) (see above). In 11 complete tetrads from a genetic cross between one transformant and JBY1, only the parental ditype \((2:2\ Ura^+Osm^R:Ura^-Osm^8)\) was observed. The tight linkage between \(HOG4\) and \(URA3\) genes are consistent with integration of the plasmid by homologous recombination at the \(hog4-I\) locus.
Generation of disruption/deletion mutants:

The plasmid used for creating a hgl disruption allele was constructed by replacing the ~400 bp Bal I-Sal I fragment containing the first half of the coding sequence of HOGI with a ~900 bp Sal I-Sma I fragment from pJJ280 (Jones and Prakash, 1990) containing the TRPI marker. Transformation of the diploid strain YPH501 strain was accomplished by digestion of the construction with Sca I and Cla I to induce replacement of the chromosomal locus of HOGI with the hgl-Δ1 construction (see text for description of gene replacement). Trp+ transformants were sporulated, tetrads dissected, printed to tryptophan depleted, NaCl, sorbitol, and YEPD plates. In each tetrad 2:2 segregation of Trp+ Osm5 was observed. The Osm5 phenotype of the disrupted strain was complemented by HOGI on a centromere plasmid. Southern analysis of the disrupted strain and YPH501 using the HOGI sequence as the probe confirmed that gene replacement had occurred as predicted. Genetic linkage between the point mutation hgl-Δ1 and the disruption (HOGI::TRPI) was established by mating strains containing the two Osm5 mutations and analyzing subsequent tetrads. Twelve complete tetrads were dissected, each showing 4:0 segregation of Osm5 to OsmR and a 2:2 segregation of Trp+ to Trp-.

To create a deletion/disruption mutant of HOG2, the following steps were taken. A 3.9 kb Cla I-Xba I fragment containing part of HOG2 was inserted into pGEM-7Zf+ (Promega). A 0.5 kb Eco RI-Sal I fragment of the hog2-complementing DNA was deleted and replaced with a 1.8 kb Eco RI-Sal I fragment from YEp24 (Carlson and
Botstein 1982) containing URA3. The URA3-marked deletion mutation of HOG2 was excised from the plasmid with Sac I-Hpa I and used to transform YPH501. Ura+ transformants were selected and sporulated. Dissected tetrads showed 2:2 segregation of Osm8 and Ura+ with all Ura+ colonies being Osm8. Southern blot analysis of the disrupted strain and YPH501 confirmed the disruption of the chromosomal HOG2 locus.

The following procedure was used to disrupt HOG4. A 5.5 kb Sal I-Spe I fragment of hog4-1 complementing genomic DNA was inserted into the Sal I-Spe I sites of pRS306 (Sikorski and Hieter, 1989). A 2.9 kb Hind III-Hind III fragment was replaced with a 1.2 kb fragment from YEpl4 containing URA3 (deletes 95% of the HOG4 coding DNA from the plasmid - see text). The construction was digested with Sac I and Spe I to generate a linear fragment containing URA3 with 1.5 and 0.5 kb of HOG4 locus genomic DNA on each side. The fragment was then used to transform the wild-type diploid strain YPH501. Ura+ transformants were sporulated, tetrads dissected and tested for growth properties and glycerol accumulation. Cosegregation of Osm8 and Ura+ was seen in all tetrads analyzed. The Osm8 of the putative hog4-ΔI was rescued by the cloned HOG4. Southern analysis of the disrupted strain and YPH501 confirmed the deletion/disruption of the chromosomal HOG4 locus. Tight linkage of Osm8 between hog4-1 and hog4-ΔI was confirmed by genetic cross.

Growth Test:

Overnight cultures of wild-type and mutant strains were grown in YEPD. The
concentration of cells was adjusted to $2 \times 10^7$ cells/ml by dilution with fresh YEPD. From each culture, 8 µl was spotted onto plates containing either YEPD, YEPD + 0.4 M NaCl, or YEPD + 0.9 M NaCl, and incubated for 36 hours at 30 °C. Plates were then photographed using T-MAX 100 film (Kodak).

**Sequence determination:**

The DNA sequence of *HOG1* was determined by Lofstrand Labs (Gaithersburg, MD). Plasmid containing *HOG1* on a 2.5 kb *Cla I-Bam HI* genomic DNA fragment was purified by banding in a CsCl density gradient and the sequence of the genomic DNA fragment determined on both strands by the method of Sanger et al. (1977).
Chapter 3:

Functional Analysis of *HOGL* and *PBS2*

The communication of signals from the cell membrane to the nucleus has long been a focal point of basic cell biology. Extracellular signals play an important role in mammalian cellular growth control as mitogens, such as nerve growth factor, platelet-derived growth factor, and epidermal growth factor bind their respective receptors. Activated receptors autophosphorylate on tyrosine to enable association with a cytoplasmic protein complex (GRB2-mSos). The activated receptor binds to one protein in the complex (GRB2), enabling the second protein (mSos) to bind the GDP-associated protein Ras. mSos then activates Ras by enabling exchange of the GDP for GTP, active Ras is then thought to initiate a MAP kinase-mediated signal transduction pathway ultimately stimulating cell growth (for review, Thomas, 1992, Howe et al, 1992, Marx, 1993).

*Saccharomyces cerevisiae* does not grow in response to mitogens as seen in multicellular systems but does respond to a small peptide pheromone secreted by yeast cells of the opposite mating type (see Chapter 1). A receptor in the cell membrane initiates the downstream cascade which results in control of growth and morphology. Cloning and characterization of the genes involved in mating has revealed a signal transduction pathway with striking similarities to receptor-mediated growth control
pathways in mammalian cells (for review: Sprague, 1992). The internal transfer of signal from the membrane to transcription factors involved in regulation of gene expression is mediated in both systems by MAP (Mitogen-Activated Protein) and MAPK (MAP kinase) kinases. The strong homology of HOG1 to MAP kinases and PBS2 to MAPK kinases fits well with the presumption that they are involved in carrying an osmotically induced signal to the downstream effectors resulting in cellular adjustments required for osmoregulation. We set out to better characterize the role of HOG1 and PBS2 in osmoregulation.

**Hog1p is phosphorylated on tyrosine in response to osmotic stress:**

A hallmark of MAP kinases is activation by phosphorylation of threonine and tyrosine residues carried in a conserved motif (Anderson et al, 1990, Boulton et al, 1991, Gartner et al, 1992, motif described below). The rarity of phosphotyrosine in the yeast cytoplasm has proven useful for detection of activating phosphorylation events on FUS3 (a MAP kinase in the mating response pathway) following treatment of haploid cells with mating factor (Ballard et al, 1991). We set out to evaluate tyrosine phosphorylation of yeast proteins following a shift to high osmolarity media.

Cells containing 0, 1, or multiple copies of HOG1 were grown up to log phase, shifted to high osmolarity media, and the cell proteins then assayed for tyrosine phosphorylation by immunoblot analysis. Figure 3-1A identifies a 47 kDa protein which is tyrosine phosphorylated in response to salt stress. Mobility of the identified band is
identical to that expected for a protein with the predicted molecular weight of Hog1p. The intensity of the band increased dramatically in cells overexpressing \textit{HOGl} while no corresponding band was seen in \textit{hog1Δ} cells. Comparison of extracts from cells grown in rich media and those exposed to a 10 minute 0.4M NaCl salt stress revealed an osmostress dependant increase in tyrosine phosphorylation of the 55 kDa band. Similar results were seen when cells were stressed with 0.7M sorbitol, indicating the tyrosine phosphorylation is activated by osmotic stress, not NaCl only. To determine if the identified band was actually Hog1p, a plasmid was constructed carrying a truncated \textit{HOGl} gene (\textit{hog1-Δ2}: 73 amino acids deleted from the COOH-terminal end). Transformation of a \textit{hog1Δ} strain with the truncation revealed no loss of complementing activity on high salt media. Immunoblot analysis of the truncation in \textit{hog1Δ} cells revealed an osmostress dependant antiphosphotyrosine activity at the lower molecular weight, proving that the identified band is Hog1p. Overexpression of \textit{HOGl} in \textit{pbs2Δ} cells resulted in no detectable tyrosine phosphorylation, though levels of Hog1p expression were shown to be unaffected using antibodies against Hog1p (not shown). These data show Hog1p to be phosphorylated on tyrosine in response to increased external osmolarity and that this phosphorylation event is \textit{PBS2} dependant.

As shown in Figure 3-1B, tyrosine phosphorylation of Hog1p was measured following exposure to increasing concentrations of NaCl in the growth media. Cells were exposed to 0.0-0.6M NaCl in the growth media for 10 minutes, cellular proteins isolated, and tyrosine phosphorylation detected by immunoblot analysis. Phosphorylation was
**Figure 3-1: Osmotic stress induced tyrosine phosphorylation of HOG1.**

Antiphosphotyrosine immunoblot analysis of cells exposed to increased osmolarity.

(A) Comparison of extracts prepared from control cultures (-), and cultures which had been treated with 0.4 M NaCl for 10 min (+). Yeast strains were; wild-type strain YPH499 (WT), *hog1Δ* strain JBY10 (hog1), and *pbs2Δ* strain JBY43 (pbs2). Cells contained the high copy plasmid pRS426 without insert (2μ), with *HOG1* (2μHOG1), or with the C-terminal truncation mutant (2μhog1-Δ2, Brewster et al, 1993). Total cellular proteins were isolated, and immunoblots performed as described in Materials and Methods. The position of p55 is indicated, as is the position of pre-stained molecular weight makers run for comparison.

(B) NaCl concentration dependence of the p55 response. NaCl was added to cultures of the strain YPH499 harboring the 2μ-*HOG1* plasmid at the indicated final concentrations for 10 min prior to immunoblot analysis.

(C) Time course of the p55 response. 0.4 M NaCl was added to a culture of the strain YPH499 harboring the 2μm-*HOG1* plasmid for increased time intervals prior to withdrawal of samples for immunoblot analysis. The length of time from the addition of NaCl to isolation of total cellular proteins is indicated.
found to be salt concentration dependant up to 0.3M NaCl. After an increase in osmolarity, phosphorylation of *HOG1* on tyrosine reached a maximum within 1 minute and maintained for 15 to 20 minutes (Figure 3-1C). Later timepoints revealed a gradual decrease in phosphorylation which stabilized at a level higher than unstimulated cells. Evaluation of Hog1p levels throughout the time course revealed no change in expression following salt stress (see Figure 3-10).

**Production and purification of antibodies against Hog1p:**

To detect the product of the *HOG1* gene, Hog1p, antibodies were generated in rabbits using Hog1p protein expressed in and purified from *E. coli*. The protein coding sequence (950 bp in size) of *HOG1* was amplified using the polymerase chain reaction (PCR). Each primer was engineered with a specific restriction endonuclease cleavage site just outside the start or stop codons. The purified PCR product was restriction digested and ligated into the bacterial expression vectors pET11a and pET15b (Novagen). pET plasmids are engineered to place the inserted gene under bacteriophage T7 transcriptional and translational controls. The bacterial strain BL21 contains a T7 RNA polymerase under an inducible promoter. Upon exposure to isopropyl-β-thiogalactopyranoside (IPTG) in the growth media, the highly active T7 RNA polymerase is expressed and dominates cellular resources for transcription. The only T7 promoter present in the cell controls expression of the gene of interest, thus up to 50% of protein synthesis is of the cloned gene. Protein accumulation usually peaks at 2-5 hours post induction at which time the cells are lysed and the protein purified. Different coding sequences in these
Figure 3-2: Overexpression and purification of Hog1p from bacteria. *HOGI* was subcloned into the bacterial overexpression plasmids, pET11 and pET15 (Novagen). Upon exposure to 1mM isopropyl-β-thiogalactopyranoside (IPTG), a significant fraction of cellular protein synthesis is converted to Hog1p synthesis. Shown are extracts prepared from induced bacterial strain BL21 DE3 pLysS (Novagen) carrying pET11a, pET11a*HOGI* (+ insert), pET15b, or pET15b*HOGI* (+ insert). Following induction, high levels of a protein at the molecular weight of Hog1p are observed. Protein produced from the pET11 construction was purified by electroelution of protein from gel slices. Protein produced from the pET15 construction was column purified using His-Bind™ resin (Novagen). See text for details.
Figure 3-3: Thrombin cleavage of the poly-histidine tag. The bacterial strain BL21 DE3 pLysS, was transformed with a pET15 plasmid carrying HOG1. Genes subcloned into pET15 are put under an inducible promoter, and fused to a NH₂-terminal poly-histidine epitope (enabling column purification). Cells were induced to overexpress Hog1p through exposure to 1 mM IPTG, and the protein purified on a His·Bind⁴ resin column (Novagen). To remove the poly-histidine epitope, the purified protein was digested with thrombin for 30 hours (at 30°C). SDS-PAGE analysis of the digestion revealed ~70% completion.
expression vectors yield different amounts of protein. A common finding is that overexpressed proteins are packaged into inclusion bodies (small insoluble cytoplasmic granules). Proteins can also be found in the cytoplasmic or soluble fraction. Lysates are therefore fractionated, run out on SDS-PAGE gels, and stained to determine the predominant location of the desired protein. HOG1 protein overexpressed from the pET11a vector was most prominent in the inclusion body fraction and was purified by electroelution from isolated gel slices (Figure 3-2A). A higher level of purification was available from the pET15b expressed protein due to an in frame NH$_2$-terminal "tag" of 20 amino acids. The additional peptide sequence contains 7 consecutive histidine residues and serves as an excellent epitope for affinity purification of the protein (Figure 3-2B). Also engineered into the polyhistidine tag is a cleavage recognition site for the protease thrombin. This allows for removal of the tag before using the protein as an antigen, thus antibodies are specific for the protein only and not against the polyhistidine tag. As shown in Figure 3-3, cleavage took 30 hours to generate ~75% completion.

We contracted with Cocalico Labs to inject purified Hog1p into rabbits to generate polyclonal antibodies as displayed in Table 4. Each bunny was test bled after 6 weeks showing good immunoreactivity in all cases. Production bleeds were then spaced over 10 day intervals (3X) followed by exsanguination. The sera was affinity purified on a Hog1p-Sepharose antigen column as previously described (Nasmyth et al, 1990, Wittenberg et al, 1989). Figure 3-4A displays a western blot of total yeast proteins as detected by affinity purified sera isolated from RC6. Some background reactivity was
detected from the secondary antibodies (Fig. 3-4B), however the prominent band, present in wild-type and absent in hog1Δ cells, is Hog1p.

Table 4: Antibody Production

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<thead>
<tr>
<th>Bunny</th>
<th>Route of Injection</th>
<th>Antibody Specificity</th>
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<tbody>
<tr>
<td>RC4</td>
<td>Electroeluted protein - 174µg total popliteal lymph node injections</td>
<td>good - significant background</td>
</tr>
<tr>
<td>RC5</td>
<td>Electroeluted protein - 500 µg total subcutaneous/intramuscular injections</td>
<td>fair - high background</td>
</tr>
<tr>
<td>RC6</td>
<td>Affinity purified/thrombin cleaved 200 µg total popliteal lymph node injections</td>
<td>excellent - very specific, low background</td>
</tr>
</tbody>
</table>

Immunolocalization of Hog1p:

The nuclear targeting of extracellular signals raises the question of how the signal crosses into the nucleus. Multiple downstream substrates for the activated kinase pathways have been identified. In mammalian cells, these include transcription factors such as myc and jun (Chou et al, 1992), but also the cytoplasmic ribosomal protein S6 which, when phosphorylated is thought to enable 40S ribosomes to initiate the protein synthesis required for growth (Olivier et al, 1988). MAP kinases were originally localized to the cytoplasm, however, recent evidence in HeLa cells has identified a significant fraction of p42 and p44 to be in the nucleus (Chen et al, 1992). One possible function of the osmotically stimulated pathway is that following activation, Hog1p goes
Figure 3-4: Affinity purified antisera is specific for Hog1p. Western blots of total cellular proteins from wild-type (YPH102) and hog1Δ (JBY13) cells. Protein isolation and immunoblots were performed as described in Materials and Methods. Following electrophoresis, proteins were transferred to nitrocellulose (Schleicher & Schuell) and incubated with rabbit anti-Hog1p sera. Rabbit antibodies were detected by probing blots with a goat anti-rabbit alkaline phosphatase conjugate (Promega). A) Blot probed with affinity purified RC6 anti-Hog1p sera identifies a band at the molecular weight of Hog1p, present in wild-type and absent in hog1Δ cells. B) To evaluate which of the background bands were due to the goat anti-rabbit secondary antibodies, a second immunoblot was performed in the absence of rabbit sera. All of the major bands except for Hog1p reappeared, indicating the affinity purified antisera is very specific for Hog1p.
Figure 3-5: Immunolocalization of Hog1p in yeast cells. To determine the intracellular localization of Hog1p, hog1Δ (JBY13) cells were transformed with pRS426 (A,C,E; Sikorski and Hieter, 1989), or pRS426 with a HOG1 insert (B,D,F). Cells were grown to log phase in selective media and fixed through addition of formaldehyde to 5% with a 1 hour incubation at 30°C. Fixed cells were then digested with zymolyase, spotted onto poly-lysine coated slides, and incubated overnight in PBS with affinity purified RC6 antisera. Bound antibodies were detected using an anti-rabbit fluorescein conjugate (Cappell). Cells were photographed using bright field (A,B) and fluorescence microscopy. C) In the absence of Hog1p, little background fluorescence was observed. D) In cells overexpressing Hog1p, fluorescent staining was uniformly distributed throughout the cell. E,F) Localization of the nucleus was determined through a 10 minute incubation in DAPI (1 μg/ml).
into the nucleus to stimulate gene expression. In vivo detection of Hog1p, both before and after salt stress, was therefore of interest.

Immunolocalization of Hog1p was attempted in wild-type cells and found to be below the level of sensitivity of the assay. Therefore, hog1Δ cells were transformed with a high copy plasmid carrying HOG1 to induce overexpression of the protein within the cell. Cells were grown to log phase, fixed in 5% formaldehyde, spheroplasted, and exposed to anti-HOG1 sera. Under normal growth conditions Hog1p was shown to be localized uniformly throughout the cell (Figure 3-5D). hog1Δ cells transformed with a high copy plasmid (no insert) were also evaluated under the same conditions revealing only basal levels of fluorescence (Figure 3-5C). Following a 10 minute, 0.4M NaCl salt stress no discernable change in localization was observed (not shown). Hog1p therefore appears to be a cytoplasmic protein, being activated and acting upon its substrate within the cellular cytoplasm. Future work on the identification and localization of the substrate protein(s) will be required to understand how the signal enters the nucleus.

Site-directed mutagenesis:

The strong homology between HOG1 and other previously characterized MAP kinases has provided important insight into the possible functional relevance of several residues. Thr174 and Tyr176 of Hog1p share striking contextual homology with residues known to be required for activation in related MAP kinases (Gartner et al, 1992; Payne et al, 1991; Anderson et al, 1990). The activated MAPK kinase phosphorylates the MAP
kinase on both residues resulting in an active kinase, while dephosphorylation of these sites eliminates activity. Identification of a phosphatase which specifically downregulates either kinase has not yet been identified, though a tyrosine phosphatase specific for the EGF receptor has been shown to be activated by the p42 kinase (Griswold-Prenner et al, 1993).

The NH$_2$-terminal portion of Hog1p shows striking homology to nucleotide binding or catalytic domains found in all kinases (Hanks, 1988). Within this domain are tightly conserved residues proposed to be involved in MgATP binding and transfer of the gamma-P0$_4$ to the peptide substrate. Structure determination of the catalytic subunit of cyclic AMP-dependant protein kinase revealed close association of three tightly conserved residues Lys$^{72}$, Asp$^{184}$, and Glu$^{91}$ with the gamma-PO$_4$ of MgATP (Knighton, 1991). Lys$^{72}$ has been proposed to be essential for the phosphotransfer reaction due to genetic studies with protein kinase A (Gibbs and Zoller, 1991). Similar crystallographic data of the cyclin-dependant kinase 2 revealed the tightly conserved Lys and Asp residues described above to have nucleotide binding dependant orientations (see Fig. 3-6, DeBondt et al, 1993). Wu et al (1991) found that a conservative substitution in the mouse MAP kinase the corresponding residue, Lys$^{52}$ to Arg results in elimination of autophosphorylation activity. Lys$^{51}$ in Hog1p corresponds to the catalytic lysine residue found in other kinases as identified by the surrounding predicted peptide sequence.
Figure 3-6: Nucleotide binding domain of cyclin-depandant kinase-2 (CDK2).

Evaluation of the nucleotide binding domain of CDK2 identifies the catalytic role of several conserved residues found in related kinases. Lys\textsuperscript{33} and Asp\textsuperscript{145}, along with several other charged residues, were shown to interact with the $\alpha$ and $\gamma$-P\textsubscript{0} of MgATP, and display different orientations in the absence of ATP. Mutagenesis of the conserved lysine has been shown to eliminate activity in several kinases (see text), suggesting a requirement for this residue in the phosphotransfer reaction. Lys\textsuperscript{33} of CDK2 corresponds to Lys\textsuperscript{51} of HOGL. Figure adapted from De Bondt et al (1993).
Figure 3-7: Site-directed mutagenesis of HOGI. Oligonucleotide directed point mutations were introduced into HOGI as described in Materials and Methods. In each case, single base changes were used, changing lysine51 to arginine (hogl-K51R, codon 51 ttc to tcc), threonine174 to alanine (hogl-T174A, codon 174 aca to gca), and tyrosine176 to phenylalanine (hogl-Y176F, codon 176 tat to ttt). Mutagenic residues are indicated with an arrow. Portions of the sequence are shown for all three mutants to reveal mutagenic changes (loading order ACGT). Mutagenesis reactions were transformed into the bacterial strain TG1, and successful mutants sequenced over the entire open reading frame.
A) ACGT... 637-666 shown

<table>
<thead>
<tr>
<th>HOG1</th>
<th>Y176F</th>
<th>K51R</th>
<th>T174A</th>
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B) AGCT... 1007-1034 shown

<table>
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<tr>
<th>HOG1</th>
<th>Y176F</th>
<th>K51R</th>
<th>T174A</th>
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A likely interpretation of the data available was that Hog1p is activated by increased extracellular osmolarity in a manner similar to other previously characterized MAP kinases. Using site-directed mutagenesis, we set out to determine if the putative phosphorylation sites, Tyr\textsuperscript{176} and Thr\textsuperscript{174}, were required for cellular growth in high salt media. A Lys\textsuperscript{51} mutant was also generated to evaluate phenotypic affects of a catalytically inactive protein. Autophosphorylation has been identified in related MAP kinases and is proposed to play a role in their activation (Seger et al, 1991, Wu et al, 1991), the Lys\textsuperscript{51} mutant was also used to evaluate autophosphorylation in Hog1p. As shown in Figure 3-7, three single residue mutations were introduced into HOG1. In each case conservative amino acid substitutions were used to avoid disruption of normal protein folding. Lysine 51 was changed to arginine (hog1-K51R), threonine 174 was changed to alanine (hog1-T174A), and tyrosine 176 was changed to phenylalanine (hog1-Y176F). Each mutant was sequenced over the entire open reading frame to confirm that only a single mutation had been introduced.

Site-directed mutants cannot rescue Osm\textsuperscript{+} of hog1Δ cells:

Plasmids carrying the mutant forms of HOG1 were transformed into a hog1Δ strain and evaluated for an ability to grow under osmotically stressed conditions. Figure 3-8 displays strains carrying a single copy vector control (pRS316), or with inserts of HOG1, or one of the mutants hog1-Y176F, hog1-K51R, or hog1-T174A. Streak outs revealed a complete elimination of complementing capacity for the hog1-T174A mutant at either 0.4 or 0.9 KCl. The hog1-K51R and hog1-Y176F mutants showed some growth
Figure 3-8: Site-directed mutants cannot rescue growth. To evaluate the ability of each point mutant to rescue cell growth under osmotically stressed conditions; HOG1, hog1-K51R, hog1-T174A, hog1-Y176F, and the control plasmid without inserted DNA (all in pRS316 - single copy) were transformed into the yeast strain hog1Δ (JBY13). Growth was evaluated by streaking cells out on YEPD and YEPD containing 0.4M or 0.9M KCl (NaCl also tested with similar results). Plates were incubated for 36 hours at 30°C and then photographed.
Vector
HOG1
Y176F
T174A
K51R
0.4 M KCl
0.9 M KCl
Figure 3-9: Overexpression of site-directed mutants. *HOG1* and each of the constructed point-mutants were subcloned into a high copy plasmid (pRS426-2µ) and transformed into *hog1Δ* (JBY13). Growth was evaluated by streaking out cells on YEPD and YEPD containing 0.4M or 0.9M KCl (NaCl also tested with similar results). Plates were incubated for 36 hours at 30°C and then photographed.
at 0.4M KCl but little or no growth at 0.9M NaCl. Growth was also evaluated in cells overexpressing each of the point mutants from a high copy plasmid (Figure 3-9). The most apparent increase in viability was seen in the hog1-K51R mutant. The general inability of the hog1-K51R mutant to rescue osmosensitivity except at intermediate osmolarities indicates the possibility that the mutant protein is an inefficient kinase with basal levels of activity. Lysine is immediately adjacent to a second lysine, which could carry out charge interactions necessary for nucleotide binding and phosphotransfer. The hog1-Y176F mutant again displayed some growth at intermediate osmolarities, indicating that the mutation did not completely eliminate protein activity. Mutation of threonine completely eliminated growth rescue in either single or high copy numbers.

**Tyrosine phosphorylation of point mutants suggests a feedback loop:**

Immunoblots were performed to evaluate the tyrosine phosphorylation state of the mutant proteins following salt stress. hog1Δ cells transformed with high copy plasmids carrying HOGL and each of the point mutants were grown to log phase, exposed to 0.4M NaCl for 10 minutes and total cellular proteins isolated. Figure 3-10B displays an anti-Hog1p probed immunoblot. HOGL and each of the point mutants were expressed to comparable levels indicating activity of the protein has no influence upon protein expression. Immunoblot detection of tyrosine phosphorylation revealed no reactivity in hog1Δ cells transformed with a control high copy (2μ) plasmid and a salt induced tyrosine phosphorylation of Hog1p in cells transformed with a high copy plasmid carrying a HOGL insert. In contrast, the hog1-Y176F mutant displays no phosphotyrosine
immunoreactivity indicating that Tyr^{176} is the phosphorylated residue in Hog1p. Following salt stress the *hog1*-K51R and *hog1*-T174A mutants show levels of tyrosine phosphorylation much higher than that of the wild-type protein. The kinase cascade therefore appears to be downregulated by an active Hog1p, similar to the Fus3p downregulation proposed in the mating arrest pathway (see section V).

Wild-type cells display a peak of salt-induced Hog1p tyrosine phosphorylation 1-10 minutes following exposure to the stress followed by a gradual decline (Figure 3-11). We evaluated the time course of phosphorylation in the *hog1*-K51R point mutant to determine if the kinetics of activation or inactivation were altered. hog1Δ cells carrying *HOGL* or *hog1*-K51R on a high copy plasmid were grown up, 0.4M NaCl added to the culture, and tyrosine phosphorylation of Hog1p (wild-type or mutant) measured at different time points. *HOGL*-carrying cells displayed a maximal phosphorylation within the first 10 minutes followed by a gradual decrease over the remaining 50 minutes (Figure 3-11). Cells overexpressing the *hog1*-K51R mutant displayed much higher levels of tyrosine phosphorylation, peaking within the first 10 minutes and remaining high throughout the time course (Figure 3-12). Blots probed with anti-Hog1p antibodies revealed no changes in protein expression throughout either time course. Thus the catalytically inactive Hog1p reveals no dephosphorylation at later timepoints, and indicates that downregulation of the kinase cascade must be mediated through an active Hog1p.
High osmolarity media delays bud emergence:

MAP kinases have been shown to stimulate a signalling cascade which results in growth arrest. As described in Chapter 1, the mating arrest pathway functions to inactivate the CDC28/G1 cyclin required for cell cycle progression from G1 to S phase. Osmotic stress has been shown to transiently arrest cultures of *S. cerevisiae* (Chowdhury et al, 1991). We therefore sought to evaluate the influence of *HOG1* and *PBS2* upon the observed growth arrest in salt stressed cells through observation of bud emergence. If the osmotically-activated kinase cascade signals the observed growth arrest, evaluation of *hog1Δ* and *pbs2Δ* mutants should reveal an absence of the arrest. Bud initiation indicates transition of cells beyond the G1/S arrest point as growth is resumed. Isolation of unbudded cells following growth arrest allows for observation of those cells to determine when growth resumes. Unbudded cells of wild-type and each of the mutants were therefore isolated and evaluated for bud initiation following salt stress.

The wild-type strain (YPH102D) was first grown to log-phase in YEPD, transferred to agar plates containing YEPD or YEPD plus 0.6 M NaCl, and the emergence of buds on unbudded cells monitored by time-lapse photomicroscopy (Figure 3-13). There was no noticeable difference in the initial appearance of cells on the two different types of media. This was not unexpected because the NaCl concentration used, 0.6 M, has an osmolarity less than that needed to induce incipient plasmolysis in yeast (Arnold, 1981). After transfer to control media, the population of unbudded cells showed some budding cells within 30 min with half of the cells budding by 1 hr. After transfer
to YEPD plus 0.6 M NaCl, the population of unbudded wild-type cells did not begin budding until shortly after 1.5 hr. Once budding started, the time course of bud emergence was similar in the two media. Thus, bud emergence in yeast is delayed by an increase in external osmolarity.

To examine the effect of the osmotic response pathway on the kinetics of bud emergence, we also monitored the budding of hog1Δ, hog2Δ, or pbs2Δ cells. On YEPD, bud emergence of the three mutants coincided with wild-type cells. However, in YEPD + 0.6M NaCl all three mutants took longer than the wild-type to start budding (Figure 3-13). The observed delay in budding was similar for each mutant, suggesting the phenotype is likely due to a general defect in osmoregulation which is shared by hog1Δ, hog2Δ, and pbs2Δ mutants. The fact that a delay in bud emergence, similar to wild type is observed, indicates that the osmotic stress induced growth arrest occurs by a mechanism independent of the HOG1Δ/PBS2Δ pathway or that multiple growth arrest signals result from osmotic stress.
<table>
<thead>
<tr>
<th>Plasmid</th>
<th>2μ</th>
<th>HOG1</th>
<th>Y176F</th>
<th>K51R</th>
<th>T174A</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>A.</td>
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**Figure 3-10: Antiphosphotyrosine analysis of point mutants.** Immunoblot analysis was performed on hog1Δ (JBY13) transformed with pRS426 (2μ), or pRS426 with an insert of HOG1, hog1-K51R, hog1-T174A, or hog1-Y176F (see text for details). Cells were grown overnight in selective media, diluted into YEPD for 3-6 hours to allow cells to reach log phase, and divided into aliquots. Total cellular proteins isolated immediately (-), or following a 10 minute exposure to 0.4M NaCl (+). Duplicate western blots were performed as described in Materials and Methods (40 µg protein loaded per lane). Primary antibodies were antiphosphotyrosine (A) or affinity purified RC6 sera specific for Hog1p (B).
Minutes in 0.4M NaCl

| 0 | 10 | 20 | 30 | 40 | 50 | 60 |

A.

B.

Figure 3-11: Tyrosine phosphorylation peaks after 10 minutes. Immunoblot analysis was performed on hog1Δ (JBY13) transformed with pRS426 (2μ) carrying HOG1 to overexpress wild type Hog1p. Cells were grown overnight in selective media, diluted into YEPD for 3-6 hours to allow cells to reach log phase, and divided into aliquots. Total cellular proteins isolated immediately (-), or following a 10, 20, 30, 40, 50, or 60 minute exposure to 0.4M NaCl (+). Duplicate western blots were performed as described in Materials and Methods (40 μg protein loaded per lane). Primary antibodies were antiphosphotyrosine (A) or affinity purified RC6 sera specific for Hog1p (B).
Figure 3-12: Western analysis of the hog1-K51R mutant. Immunoblot analysis was performed on hog1Δ (JBY13) transformed with pRS426 (2µ) carrying hog1-K51R to overexpress mutant Hog1p. See figure legend 3-11 and text for details. Primary antibodies were antiphosphotyrosine (A) or affinity purified RC6 specific for Hog1p (B).
Figure 3-13: Osmotic-dependence of bud emergence in wild-type and mutant strains. The time course of bud emergence was evaluated by time-lapse photomicroscopy of cells isolated by micromanipulation. Cells were grown in liquid YEPD and transferred to YEPD or YEPD + 0.6M NaCl plates. Unbudded cells (35 for each strain) were isolated and scored every 30 minutes for the percentage of cells with a new bud. Diploid strains were used; wt, YPH102D, hog1, JBY13D; hog2, JBY23D, pbs2, JBY43D (genotypes same as related haploid strain shown in table 3; YPH102D = YPH102/YPH102).
Materials and Methods: III

Western Blot Analysis:

Analysis of tyrosine phosphorylation in yeast extracts was performed essentially as described by Ballard et al (1991). Cells were grown overnight in selective media, pelleted by centrifugation at 2500 rpm, and resuspended in rich media at an A_{600} = .1 to .3. The cultures were grown another 2-6 hours, to a density of ~ .6 where they were divided into 5 ml aliquots and NaCl was added. Following the salt stress (10 minutes in most cases), the cultures were transferred to ice and all subsequent manipulations performed in a cold room. Cells were then pelleted and for every 5 ml of culture were resuspended in 120 µl of lysis buffer (50mM Tris·HCl pH7.5, 1% sodium deoxycholate, 1% Triton X-100, 1% SDS, 5mM sodium pyrophosphate, 1mM sodium metavanadate, and 50mM sodium fluoride). Protease inhibitors were added (.02% phenylmethylsulfonyl fluoride-PMSF, 1 µg/ml leupeptin, 1 µg/ml pepstatin, and 12 µg/ml aprotinin), followed by addition of a 3/4 volume of pre-chilled glass beads. The cells were lysed by 3-4, 30 second cycles on the bead-beater homogenizer (Bio-Spec Products), followed by a 15 minute centrifugation (12,000 rpm). The supernatant stored away at -20°C.

Extracts from each strain were assayed for total protein concentration using a Biuret Protein Assay Kit (Sigma). Samples were diluted 1:1 with Laemmlli sample buffer, and a volume corresponding to 60 µg of protein exposed to standard SDS-PAGE analysis (Sambrook et al, 1989). Protein gels were washed for 15 minutes in Towbin transfer buffer (25mM Tris·HCl, 192mM glycine, 20% methanol, ph 8.3) and
electrophoretically transferred to nitrocellulose membrane (Schleicher & Schuell) using the Trans-Blot SD Semi-Dry Electrophoretic Cell (Bio-Rad) according to manufacturers instructions. The nitrocellulose was then washed for 15 minutes in ANT buffer (50mM Tris·HCl, pH 8.0, 150mM NaCl, 0.02% NaN₃, and 0.05% Tween-20), and then blocked for 45 minutes in ANT + 3% bovine serum albumin (BSA). Primary antibody was added in a small volume of blocking buffer and slowly washed over the nitrocellulose on a rocker platform for 1-2 hours. The blot was washed, and the secondary antibody alkaline phosphatase conjugate added in a small volume followed by a 30-45 minute incubation with slow rocking. The blots were again washed and developed using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate reagents (NBT/BCIP-Promega).

**Antibody Production:**

To insert *HOG1* into a bacterial expression vector, the polymerase chain reaction using oligonucleotide primers was used to introduce restriction sites at the start and stop codons of the gene, (5'-gga cat atg acc act aac gag-3', containing an Nde I site; 5'-cgt gga tcc tca ttt gca gct aca-3', containing a Bam HI site). To purify the PCR product, samples were separated from remaining primers by size exclusion chromatography columns (G-100, Clonetech), the eluate extracted with CPI (50:48:2 of phenol/chloroform/isoamyl alcohol), and ethanol precipitated. Restriction digests and ligations into pET vectors (Novagen) were performed according to standard techniques.
(Sambrook et al, 1989) and the ligation products transformed into *E. coli* Sure™ cells (Stratagene).

Induction of protein overexpression was done essentially as described by Novagen. BL21 cells carrying the plasmid construction were grown in M9ZB media (Studier et al, 1991) under antibiotic selection with chloramphenicol (25 μg/ml) and ampicillin (100 μg/ml). An overnight culture was diluted 1/100 in M9ZB + antibiotics and allowed to grow to an A_{600} of 0.8. IPTG was added to a final concentration of 1mM and the induced cells cultured for additional 4 hours. Inclusion bodies were prepared as described by Harlow and Lane (1988) and solubilized in 6M urea.

Protein purification was accomplished by electroelution of the stained Hog1p protein from gel slices following electrophoresis. Protein induced from the pET15b construction carrying the unique poly-histidine tag was purified using His Bind™ Resin (Novagen). The inclusion body pellet was incubated in 6M urea, and the solubilized protein purified over a nickel affinity column to obtain proteins containing multiple histidines, and eluted by competition with imidazole. Following purification of the protein (mainly Hog1p), the poly histidine tag was removed by cleavage of the protein with thrombin overnight at 30°C at which time ~70% of the protein had been cleaved. Purified protein was quantitated, lyophilized, and used to inoculate New Zealand white rabbits at Cocalico, Inc. (Reamstown, PA). Rabbits were injected either intramuscularly or into popliteal lymph nodes if less protein was available. Boost inoculations were given
after two weeks and test bleeds performed after six weeks.

Affinity purification of RC6 polyclonal sera was performed essentially as described by Wittenberg et al (1987). CNBr activated Sepharose (Pharmacia) was hydrated and washed in 1 mM HCl. In a sterile tube, 3 mls of purified Hog1p (~1mg) was added to 1 gram of activated Sepharose in 2 mls of coupling buffer (0.1M NaHCO$_3$, 0.5M NaCl, pH 8.3). The tube was slowly rotated end-over-end for 2 hours to allow binding of the protein. Beads were then washed in 10 mls of coupling buffer to wash off any unbound proteins and any remaining CNBr inactivated by a 2 hour incubation in 0.1M Tris (pH 8.0). The Hog1p-Sepharose was then used to make an affinity column and crude RC6 sera diluted in .01M Tris (pH 7.5) run over the column five times to ensure binding. The column was then washed several times and the antibodies eluted using acidic (pH 2.5) and basic (pH 11.3) solutions. Fractions eluted from either condition were immediately neutralized. Elution procedures yielded both acidic and basic fractions with equally strong anti-Hog1p reactivity, therefore fractions were combined for subsequent immunoblots.

**Immunolocalization:**

The intracellular localization of Hog1p was determined using affinity purified RC6 antibodies. Yeast cells were grown in minimal media lacking uracil to a density of 10$^7$-10$^8$ cells/ml and fixed through the addition of formaldehyde to a concentration of 5%.
The cells were fixed in formaldehyde at 30°C for 1-1.5 hours. Cells were then washed in 0.1M KPO₄ (pH 7.5) and resuspended in a Zymolyase solution (0.1M KPO₄, pH 7.5, 25mM 2-mercaptoethanol, 1.2M sorbitol, and 25 ug/ml Zymolyase) to remove the cell wall. Optimal digestion times varied depending upon the strain but generally were around 1-1.5 hours for best results. Cells were spun down, resuspended in 0.1M KPO₄ (pH 7.5) and spotted onto polylysine-coated slides. Primary antibodies were added at various concentrations in PBS-I (40mM K₂HPO₄, 10mM KH₂PO₄, and 150mM NaCl) containing 1 mg/ml BSA, and the slides incubated overnight at 4°C. The cells were washed 3X in PBS-I and followed by secondary antibody incubation with an anti-rabbit fluorescein conjugate (Cappel) diluted 1/200 in PBS-I. Secondary antibody staining was allowed to proceed for 2 hours at room temperature (in dark room) followed by several washes with PBS-I. Nuclear localization was determined by incubating cells in PBS-I + DAPI (1 ug/ml) for 10 minutes followed by several washes in PBS-I. For fluorescence microscopy, 2-4 μl of mounting media (0.01% p-phenylene diamine, 90% glycerol, 4mM K₂HPO₄, 1mM KH₂PO₄, and 15mM NaCl) was dropped onto the cells to decrease photobleaching.

**Site Directed Mutagenesis:**

Point mutations were introduced into *HOG1* using protocols and materials provided in the oligonucleotide-directed *in vitro* mutagenesis system (Amersham). Single stranded DNA was generated by standard techniques (Sambrook et al, 1989). Mutant oligonucleotides with single base pair changes from the *HOG1* sequence were annealed...
to single stranded wild-type template DNA. Mutant oligonucleotides, with mutagenic residues underlined, were; *hogI*-K51R, 5'-g ttt cat gat ttt cct aat gcc aac tgg-3', *hogI*-T174A, 5'-ga aac ata gcc tgc cat tgg agg gtc-3', *hogI*-Y176F, 5'-tct agt gga aac aga gcc tgt cat ttg-3'. A mutant strand was initiated from the primer-template hybrid using modified phosphorothioate nucleotides which are restriction endonuclease resistant. The double stranded plasmid was then digested with Nci I to introduce nicks into the wild-type sequence, followed by Exonuclease III treatment. Priming off of the remaining wild-type DNA fragments and using the mutant strand as a template, T4 DNA polymerase was added to synthesize a second strand of DNA. Following the second round of DNA synthesis the plasmid DNA was used to transform E. coli strain TG1 which is efficiently transformed with phosphorothioate modified nucleotides. Transformants were first screened phenotypically by transformation into a *hogI*Δ strain, and colonies showing salt sensitivity were sequenced over the entire reading frame to ensure that only a single base change has occurred. An efficiency of ~50-60% was seen with successful mutants.
Chapter 4:

Influences of \textit{HOG1} and \textit{PBS2}

upon cellular morphology and bud site selection

Cell growth in \textit{Saccharomyces cerevisiae} proceeds by a tightly regulated progression from bud initiation to cell separation. Mutational analysis of yeast budding has revealed a genetic program for bud morphogenesis and cell division (Hartwell, 1971). The budding mechanism of cell division is unique, raising the question of relevance of yeast studies to growth in more complex systems. However, eucaryotic cells have revealed tightly conserved regulatory mechanisms for cell cycle progression identified initially through the use of the simpler yeast and frogs. Already, \textit{CDC42}, a gene isolated in \textit{S. cerevisiae} as required for bud initiation, has identified a closely related gene in mammalian cells (Johnson and Pringle, 1990, Munemitsu et al, 1990).

Haploid yeast initiate new buds adjacent to the previous bud site, a pattern termed axial or polar budding. Diploid yeast display bipolar budding with each new bud appearing adjacent to the previous one or, more frequently, on the opposite side of the mother cell. Five genes, \textit{BUD1-BUD5}, have been identified as required for normal budding patterns (for review, Drubin, 1991, Chant and Herskowitz, 1991). The mechanism of bud initiation and elongation requires another set of genes and is not yet well understood (Bender et al, 1991, Chenevert, 1992). Within budding cells, the actin
cytoskeleton appears as long filamentous cables and small cortical patches generally associated with the bud or growing region (Adams and Pringle, 1984). The actin filaments are thought to mediate delivery of vesicles to the bud resulting in the generation of new cell membrane.

Extracellular signals use MAP kinase-containing signaling pathways in animal cells to regulate not only cell proliferation but also cell morphogenesis. We noted that although hog1, pbs2, and hog2 mutants have similar defects in osmoregulation, hog1 and pbs2 cells show morphological abnormalities not shared by hog2 cells. This suggested that the osmotic response pathway may play a role in regulating cell morphogenesis. Therefore, I have carefully examined the events of bud morphogenesis in wild-type and mutant cells after transfer to high osmolarity media. Cells with hog1Δ and pbs2Δ mutations had osmotic-dependent defects in cell-type specific patterns of bud site selection and in cytokinesis. One unexpected finding was that hog1Δ and pbs2Δ mutants "forget" where to resume growth after recovery from osmotic stress. These results suggest a novel concept in cell morphogenesis: homeostatic control of cell morphogenesis by a stress-specific signaling pathway.

Recovery of cell growth and division after osmotic stress:

A sudden increase in external osmolarity induces a short growth arrest, and is associated with a disassembly and delocalization of actin filaments and cortical patches from the bud to the mother cell (Chowdhury et al, 1992). Yeast cultures that resumed
growth in high osmolarity media contained cells that again had actin patches localized to the bud. Because the location of actin patches is generally correlated with sites of active cell growth, one could assume that yeast cells resume the process of cell growth and division in the same location as that used before osmotic stress was applied. To test this assumption, I used time-lapse photomicroscopy to follow the growth of individual cells after transfer from YEPD, a commonly used rich media, to YEPD containing 400 mM NaCl. This increase in osmolarity is less than that needed to induce cell shrinkage (Arnold, 1981), which requires NaCl concentrations greater than 600 mM. The small bud on each of the diploid wild-type cells remained the same size and shape for 1-2 h on this high osmolarity media and then began to enlarge (Figure 4-1A,B). In a larger sample taken from several experiments, all wild-type cells resumed growth of the same bud, designated the 1\textsuperscript{st} bud, after recovery from osmotic stress (Table 5, top row). Similar results were obtained when the high osmolarity test plate contained 700 mM sorbitol or 600 mM glucose instead of NaCl or when haploid cells were tested.

The return of actin patches and growth back to the original bud during the recovery from osmotic stress could be explained if one or more of the functions required for selecting a bud site or initiating a bud were retained in cells whose growth has been arrested by osmotic stress. These morphogenetic functions would guide the return of the actin cytoskeleton and growth to the original bud. To test part of this hypothesis, I followed the growth of individual cells (haploid and diploid) containing mutations in the five \textit{BUD} genes, \textit{BUD1} to \textit{BUD5}, after transfer from YEPD to YEPD plus 400 mM
NaCl. Each of the ten bud mutants tested (see Methods) behaved like wild-type, resuming the growth of the same bud they had initiated before transfer to high osmolarity media. Thus, although the BUD genes are required for defining the position of each new cell division on the cell surface, these genes are not required for retention of the original location of cell division following osmotic stress.

Resumption of bud growth after osmotic stress in hog1Δ and pbs2Δ mutants:

The HOG1 and PBS2 genes, implicated in the response of yeast cells to osmotic stress, are related to genes that mediate growth factor-induced changes in cell morphogenesis. To determine whether a HOG1- and PBS2-dependent morphogenetic response is part of the adaptation of yeast cells to osmotic stress, we studied the morphogenesis of hog1Δ and pbs2Δ mutants before and after a change in osmolarity. The hog1Δ and pbs2Δ mutants grew well on YEPD, at reduced rates on YEPD plus 400 mM NaCl, and did not grow on YEPD plus 900 mM NaCl. Grown in YEPD, both mutants have similar cell morphologies as wild-type cells, with the only difference being slightly longer buds in the mutants (see below). When hogΔ cells were transferred to YEPD plus 400 mM NaCl, mutant cells with a small bud (Figure 4-1B) retained their initial shape for up to 6 h, but then, remarkably, resumed growth by initiating a new bud rather than enlarging the pre-existing 10 bud (Figure 4-1D). Table 5 (rows 2 and 3) shows that ~90% of the hog1Δ and pbs2Δ cells that resumed growth after transfer to high osmolarity re-initiated cell division in a new location. Similar results were obtained when 700 mM sorbitol or 600 mM glucose were used instead of NaCl to raise the osmolarity.
Haploid hog1Δ and pbs2Δ strains also failed to resume growth of their 1^0 buds following an increase in osmolarity.

The failure of hog1Δ and pbs2Δ mutants to resume cell division in the proper location could reflect either a non-specific response to any environmental stimulus that induces growth arrest or a specific defect in a response to osmotic stress. Shifting the temperature rapidly from 30^0 to 37^0 or changing the carbon source by substituting galactose for glucose results in a short growth arrest. During either of these stress-induced growth arrests, cortical patches of actin filaments re-distribute from bud to mother cell and back again when growth resumes. Thus, these responses are similar to that seen in cells exposed to osmotic stress. When hog1Δ and pbs2Δ cells were exposed to either the 30 to 37^0C shock or switched from YEPD to galactose media (YEPgalactose), almost all of the mutant cells resumed growth of the 1^0 bud after recovery from the growth arrest (Table 5, columns 4 and 5). Thus, the failure of hog1Δ and pbs2Δ mutants to resume cell division at the proper location occurs only after osmotic stress and is not due to growth arrest per se.

The hog1- and pbs2-induced defect in restoring cell growth and division to the original location after an increase in osmolarity could be an indirect effect of defective osmoregulation or it could reflect the loss of a function unrelated to osmoregulation. To distinguish between these possibilities, we used the a diploid hog2Δ mutant as a control. High osmolarity-induced glycerol accumulation in strains containing this mutation (like
TABLE 5: Resumption of growth in a bud after an increase in external osmolarity.

The percentage of cells that resume growth in a pre-existing bud (the 1\textsuperscript{st} bud) within 6 h after transfer to YEPD or YEPD plus 0.4 M NaCl were determined using the method described in the legend to Fig. 2. Diploid strains used are: wild-type, YPH102D (YEPD, 34,0; YEPD/NaCl, 59,0); hog1, JBY13D (YEPD, 33,0; YEPD/NaCl, 121,14); hog2, JBY23D (YEPD, 35,0; YEPD/NaCl, 75,15); pbs2, JBY43D (YEPD, 34,0; YEPD/NaCl, 109,26). In parentheses are, respectively, the number of cells observed which resumed growth and those that did not grow during the 6 h period. Only the former group of cells was used to calculate the percentage of cells that resume growth in a pre-existing bud. For example, 90 JBY23D cells were watched on YEPD plus 0.4 M NaCl. Of these, 15 cells failed to grow by the end of 6 h, 67 cells resumed growth in the 1\textsuperscript{st} bud, and 8 cells resumed growth by initiating a new bud rather than resuming growth of the 1\textsuperscript{st} bud. 67/(8+67) x 100\% = 89\%.
Table 5: Resumption of 1° bud growth

<table>
<thead>
<tr>
<th>Strain</th>
<th>YEPD</th>
<th>YEPD 400 mM NaCl</th>
<th>YEP galactose</th>
<th>YEPD 37°</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>hog1</td>
<td>100%</td>
<td>12%</td>
<td>100%</td>
<td>87%</td>
</tr>
<tr>
<td>pbs2</td>
<td>100%</td>
<td>13%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>hog2</td>
<td>100%</td>
<td>89%</td>
<td>100%</td>
<td>N.D.</td>
</tr>
</tbody>
</table>
Figure 4-1: Bud growth of small budded wild-type and \textit{hog1Δ} mutant cells after osmotic upshift. Cells were grown to log phase in YEPD, spotted onto YEPD + 0.4M NaCl plates, and individual small budded cells isolated by micromanipulation. Cells were photographed every hour for 6 hours and incubated at 30°C. (A) Small-budded wild-type (YPH102D) cells 15 minutes after transfer from liquid YEPD to plate containing YEPD + NaCl, (C) Same wild-type cells 1 hr later. (B) Small-budded \textit{hog1Δ} (IBY13D) cells 15 min. after transfer from liquid YEPD to plate containing YEPD + 0.4 M NaCl, (D) Same \textit{hog1Δ} cells 4 hr later. See Methods and the text for other experimental details.
those carrying \textit{hog1}\(\Delta\) or \textit{pbs2}\(\Delta\) is half the normal response. Despite this osmoregulation
defect, \(\sim 90\%\) of the \textit{hog2}\(\Delta\) cells that resumed growth after transfer from YEPD to
YEPD plus 400 mM NaCl did so by enlarging the pre-existing \(1^0\) bud rather than starting
a new one (Table 5, last row). Thus, the failure to retain the same plane of cell division
following osmotic stress is not the indirect result of a defect in osmoregulation.

\textbf{Re-localization of actin filaments is defective in \textit{hog1}\(\Delta\) and \textit{pbs2}\(\Delta\) mutants:}

Osmotic stress-induced growth arrest coincides with a temporary delocalization of
actin patches from the bud to the mother cell (Chowdhury et al, 1992). To determine
whether \textit{hog1}\(\Delta\) cells show a similar osmotic-sensitive redistribution of actin filaments,
cells were grown to log-phase in YEPD and the osmolarity of the media increased by the
addition of glucose to 0.6 M. Cultures were fixed at different times after changing
osmolarity and the cells stained with rhodamine phalloidin to reveal actin filaments
(Cooper et al, 1987). Before a change in osmolarity, \textit{hog1}\(\Delta\) cells have a normal
distribution of actin filaments (Adams and Pringle, 1984, Barnes et al, 1990, Kilmartin
et al, 1984) with actin patches localized to the bud and actin cables extending between
the mother cell and bud (Figure 4-2D). At 30 min after increasing osmolarity, mother
cells had no actin cables and a high density of actin patches (Figure 4-2E). The
distribution of actin filaments in \textit{hog1}\(\Delta\) cells in Figure 4-2D, E is similar to that observed
in wild-type cells.
Figure 4-2: Effect of osmotic stress on the actin cytoskeleton of a *hog1Δ* mutant. A culture of *hog1Δ* (JBY13D) cells in YEPD was transferred to YEPD + 0.4M NaCl. Aliquots of cells were taken at time points before (A,D), 30 minutes after (B,E) and 2 hours after (C,F) the shift to high osmolarity media. Cells were fixed in 3.7% formaldehyde, and the cellular actin filaments stained by adding rhodamine-phalloidin (see Methods). Representative cells were then photographed using both phase contrast (A,B,C) and fluorescence (D,E,F) microscopy.
Wild-type cells with two buds of different size generally have a higher cortical density of actin filament patches in the smaller, growing bud. The larger bud has finished enlarging and has a lower patch density. If \textit{hog1Δ} cells abandon small buds after osmotic stress to initiate a new round of budding, this ought to result in two-budded cells with an unusual distribution of actin filament patches: a small bud without patches and a larger bud with patches. To test this prediction, we examined a \textit{hog1Δ} culture 2 hr after addition of glucose to 0.6 M, a time when most cells had resumed growing. This culture contained numerous examples of cells with two different-sized buds (Figure 4-2C) with actin filament patches localized preferentially to the larger bud (Figure 4-2F). Two noteworthy features of this cell are the complete lack of actin filaments in the small bud and the elongated appearance of the larger bud with actin filament cables oriented into the bud. The same types of cells were observed in a \textit{pbs2Δ} culture but not in \textit{hog2Δ} or wild-type cultures (data not shown).

Other stresses such as an increase in temperature from 30 °C to 37 °C or a change in carbon source from glucose to galactose also cause a temporary loss of actin cables and redistribution of actin filament patches to the mother cell. When small-budded \textit{hog1Δ} or \textit{pbs2Δ} cells were exposed to a 30 °C to 37 °C shift or to a glucose to galactose shift, the mutants were able to efficiently resume growing the small pre-existing bud instead of initiating growth of a new bud.
Effect of osmotic stress on bud site selection:

The failure of *hog1* and *pbs2* mutants to correctly reposition actin filaments back into the bud after osmotic stress prompted an evaluation of bud site selection to determine if this function, also requiring positional information, is retained in the mutant cells. Bud site selection in different strains was examined before and after cells were exposed to an increase in osmolarity. Small-budded cells from a log-phase YEPD culture were placed on either YEPD or YEPD plus 0.4 M NaCl and followed by time-lapse photomicroscopy. The position of the new bud (the $2^0$ bud) relative to the initial bud (the $1^0$ bud) was noted. The $2^0$ bud was scored as appearing in one of four quadrants of the mother cell (Figure 4-3A), where the position of the $1^0$ bud was defined as the midpoint of the A quadrant. As shown in Figure 4-3B and C, 100% of the haploid wild-type cells displayed an axial pattern, i.e. $2^0$ buds in quadrant A (termed A quadrant cells), in both media. The *hog2Δ* strain also showed $\sim$100% A quadrant cells on both types of media. *hog1Δ* and *pbs2Δ* mutants also had $\sim$100% A quadrant cells on YEPD. However, on YEPD plus 0.4 M NaCl, the mutant budding pattern was significantly different from wild type. Similar results were obtained on YEPD plus 0.6 M glucose or 0.7 M sorbitol. To determine the effect of $1^0$ bud size, we repeated the above procedure using cells with a bud greater than 1/2 their size. *hog1Δ* and *pbs2Δ* cells with a large bud formed a $2^0$ bud in quadrants other than the A quadrant with 50% frequency (data not shown), indicating that the *hog1*- and *pbs2*-defect in bud site selection at high osmolarity is independent of bud size.
Figure 4-3: Bud site selection pattern in haploid cells before and after osmotic stress. In a wild type cell, the 2° buds are the buds in the second cell cycle, while in hog1- or pbs2- mutants after osmotic shock, the secondary buds are usually the second buds in the first cell cycle. Cells with a small bud were followed over time using time-lapse photomicroscopy to observe the position of the next bud (the 2° bud) to emerge. To evaluate the budding pattern of osmotically stressed cells each mutant and a wild type parental strain were scored according to the bud site selected, either in quadrant A, B, C, or D. (Panel A) Diagram of the four possible quadrants relative to the 1° bud where emergence of a 2° bud could occur (see text for explanation). (B) Budding pattern on YEPD. (C) Budding pattern on YEPD plus 0.4 M NaCl. The strains were: wt, YPH102 (YEPD, 38; YEPD/NaCl, 39); hog1, JBY13 (YEPD, 99; YEPD/NaCl, 127); hog2, JBY23 (YEPD, 40; YEPD/NaCl, 37); pbs2, JBY43 (YEPD, 99; YEPD/NaCl, 81). Numbers in parentheses are the total number of growing cells observed. After correcting for sample size, chi-squared ($X^2$) analysis was performed to determine if bud site selection in the mutants differed significantly from that of wild type cells. On YEPD, no significant difference was observed. On YEPD + 0.4M NaCl, $X^2$ values for all quadrants were the following; hog1, 165 (significant at $p < 0.001$); hog2, 1 ($p > 0.05$, not significant); pbs2, 220 (significant at $p < 0.001$).
A

B  YEPD, 30°C

C  YEPD + 0.4M NaCl, 30°C
Figure 4-4: Bud site selection pattern in diploid cells before and after osmotic stress.

See legend to Figure 4-3 for experimental protocol. (A) Budding pattern on YEPD. (B) Budding pattern on YEPD plus 0.4 M NaCl. The strains were: wt, YPH102D (YEPD, 59; YEPD/NaCl, 60); hog1, JBY13D (YEPD, 56; YEPD/NaCl, 123); hog2, JBY23D (YEPD, 65; YEPD/NaCl, 85); pbs2, JBY43D (YEPD, 59; YEPD/NaCl, 111). Numbers in parentheses are the total number of growing cells observed. After correcting for sample size, $X^2$ analysis was performed to determine if bud site selection in the mutants differed significantly from that of wild type cells. On YEPD, no significant differences were observed. On YEPD + 0.4M NaCl, $X^2$ values for all quadrants were the following: hog1, 63 (significant at $p < 0.001$); hog2, 45 (significant at $p < 0.001$); pbs2, 182 (significant at $p < 0.001$). When only the frequency of budding in the A and C quadrants was used to compare mutant to wild-type, the $X^2$ values were: hog1, 33 (significant at $p < 0.001$); hog2, 2 ($p > 0.05$, not significant); pbs2, 182 (significant at $p < 0.001$).
A  YEPD, 30°C

B  YEPD + 0.4M NaCl, 30°C
Also examined were the effects of increasing osmolarity on the diploid budding pattern. On YEPD, the $2^0$ buds on diploid wild-type cells were formed in predominantly the A or C quadrant, with the number of C quadrant cells exceeding that of A quadrant cells (Figure 4-4A). This bipolar budding pattern is typical of diploid cells. On YEPD plus 0.4 M NaCl, the percentage of C quadrant cells increased while that of A quadrant cells decreased (Figure 4-4B). Similar effects were observed with high concentrations of glucose or sorbitol, showing that increasing osmolarity increases the probability of budding at a site on the opposite side of the diploid cell from the previous bud site. This effect was not observed in haploid cells. Compared to wild-type the $hog1\Delta$, $hog2\Delta$, and $pbs2\Delta$ mutants each showed a significantly different budding pattern on NaCl plates. However, when the relative frequency of budding at same-side (A quadrant) versus opposite-side (C quadrant) was examined, only $hog1\Delta$ and $pbs2\Delta$ mutants showed a significantly different distribution from wild-type. Thus, $HOG1$ and $PBS2$ are required for retention of the normal positioning of emerging buds on the surface of the mother cell after osmotic stress.

Cytokinesis defect in $hog1\Delta$ and $pbs2\Delta$ mutants:

When shifted to YEPD plus 400 mM NaCl, $hog1\Delta$ and $pbs2\Delta$ cells form elongated buds. Cells with an elongated bud often have an associated defect in separating cells following mitosis (Gimeno et al, 1992, Healy et al, 1991). To analyze the effect of osmotic stress on cell separation, the percentage of unbudded cells was determined in asynchronous cultures at various times after addition of NaCl to 0.4 M (Figure 4-5A).
Figure 4-5: Time course of bud emergence before and after osmotic stress. A) Samples of liquid cultures in YEPD were transferred to YEPD containing 0.4M NaCl. Aliquots of the cultures were taken before and every hour after shifting to high salt. The percentage of unbudded cells was determined microscopically using a hemocytometer. (B) Flow cytometry analysis of cellular DNA content in wild-type and mutant cultures after osmotic upshift. The duration of time from the transfer of cells into YEPD plus 0.4 M NaCl (from YEPD) to the time ethanol was added to fix the cells (see Methods). The procedure for propidium iodide staining of cellular DNA is in Methods. Diploid strains were: wild-type (YPH102D), hog1 (JBY13D), hog2 (JBY23D), or pbs2 (JBY43D).
A

% UNBUDDED CELLS

HOURS IN 400 mM NaCl

B

Cell Number

0h  2h  4h

Fluorescence

wt  hog1  hog2
Wild-type and hog2Δ cultures showed a small but significant increase in the percentage of unbudded cells at five hours after NaCl addition. The increase in unbudded cells most likely reflects a slower growth rate. In contrast, the percentage of unbudded cells in hog1Δ and pbs2Δ cultures decreased until all cells had at least one bud. This decrease reflected continued bud initiation plus a failure of large buds to separate from the mother cell.

To determine whether the multiple rounds of budding of hog1Δ mutant cells in high osmolarity medium are accompanied by rounds of DNA replication and mitosis, cells were stained with propidium iodide and the distribution of cellular DNA content determined by flow cytometry (Figure 4-5B). Cells that had finished cytokinesis but were still attached to one another were separated by sonication after fixation. Immediately after transfer from YEPD to YEPD plus 0.4 M NaCl, wild-type, hog1Δ, and hog2Δ cultures had similar amounts of cells with a 2N (left peak) and 4N (right peak) DNA content. At 2 and 4 hours after adding 0.4 M NaCl, the wild-type and hog2Δ cultures contained a higher percentage of 2N cells. In contrast, the hog1Δ culture exposed to NaCl accumulated cells with a 4N or higher content of DNA. Propidium iodide-stained DNA in cells from this latter culture was viewed by fluorescence microscopy, revealing cells with multiple nuclei, usually with one nucleus per cell body (Figure 4-6). pbs2Δ cells gave similar results to hog1Δ (not shown). hog1Δ and pbs2Δ mutant cells therefore go through multiple rounds of bud formation and nuclear division in high osmolarity media without completing cytokinesis.
Cell morphogenesis of *hog1Δ* and *pbs2Δ* mutants:

When grown on the rich medium YEPD, the cell morphology of the diploid *hog1Δ* and *pbs2Δ* strains was similar to that of a congenic diploid wild-type strain (Figure 4-7A). However, when the same strains were transferred to YEPD supplemented with 0.9 M NaCl, *hog1Δ* and *pbs2Δ* cells arrested growth after 1-2 days (wild-type continued to grow) with a similar terminal cell morphology (Figure 4-7B): large cells with multiple elongated buds with branches or constrictions along the length of the buds. This aberrant cell morphology was also observed when *hog1Δ* or *pbs2Δ* cells were transferred to YEPD medium supplemented with 1.2 M KCl or 1.8 M sorbitol. In contrast to *hog1Δ* and *pbs2Δ*, the *hog2Δ* diploid strain arrested growth in high osmolarity medium as unbudded cells.

The aberrant cell morphology of *hog1Δ* and *pbs2Δ* mutants was only observed in high osmolarity medium. Abnormally-shaped cell projections developed more rapidly when cells were exposed to smaller increases in external osmolarity. When transferred from YEPD to YEPD plus 0.4 M NaCl, the *hog1Δ* and *pbs2Δ* mutant cells grew within hours into abnormally-shaped cells similar to those in Figure 4-7B. The mutants continued to grow in the latter medium but at a rate slower that wild-type. The *hog1*- or *pbs2*-dependent development of abnormally-shaped cells was observed in both liquid and solid high osmolarity medium, at growth temperatures between 21-37°C and in haploid (*MATa* or *MATα*) or diploid (*MATa/MATα*) cells. The cell morphology of *hog1Δ* and *pbs2Δ* is therefore dependent upon the osmolarity of the media. The reason for this
highly aberrant morphology is not known for certain, but may result from a combination of changes in cell growth and division (Figures 4-1 through 4-6) occurring immediately after osmotic stress was applied.

**Summary:**

The two major findings of the morphological characterization of *hog1Δ* and *pbs2Δ* are the following: (a) after a short adjustment period, bud growth of yeast cells is relatively unaffected by an increase in osmolarity and (b) cells lacking a functional osmotic response pathway have several osmotic-dependent morphogenetic defects. We suggest an explanation for these results is that the MAP kinase-mediated osmotic response pathway is responsible for homeostatic adjustment of cell morphogenesis in response to osmotic stress. These findings and their interpretation are discussed in Chapter 5.
Figure 4-6: The *hog1A* and *pbs2A* mutants display multiple nuclei under osmotically stressed conditions. Cells were grown in liquid YEPD + 0.6 M NaCl for 24 hours, fixed in 70% ethanol, incubated with RNaseA, and the nuclear DNA stained with propidium iodide. Photographs were taken of cells using phase contrast (A,C), or fluorescence (B,D) microscopy under 1000X magnification. Strains were *hog1A* (JBY13D; A,B) and *pbs2A* (JBY43D; C,D).
Figure 4-7: Osmotic-sensitive \textit{hog} mutants have different terminal cell morphologies in high osmolarity media. The \textit{hog} mutants are morphologically normal in rich media but are aberrant under osmotically stressed conditions as multibudded, elongate cells (\textit{hog1}Δ and \textit{pbs4}Δ) or as unbudded cells (\textit{hog2}Δ). All cells shown are diploid: wild-type (YPH501), \textit{hog1}Δ (JBY13D), \textit{hog2}Δ (JBY23D), or \textit{pbs2}Δ (JBY43D). Cells were grown on plates composed of A) YEPD or B) YEPD + 0.6 M NaCl for 24 hours at 30 °C. Cells were photographed under 400X magnification.
**Materials and Methods:**

**Photomicroscopy:**

Yeast cultures were grown to log-phase (\(\sim 1 \times 10^7\) cells/ml) in YEPD at 30\(^\circ\)C. 20 \(\mu l\) of each culture was spotted onto an agar plate containing a specific growth medium. Fifteen to twenty individual cells with a common morphology were then moved by micromanipulation to a small area of the plate where cells were placed 3-5 cell diameters apart. Plates were incubated at 30\(^\circ\)C and the same cells photographed every hour for up to 6 hours using Kodak T-MAX film. Media used in agar plates was either YEPD or YEPD supplemented with 0.4M NaCl, 0.6 M NaCl, 0.6 M sorbitol, or 0.6 M glucose. In experiments where galactose was the carbon source, the medium contained 2% galactose in place of 2% glucose.

To determine the percentage of unbudded cells in osmotically stressed cells, log-phase cultures were shifted from YEPD to YEPD supplemented with 0.4 M NaCl by a 1/1 dilution of the culture with YEPD supplemented with 0.8M NaCl. Aliquots from 1 hour time points were fixed by addition of formaldehyde to 3.7%, sonicated to separate cells, and the number of total cells and unbudded cells determined using a hemocytometer. The percentage of unbudded cells was determined from a total of \(\sim 100\) cells for each time point. Three independent cultures of each strain was used to analyze the time course.
Rhodamine-phalloidin staining of actin filaments:

The staining of actin filaments in wild-type and mutant cells was performed as described (Adams et al., 1991, Chowdhury et al., 1992). Log-phase cultures in YEPD were shifted to fresh YEPD plus 0.4M NaCl as described above. At 30 minute intervals, 1 ml aliquots were removed and 110 µl of 37% formaldehyde added. Following a 30 minute incubation at room temperature, cells were collected by centrifugation, resuspended in PBS plus 3.7% formaldehyde, incubated for an additional hour, and then washed 3X with PBS. Aliquots of the final cell suspension in PBS were centrifuged, the cell pellet resuspended in 10 µl of a 300 U/ml rhodamine-phalloidin solution and incubated at room temperature in the dark for 90 minutes. Stained cells were washed 3X in PBS, resuspended in 20 µl of mounting media (70% glycerol saturated with p-phenylenediamine), and 2 µl aliquots of this suspension were viewed by phase contrast and fluorescence microscopy. Kodak T-MAX 100 was used for photography.

Propidium iodide staining of cell DNA:

The procedure for fixation of cells and propidium iodide staining of DNA was provided by M. Winey (University of Colorado-Boulder, CO). Log-phase cultures in YEPD were shifted to YEPD plus 0.4M NaCl as described above. 1 ml aliquots were removed at various time points, spun down, and resuspended in 70% ethanol. Following a 1 hour incubation at room temperature, the cells were washed twice in FACS buffer (200 mM Tris, 20 mM EDTA, pH 7.5), resuspended in 100 µl of FACS buffer containing 1 mg/ml RNase, and incubated at 37°C for 2 hours. The cells were then
washed twice with PBS and resuspended in 100 \( \mu l \) PBS. Propidium iodide was then added to a final concentration of 50 \( \mu g/\mu l \) and the staining allowed to proceed for 30 minutes at room temperature in the dark. Cells were then diluted with 900 \( \mu l \) of PBS and either observed by fluorescence microscopy or used to determine the distribution of cellular DNA content by flow cytometry.
Chapter 5: Discussion

At least three genes are required for yeast osmoregulation:

This study has identified three genes, \textit{HOG1}, \textit{HOG2}, and \textit{PBS2} in \textit{Saccharomyces cerevisiae} that are required for cellular growth under osmotically stressed conditions. Each gene was cloned by complementation of recessive mutations and its identity confirmed through standard molecular genetic techniques. Disruption mutations generated for all three genes revealed sensitivity to as little as 0.2M NaCl and salt induced glycerol accumulation only $\sim 35\%$ of that seen in wild-type cells. Double mutant analysis of growth and glycerol accumulation under osmotically stressed conditions suggested a similar role for \textit{HOG1} and \textit{PBS2}, as cells carrying mutations in both genes were phenotypically indistinguishable from either single mutant. However, double mutants containing \textit{hog1}$\Delta$ or \textit{pbs2}$\Delta$ with \textit{hog2}$\Delta$ displayed lower glycerol accumulation and increased salt sensitivity than any of the three single mutants. \textit{HOG2} was therefore shown to have a role in osmoregulation which is independent of \textit{HOG1} and \textit{PBS2}, identifying at least two mechanisms required for yeast response to osmotic stress. Recent work has identified that osmotic-stress induction of \textit{HOG2} mRNA levels (described below, Nye et al, 1993), is \textit{HOG1} dependant. These data combined with the glycerol assays suggest that basal levels of \textit{HOG2} mRNA expression, observed in \textit{hog1}$\Delta$ cells, aids the accumulation of glycerol to some degree, making \textit{hog1}$\Delta$ (or \textit{pbs2}$\Delta$) less osmosensitive than the \textit{hog1}$\Delta$\textit{hog2}$\Delta$ (or \textit{pbs2}$\Delta$\textit{hog2}$\Delta$) double mutant.
Sequence analysis revealed *HOG1* to be a previously unidentified gene with 50% amino acid identity to *FUS3* and *KSS1*, MAP kinases in the yeast mating response pathway. *HOG4* was identified by several tests including reciprocal cross complementation, restriction mapping, and partial sequence analysis to be identical to the previously identified *PBS2* (Brewster et al, 1993). The DNA sequence of *HOG4/PBS2* revealed strong homology between *PBS2* and *STE7*, a MAP kinase kinase or MAP activator. *STE7* has been identified as the kinase upstream of *FUS3* in the mating response pathway and has been shown to activate *FUS3* through phosphorylation of tyrosine and threonine (Gartner et al, 1992). *HOG2* was identified by sequence identity as identical to *TPS2* (Nye et al, 1993), a subunit of the enzyme trehalose synthase (de Virgillo, et al 1993). Characterization of the *HOG2* role in stress osmoregulation and stress response is currently underway.

**HOG1 is tyrosine phosphorylated in response to salt stress:**

The sequence homologies of *HOG1* and *PBS2* to kinases involved in the mating response pathway suggested a role for these genes in signal transduction. The predicted Hog1p amino acid sequence identified the highly conserved M-T*-X-Y*-V-X-T-R-X-Y-R-A-P-E found in all MAP kinases. Phosphorylation of threonine and tyrosine (indicated with an asterisk), has been identified in several MAP kinases as required for activation (see section III). Immunoblots of wild-type cells and cells overexpressing Hog1p revealed a protein (mobility ~55kDa) which is reversibly phosphorylated on tyrosine following osmotic stress. The phosphorylation is very fast, occurring to near maximal
levels within the first 60 seconds following salt stress. The identity of the band was confirmed as being Hog1p through the overexpression of a truncated protein (10 kDa removed) which displayed similar salt dependant tyrosine phosphorylation. Overexpression of Hog1p in a pbs2Δ mutant revealed no tyrosine phosphorylation, a result consistent with Pbs2p being an upstream activator of Hog1p in a relationship similar to that between STE7 and FUS3.

**Amino acid residues required for HOG1 function:**

Site-directed mutagenesis was employed to determine whether residues in Hog1p, that are homologous to residues required for regulation of activity in related kinases, play a similarly-important role in Hog1p function. **HOG1**-tyrosine\(^ {176} \), which is predicted by homology to be phosphorylated as a requirement for activation of kinase activity, was mutated to phenylalanine. Immunoblots of cells carrying the hog1-Y176F mutant in either single or high copy, revealed no detectable tyrosine phosphorylation of Hog1p. This is consistent with tyrosine\(^ {176} \) being the residue phosphorylated in response to osmotic stress. Activation of MAP kinases requires a second phosphorylation event on the conserved upstream threonine (T\(^ \tau \)-X-Y\(^ \tau \)). A conservative substitution was introduced into **HOG1** at threonine\(^ {174} \), which corresponds to the residue required for function in related MAP kinases. Autophosphorylation on tyrosine and threonine has been identified in the rat MAP kinases, **ERK1** and **ERK2**, and is proposed to play a role in their activation (Seger et al, 1991). To evaluate tyrosine autophosphorylation of Hog1p following salt stress, a conserved residue required for catalytic activity in related kinases, lysine\(^ {51} \), was
mutated to arginine. Immunoblots of hog1Δ cells containing the hog1-K51R mutant revealed a salt-stress inducible tyrosine phosphorylation of Hog1p, consistent with a second kinase phosphorylating Hog1p in response to salt stress.

All three mutants were evaluated for an ability to rescue growth of hog1Δ cells under osmotically stressed conditions. Cells carrying the hog1-T174A mutant, in single or high copy, displayed no detectable increase in cell viability on YEPD containing 0.4 or 0.9 KCl (NaCl gave similar results). However, cells carrying the hog1-Y176F and hog1-K51R mutants in single or high copy, did display some growth on YEPD containing 0.4M NaCl or KCl. When these cells were tested on YEPD containing 0.9M NaCl or KCl little or no growth was observed, which was similar to hog1Δ cells transformed with a control plasmid. Each mutant resulted in cells that were osmotically sensitive and displayed the hog1Δ terminal morphology following salt stress. Lysine51, threonine174, and tyrosine176 are therefore required for HOG1 dependant cellular responses to osmotic stress and likely function in manner described in related MAP kinases.

A HOG1 dependant feedback loop downregulates signalling cascade:

Western analysis of the hog1-K51R and hog1-T174A mutants revealed an inducible hyperphosphorylation of each protein following salt stress (Figure 3-10). Wild-type Hog1p phosphorylation is assayed on the same blot and reveals an intensity dramatically lower than either mutant. This result identifies a down regulation of the signal transduction pathway which is dependant upon an active HOG1 protein. MAP
kinase cascade down regulation is currently not well understood though results from the mating arrest pathway in *S. cerevisiae*, and the EGF stimulated cascade in mammalian cells have offered some insight.

Haploid yeast cells will recover from growth arrest to resume mitotic growth, even in the presence of pheromone, if mating doesn’t occur within a certain amount of time (Moore, 1984). Desensitization occurs through multiple mechanisms including pheromone degradation by secreted proteases (Ciejek and Thorner, 1979), endocytosis of bound receptors (Chvatchko et al, 1986), and ligand induced phosphorylation of the receptor (Blumer et al, 1988, Reneke et al, 1988). Deletion of the receptor carboxy terminus, identified as phosphorylated in pheromone stimulated cells, results in cells that are 100-times more sensitive to pheromone and are unable to recover from the induced growth arrest (N. Davis and G. Sprague, unpublished results). Whether the observed receptor phosphorylation is responsible for lowering cellular sensitivity to mating factor is not yet clear. *FUS3*, a MAP kinase in the mating arrest pathway, has been shown to regulate its own phosphorylation. This suggests a feedback loop in which Fus3p down regulates the upstream kinase or the membrane-associated receptor through phosphorylation events (Gartner et al, 1992). The mammalian MAP kinase pp42, has been shown to regulate the activity of a tyrosine phosphatase which acts on the EGF receptor (Griswold-Prenner et al, 1993). Identification of how the MAP dependant tyrosine dephosphorylation influences receptor activity is under investigation. Both of these examples point towards a role of kinases in downregulation of signalling pathways.
Results from the immunoblots of the \textit{hog1-K51R} and \textit{hog1-T174A} mutants indicate a similar role for either Hog1p or a downstream component of the cascade in the formation of a feedback loop to turn down the signalling pathway.

\textbf{The osmotic stress response pathway affects gene expression:}

Following exposure to osmotic stress the activities of several enzymes have been shown to increase. Expression of catalase, which converts H\textsubscript{2}O\textsubscript{2} to water and oxygen, is derepressed in response to nitrogen starvation, and activated following heat shock, osmotic stress, or oxidative stress (Marchler et al, 1993). \textit{hog1Δ}, \textit{hog2Δ} and \textit{pbs2Δ} mutants were assayed for catalase induction following exposure to a osmotic stress. Both \textit{hog1Δ} and \textit{pbs2Δ} cells displayed only basal levels of catalase activity while wild-type and \textit{hog2Δ} cells displayed a 40-fold induction (C. Schuller and M. Gustin, unpublished results). \textit{HOG2} mRNA levels increase in response to heat shock, and osmotic stress (Nye et al, 1993). In \textit{hog1Δ} cells, osmotic stress has no affect on \textit{HOG2} mRNA while heat shock induces levels as in wild-type cells (Nye and Gustin, unpublished results).

Glycerol-3-phosphate dehydrogenase and glycerol-3-phosphatase are also induced by increases in external osmolarity (Blomberg and Adler, 1989). Analysis of \textit{hog1Δ} cells (\textit{pbs2Δ}, \textit{hog2Δ} not yet tested), revealed a salt inducible increase in glycerol-3-phosphate dehydrogenase activity only \(\sim 50\%\) of that observed in wild-type cells (L. Adler, unpublished results). Both wild-type cells and \textit{hog2Δ} cells displayed glycerol-3-phosphatase activity which increased incrementally with external osmolarity. Suprisingly,
similar assays in hog1Δ and pbs2Δ cells detected a decrease in glycerol-3-phosphatase activity following salt stress (L. Adler, unpublished results). These results show that the osmotic stress response pathway affects expression of at least four genes. One target of the kinase cascade must therefore be the nucleus.

Influences of HOG1 and PBS2 upon cell morphogenesis:

When osmotically stressed, cells lacking HOG1 or PBS2 failed to show the same patterns of cell growth and division as wild-type cells to osmotic stress. The most striking changes in bud morphogenesis occurring in hog1Δ and pbs2Δ mutants were (a) a failure to remember where to resume bud growth after an increase in osmolarity and (b) an osmotic-dependent defect in bud site selection. This novel phenotype, not previously described in yeast, suggests that the HOG pathway regulates cell morphogenesis in response to an increase in external osmolarity.

Wild-type cells with a small bud temporarily stopped bud growth after an upshift in osmolarity, but then resumed growth of the same bud at a later time. In contrast, hog1Δ and pbs2Δ cells with a small bud almost never resumed growth of this bud after an increase in osmolarity, but initiated the growth of a new bud (Table 5). These temporal and spatial changes in the growth of wild-type and mutant cells after osmotic stress were correlated with the intracellular organization of actin filaments, as summarized in Figure 5-1. An increase in external osmolarity induced the redistribution of actin patches out of small buds in both mutant and wild-type cells. Later, actin patches
returned to pre-existing buds of the wild-type but not the \textit{hog1\Delta} or \textit{pbs2\Delta} cells. These observations suggest that topological information is retained in the bud of the wild-type cell after initial exposure to osmotic stress; this information is required for the post-recovery restoration of actin filaments and growth to the bud. \textit{hog1\Delta} and \textit{pbs2\Delta} cells lack this information, forget where to resume growth, and abandon the pre-existing bud. The increased randomness of the budding pattern in haploid \textit{hog1\Delta} and \textit{pbs2\Delta} cells after an increase in external osmolarity (Figure 4-3, 4-4) also points to a loss of specific topological information. The biochemical nature of this spatial information is unknown but could involve changes in the amounts or activities of proteins that regulate bud emergence or bud site selection. The ability of the \textit{bud} mutants to correctly resume growth of their buds after osmotic stress argues against the latter possibility. One interpretation of these results is that the osmotic stress response pathway is directly responsible for retaining information about where to resume growth after a change in osmolarity. Loss of \textit{HOG1} or \textit{PBS2} also leads to other osmotic-dependent defects such as reduced cytokinesis and cell elongation. Our results cannot distinguish whether these later-appearing defects are related to the \textit{hog1\Delta}- and \textit{pbs2\Delta}-induced loss of positional information or represent a separate function of \textit{HOG1} and \textit{PBS2}.

Different stresses induce similar changes in actin filament distribution in yeast cells. Osmotic stress, an upshift in temperature, and change of carbon source all induce the reversible loss of actin filament cables and the redistribution of cortical patches of actin filaments from bud to mother cell and, later, back to the bud. \textit{hog1\Delta} and \textit{pbs2\Delta}
Figure 5-1: Overview of the effect of an osmolarity upshift on the yeast actin cytoskeleton and bud growth in a wild-type and a *hog1Δ* or *pbs2Δ* mutant. Following osmotic stress, growth is arrested with an associated delocalization of actin patches and filaments from the bud. Growth later resumes in wild-type cells, with actin reoriented into the previously initiated bud. *hog1Δ* and *pbs2Δ* mutants also attempt to grow following an arrest, but initiate growth in a new, aberrantly shaped bud.
normal growth

osmotic stress

growth arrest

HOG1 PBS2

recovery

hog1 pbs2
cells behave like wild-type cells in response to heat stress and changes in carbon source. Only in response to osmotic stress are \textit{hog1}\textDelta and \textit{pbs2}\textDelta cells defective in restoring actin filaments and growth to the pre-existing bud. Thus, the \textit{hog1}\textDelta and \textit{pbs2}\textDelta mutations do not interfere with all stress-induced morphogenetic responses, but affect specifically those responses to osmotic stress. It is also important to note that other more indirect mechanisms could explain the effect of \textit{hog1}\textDelta and \textit{pbs2}\textDelta on bud morphogenesis in high osmolarity media. For example, \textit{HOG1} and \textit{PBS2} are also required for normal osmoregulation: the increase in intracellular glycerol after an increase in external osmolarity. The morphogenetic changes observed in \textit{hog1}\textDelta and \textit{pbs2}\textDelta cells could be a secondary result of reduction in osmotic-dependent glycerol accumulation in these mutants. We have used a \textit{hog2}\textDelta mutant to attempt to address this possibility. All three mutants have similar effects on osmotic-dependent glycerol production (see section II), yet the morphogenesis of \textit{hog2}\textDelta cells in high osmolarity media was very similar to that of wild-type cells and not like that of \textit{hog1}\textDelta and \textit{pbs2}\textDelta cells. We conclude therefore that a reduction in the amount of osmotic-sensitive glycerol accumulation is not correlated with osmotic-sensitive changes in bud morphogenesis.

\textbf{Summary:}

As summarized in Figure 5-2, the osmotic stress response pathway influences a variety of cellular responses. Activation of the kinase cascade results in increased expression of several genes, an accumulation of intracellular glycerol, and influences upon bud initiation and morphogenesis. The signal most likely begins at the cell
membrane and may employ stretch-activated channels which have been detected in a variety of systems. Recent reports have linked a stretch-activated channel to detection/signalling of osmotic stress in rat neurons (Ollet and Bourque, 1993). The complexity of related kinase cascades (mating arrest, protein kinase C), will likely be seen in the osmotic stress response pathway, especially due to the multiple phenotypes thus far identified. Future studies will hopefully identify the remaining components of the pathway to benefit an understanding of stress response and growth control in all cells.
Figure 5-2: Summary of osmotically induced signal transduction in *Saccharomyces cerevisiae*. Activation of the *HOG1* and *PBS2* dependant signal transduction signalling pathway results in a variety of downstream affects (osmoregulation, gene expression, bud initiation, bud morphology). See text for discussion.
References


Thomas, G., MAP kinase by any other name smells just as sweet, *Cell*, 68:3-6, 1992.


