EUKARYOTIC CELL, Aug. 2005, p. 1343–1352 Vol. 4, No. 8
1535-9778/05/$08.00+0 doi:10.1128/EC.4.8.1343–1352.2005
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Genomewide Identification of Sko1 Target Promoters Reveals a Regulatory Network That Operates in Response to Osmotic Stress in *Saccharomyces cerevisiae*†

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Received 25 April 2005/Accepted 3 June 2005

In *Saccharomyces cerevisiae*, the ATF/CREB transcription factor Sko1 (Acr1) regulates the expression of genes induced by osmotic stress under the control of the high osmolarity glycerol (HOG) mitogen-activated protein kinase pathway. By combining chromatin immunoprecipitation and microarrays containing essentially all intergenic regions, we estimate that yeast cells contain approximately 40 Sko1 target promoters in vivo; 20 Sko1 target promoters were validated by direct analysis of individual loci. The ATF/CREB consensus sequence is not statistically overrepresented in confirmed Sko1 target promoters, although some sites are evolutionarily conserved among related yeast species, suggesting that they are functionally important in vivo. These observations suggest that Sko1 association in vivo is affected by factors beyond the protein-DNA interaction defined in vitro. Sko1 binds a number of promoters for genes directly involved in defense functions that relieve osmotic stress. In addition, Sko1 binds to the promoters of genes encoding transcription factors, including Msn2, Mot3, Rox1, Mga1, and Gat2. Stress-induced expression of *MSN2*, *MOT3*, and *MGA1* is diminished in *sko1* mutant cells, while transcriptional regulation of *ROX1* seems to be unaffected. Lastly, Sko1 targets *PTP3*, which encodes a phosphatase that negatively regulates Hog1 kinase activity, and Sko1 is required for osmotic induction of *PTP3* expression. Taken together our results suggest that Sko1 operates a transcriptional network upon osmotic stress, which involves other specific transcription factors and a phosphatase that regulates the key component of the signal transduction pathway.

The response of eukaryotic cells to environmental stress involves complex changes of gene expression. For example, the adaptation of *Saccharomyces cerevisiae* to hyperosmotic shock leads to the transient transcriptional induction of more than 150 genes (32, 38, 49). The HOG (high osmolarity glycerol) mitogen-activated protein (MAP) kinase signaling pathway is essential for the efficient up-regulation of the vast majority of genes in response to osmotic stress (5). The terminal MAP kinase, Hog1, accumulates in the nucleus within minutes after exposure to hyperosmolarity (7, 36), whereupon it interacts with and phosphorylates transcription factors Sko1 (33), Hot1 (40), and Smp1 (6). Additionally, the general stress-responsive transcriptional activators Msn2 and Msn4, as well as Msn1, have been genetically placed downstream of Hog1 kinase (25, 37). Therefore, considerable complexity exists at the level of the specific transcription factors which participate in the yeast osmostress response.

The molecular functions of activated Hog1 kinase in transcriptional stimulation are surprisingly complex. Activated Hog1 is recruited to the promoters of osmoinducible genes by specific transcription factors (1, 35). In the case of Sko1, which acts as a repressor of osmoinducible genes via the general corepressor complex Cyc8-Tup1 (34), the association with Hog1 leads to its phosphorylation and the conversion to an activator (33, 35). This repressor/activator switch involves the additional recruitment of the chromatin-modifying complexes SAGA and Swi/Snf, but it does not result in the dissociation of Cyc8-Tup1 (35). Furthermore, the nuclear localization and repressor functions of Sko1 are regulated by protein kinase A (31), which phosphorylates Sko1 at multiple sites in vitro (33).

The redundant operation of various transcription factors in the transcriptional osmostress program makes this adaptive response difficult to describe by genomic profiling experiments. Subsets of Hot1- and Msn2/4-dependent genes have been identified by expression-based microarrays (38), but these represent a small proportion of the genes induced by hyperosmotic shock. It is clear that understanding the yeast osmostress response will require knowledge of the physical interactions of the transcriptional regulators with their genomic targets during stress.

Here, we used genomewide location analysis to identify the in vivo target promoters of Sko1. Our results differ substantially from an initial genome-wide location analysis (22), but are in good accord with a report that appeared after the work described here was completed (11). We validate 20 Sko1 target sites by direct chromatin immunoprecipitation experiments and demonstrate that Sko1 is important to various extents for osmotic induction of genes encoding transcription factors involved in other stress responses and Ptp3, which encodes a phosphatase that regulates Hog1 kinase. Our results reveal important information about the biological function of Sko1 and about transcription factor networks that operate after osmotic stress.

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MATERIALS AND METHODS

Yeast strains. Yeast strain MAP37 (33) expressing a fully functional trihemagglutinin [$(HA)_3$]-Sko1 fusion was used for chromatin immunoprecipitation experiments. Control chromatin immunoprecipitation assays were performed with the untagged parental strain W303-1A (*MAT***a** *ura3 leu2 trp1 his3 ade2*). Analysis of mRNA levels was performed using wild-type W303-1A, *sko1* (MAP19), and *hog1* (MAP32) mutant strains (33).

Chromatin immunoprecipitation. Cells were grown in rich medium (YPD) to an optical density at 600 nm of 0.8. Chromatin immunoprecipitation was performed essentially as described previously (20), except that insoluble material was removed from the broken cells by 2 min centrifugation in a minicentrifuge. After sonication, soluble chromatin fragments were obtained by spinning for 30 min in a minicentrifuge. $(HA)_3$-Sko1 was immunoprecipitated with antibodies against the HA epitope (12CA5 ascites). Input and immunoprecipitated samples were assayed by quantitative PCR in real time using the Applied Biosystems 7700 sequence detector. An internal fragment of the *POL1* gene (nucleotides 2499 to 2717) was used as a negative control.

For targeted chromatin immunoprecipitation experiments, immunoprecipitations were performed on three independent chromatin preparations. Immunoprecipitation efficiencies were calculated in triplicate by dividing the amount of PCR product in the immunoprecipitated sample by the amount of PCR product in the input sample. All data (Fig. 1 and Table 1) are presented as fold immunoprecipitation over the *POL1* coding sequence control.

Primers were used to quantitate the following DNA regions: *ALD6* (−647/−317, STR6486/6487), *YOR246C* (−479/−288, STR9973/9974), *PUT4* (−617/−372, STR6989/6990), *FAA1* (−827/−576, STR6993/6994), *CWP1* (−413/−187, STR6484/6485), *RPI1* (−606/−451, STR6578/6579), *STL1* (−698/−416, STR4680/4681), *PTP3* (−649/−338, STR6478/6479), *MGA1* (−563/−421, STR6582/6583), *SED1* (−836/−510, STR6476/6477), *ROX1* (−843/−629, STR9969/9970), *UTH1* (−304/−108, STR9975/9976), *YAP1802* (−608/−371, STR9991/9992), *MOT3* (−677/−413, STR7321/7322), *GAT2* (−788/−643, STR6588/6589), *MSN2* (−846/−631, STR6482/6483), *SOR1* (−843/−601, STR6987/6988), *HXT5* (−573/−173, STR6983/6984), *SPO20* (−590/−265, STR7895/7896), *CPA1* (−752/−553, STR9959/9960), *ICY1* (−887/−655, STR9967/9968), *HOR7* (−291/−73, STR9963/9964), *YPR127W* (−269/−54, STR9977/9978), *TYE7* (−645/−409, STR6580/6581), *GAC1* (−791/−568, STR6981/6982), *DPM1* (−687/−481, STR9985/9986), *POS5* (−448/−224, STR9989/9990), *RSN1* (−403/−169, STR9979/9980), *EFT1* (−798/−568, STR9981/9982), *MFα1* (−318/−127, STR9987/9988), *HIR2* (−274/−38, STR9983/9984), *MLH3* (−886/−669, STR9965/9966), *FOL1* (−338/−159, STR9961/9962), and open reading frame free region on chromosome V (STR5332/5333). Primer sequences are available upon request.

Microarray hybridization. Microarray preparation, amplification of total and immunoprecipitated DNA, fluorescent labeling and microarray hybridization were performed as described previously (29). For chromatin immunoprecipitation analysis of Sko1, three independent chromatin samples were immunoprecipitated and the resulting DNA fragments amplified and labeled with Cy5 fluorescent dye (Amersham Biosciences). Three independent total chromatin samples were similarly amplified and labeled with Cy3 fluorescent dye. Microarrays were hybridized with a mixture of labeled fragments from immunoprecipitated and total chromatin samples. Slides were scanned on an Axon scanner, and data were analyzed with Axon GenePix 4.0 software. Raw data were then further analyzed in Microsoft Excel. Poor-quality or undetectable DNA spots were removed and the data were normalized for equal background-subtracted median fluorescence of Cy3 and Cy5 over the entire arrays. Spots were ranked in descending order by their median Cy5/Cy3 ratios (see the supplemental material).

Feature names were obtained from the *Saccharomyces* Genome Database (14). Statistical significance was computed for each spot using the Chipper software (9) for each spot to describe their likelihood of being bound by Sko1. Individual *P* values were then combined by Stouffer's method and are given in Fig. 1 and Table 1.

We tested some of the intergenic regions that were identified over different *P* value ranges by standard chromatin immunoprecipitation analysis. We regarded a DNA sequence to be bound by Sko1-HA when the respective fragment was at least 2.5-fold enriched over the unbound *POL1* control and when there was no enrichment in the absence of the HA epitope. Some intergenic regions showed slight enrichment (about twofold) independently on the presence of the HA antibody and therefore represent unspecific antibody recognition or just "sticky" DNA fragments.

Sequence analysis. The AlignAce program (13, 41) was used to identify common motifs across multiple promoter regions. CRE sites were searched using the DNA pattern feature of the RSA-tools webpage (http://rsat.ulb.ac.be/rsat/dna-pattern.cgi).

Analysis of mRNA levels. Yeast strains were grown in YPD to an optical density at 600 nm of 0.8 and were treated or not with 0.4 M NaCl for 10 min. Total RNA was extracted from 50 ml of culture by acid phenol treatment (15) and DNase digested (RQ1 DNase, Promega). Total RNA was reverse transcribed using Superscript II reverse transcriptase (Invitrogen) following the manufacturer's instructions and RNase treated (Roche). As a control, each sample was additionally mock treated (without reverse transcriptase). Appropriately diluted samples were analyzed by quantitative PCR in real time using primers amplifying *TBP1* (nucleotides 521 to 677), *MSN2* (nucleotides 1671 to 1886), *MSN4* (nucleotides 1505 to 1658), *PTP3* (nucleotides 2513 to 2680), *PTP2* (nucleotides 1997 to 2201), *MOT3* (nucleotides 1127 to 1432), *MGA1* (nucleotides 1143 to 1280), and *ROX1* (nucleotides 903 to 1088).

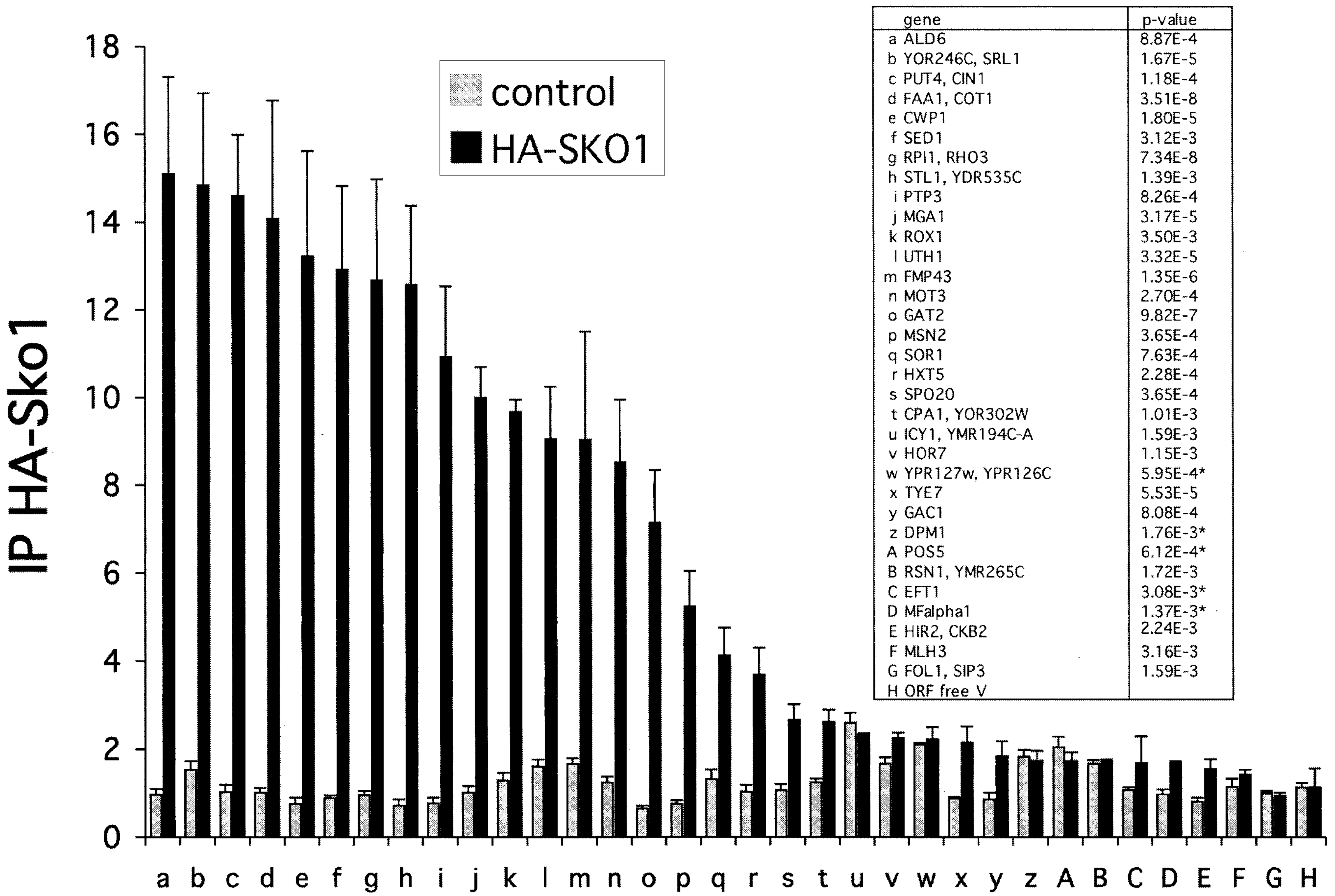
RESULTS

Genomewide location analysis reveals approximately 40 Sko1 target regions in vivo. To define Sko1 targets in vivo, we performed chromatin immunoprecipitation of an $(HA)_3$-Sko1-expressing strain under exponential growth in rich medium. Input and immunoprecipitated samples from three independent experiments were amplified and hybridized with microarrays, which contained almost all yeast intergenic regions (29). A *P* value measuring the statistical significance of the Sko1 interaction with each intergenic region was obtained using the Chipper data analysis program (9). As our experiments are performed under conditions in which Sko1 acts as a repressor, it is formally possible that we might miss targets that are observed only under osmotic stress, when Sko1 functions as an activator. However, this is unlikely to be a major problem, because the Sko1 target promoters previously tested show similar binding under repressing and inducing conditions (35), and the Sko1 phosphorylation sites map far from the DNA-binding domain (33).

We created a ranked list of putative Sko1-promoter interactions, which contains 33 intergenic regions with a *P* value of $<10^{-4}$ (group I) and 34 intergenic regions with a *P* value of between 10^{-4} and 0.004 (group II) based on a minimum of two independent replicate experiments.

Direct analysis by standard chromatin immunoprecipitation (Fig. 1) confirmed Sko1 binding to 16 out of 18 group I targets (89%) and 4 out of 10 group II (40%) as defined by >2.5-fold enrichment of the respective locus comparing HA-Sko1-tagged and untagged control strains. The two group I regions that did not pass the test (*TYE1* and *GAC1*) were weakly enriched by HA-Sko1, but at a level just below the 2.5-fold cutoff. HA-Sko1 binding was not observed to five regions characterized as being in group I or II based on just one experiment, indicating that results based on one experiment are not reliable. Three group II regions (*ICY1*, *HOR7*, and *RSN1*) were enriched independently of Sko1-HA (about twofold), probably due to cross-reactivity of the HA antibody with other cross-linked proteins in the chromatin preparations or to the general stickiness of these genomic regions during the immunoprecipitation procedure. The existence of these nonspecifically enriched regions emphasizes the importance of performing comparisons between tagged and untagged strains.

Based on the percentage of confirmed targets of those tested from groups I and II, we estimate that there are approximately 40 Sko1 target loci under the conditions tested in vivo. In addition, the experimentally determined false discovery rates of specifically or nonspecifically enriched regions for groups I



gene	p-value
a ALD6	8.87E-4
b YOR246C, SRL1	1.67E-5
c PUT4, CIN1	1.18E-4
d FAA1, COT1	3.51E-8
e CWP1	1.80E-5
f SED1	3.12E-3
g RPI1, RHO3	7.34E-8
h STL1, YDR535C	1.39E-3
i PTP3	8.26E-4
j MGA1	3.17E-5
k ROX1	3.50E-3
l UTH1	3.32E-5
m FMP43	1.35E-6
n MOT3	2.70E-4
o GAT2	9.82E-7
p MSN2	3.65E-4
q SOR1	7.63E-4
r HXT5	2.28E-4
s SPO20	3.65E-4
t CPA1, YOR302W	1.01E-3
u ICY1, YMR194C-A	1.59E-3
v HOR7	1.15E-3
w YPR127w, YPR126C	5.95E-4*
x TYE7	5.53E-5
y GAC1	8.08E-4
z DPM1	1.76E-3*
A POS5	6.12E-4*
B RSN1, YMR265C	1.72E-3
C EFT1	3.08E-3*
D MFalpha1	1.37E-3*
E HIR2, CKB2	2.24E-3
F MLH3	3.16E-3
G FOL1, SIP3	1.59E-3
H ORF free V	

FIG. 1. In vivo occupancy of Sko1 at various intergenic regions. From $(HA)_3$-Sko1-expressing strain MAP37 (HA-SKO1) and the untagged parental strain (control), cross-linked chromatin was immunoprecipitated with anti-HA antibodies followed by quantitative PCR analysis in real time of the indicated regions. Occupancy levels are measured compared to an internal region of the *POL1* gene, which is not bound by Sko1. The intergenic regions are ordered by their Sko1 occupancy levels determined by the targeted chromatin immunoprecipitation assays. In the cases where the intergenic region corresponds to the upstream regulatory sequence of more than one gene, all gene names are listed. The *P* values are derived from the genomewide location analysis. In general, these *P* values are based on data from at least two or three independent experiments; *P* values based on one experiment are indicated by asterisks.

TABLE 1. Sko1 target promoters confirmed by in vivo chromatin immunoprecipitation

Rank	Intergenic region[a]	Gene name[b]	P value[c]	ChIP[d]	Osmostress induced[e]	Function
1	iYOR316C-1	FAA1	3.51E-8	14	Yes	Long-chain fatty acyl-CoA synthetase
	iYOR316C-1	COT1	3.51E-8	1.6		Vacuolar zinc transporter
2	iYIL119C-0	RHO3	7.34E-8	2.0		GTP binding protein; ras homolog
	iYIL119C-0	RPI1	7.34E-8	13		Putative transcriptional regulator; multicopy suppressor of RAS2 overexpression
3	iYMR135W-A	GAT2	9.82E-7	7.1	Yes	Putative transcription factor
4	iYOR246C	SRL1	1.67E-5	15		Suppressor of Rad53 null lethality
	iYOR246C	YOR246C	1.67E-5	15		Unknown
5	iYKL097W-A-1	CWP1	1.80E-5	13	Yes	Cell wall protein, involved in O and N glycosylation
6	iYPR063C	ROX1	3.50E-3	9.7	Yes	Heme-dependent transcriptional repressor of hypoxic genes
	iYPR063C	YPR063C	3.50E-3	9.7		Unknown
7	iYGR248W	MGA1	3.17E-5	10		Putative transcriptional regulator
8	iYGR242W	FMP43	1.35E-6	9.0	Yes	Unknown
	iYGR242W	YAP1802	1.35E-6	9.0		Yeast assembly polypeptide, member of AP180 protein family
9	iYOR348C	CIN1	1.18E-4	15		Tubulin folding factor D
	iYOR348C	PUT4	1.18E-4	15	Yes	Proline-specific permease
10	iYHR096C	HXT5	2.28E-4	3.7	Yes	Hexose transporter
11	iYMR069W	MOT3	2.70E-4	8.5	Yes	DNA-binding protein implicated in heme-dependent repression
12	iYMR016C-0	SPO20	3.65E-4	2.7		Meiosis-specific subunit of the t-SNARE complex
	iYMR016C-0	SOK2	3.65E-4	1.6		Putative transcription factor, multicopy suppressor of mutants of cAMP-dependent protein kinase
13	iYMR037C	MSN2	3.65E-4	5.2	Yes	Transcription factor; regulator of stress-responsive gene expression
14	iYKR041W	UTH1	3.32E-5	9.0		Involved in determining yeast longevity
15	iYJR158W-1	SOR1	7.63E-4	4.1		Sorbitol dehydrogenase
16	iYER075C	PTP3	8.26E-3	11		Protein tyrosine phosphatase, negative regulator of Hog1 MAPK
17	iYPL062W	ALD6	8.87E-3	15	Yes	Cytosolic acetaldehyde dehydrogenase
18	iYOR301W	CPA1	1.01E-3	2.6		Carbamoylphosphate synthetase
19	iYDR535C	STL1	1.39E-3	13	Yes	Sugar transporter-like protein
20	iYDR076W	SED1	3.12E-3	13	Yes	Cell wall protein

[a] Refers to the 3-adjacent region of the indicated ORF.
[b] Two gene names indicate divergently transcribed ORFs sharing the same intergenic region.
[c] Based on the genomewide location analysis.
[d] Occupancy levels (relative to the internal region of the *POLI* gene) obtained by targeted ChIP analysis. At the long intergenic regions of *FAA1/COT1* and *RHO3/RPI1* the Sko1 association was monitored at two locations closer to the ATG of the indicated gene.
[e] At least threefold induced by NaCl in genomic profiling datasets (32, 38).

and II are in good accord with false discovery rates predicted by the *P* values. As such, this analysis indicates that Chipper provides relatively accurate *P* values for identifying enriched genomic regions. Our results differ significantly from an initial genomewide location analysis (22), but are in good accord with a later report that appeared after the work described here was completed (11). The existence of this later report permits us to combine *P* values from experiments performed in two different laboratories, thereby providing a more reliable assessment of Sko1 target sites. With respect to the data generated here, this combined analysis casts doubt on a few group I regions (*PRR2* and *MNN9*) and it provides strong evidence that some untested group II (*FSH1*, *YAP6*, *RPS4A*, and *PET9*) or even lower-ranked (*CIN5* and *ALD4*) regions are bona fide Sko1 targets.

Sko1 targets include genes encoding stress-defense proteins, putative regulators, and transcription factors. Sko1 has been characterized as a repressor/activator protein with an important function in the transcriptional response to hyperosmotic stress. Among the 39 group I and II intergenic regions bound by Sko1 with the most significant *P* values, 20 (50%) were associated with a gene induced >3-fold by osmotic stress in at least one transcriptional profiling experiment (32, 38). This relationship between Sko1 binding in vivo and transcriptional induction in response to osmotic stress is highly significant ($P = 10^{-7}$).

Table 1 summarizes the 20 intergenic regions associated with Sko1 that were confirmed by targeted chromatin immunoprecipitation experiments. Although there is a correlation between Sko1 binding and transcriptional response to osmotic shock (represented by *FAA1/COT1*, *GAT2*, *FMP43/YAP1802*, *CWP1*, *ROX1/YPR063c*, *CIN1/PUT4*, *HXT5*, *MOT3*, *MSN2*, *ALD6*, *STL1*, and *SED1*), we identified a number of promoters that are unknown to respond to osmolarity (represented by *RHO3/RPI1*, *SRL1/YOR246c*, *MGA1*, *UTH1*, *SPO20/SOK2*, *SOR1*, *PTP3*, and *CPA1*). These promoters may respond weakly to osmotic stress (i.e., less than threefold) or a Sko1-dependent effect might be masked by other factors that regulate the expression of these genes. Conversely, we and others (11) did not detect Sko1 binding to three genes, *SFA1*, *GLR1*, and

YML131W, shown to have a modest Sko1-dependent effect on transcription (39). This observation suggests the possibility that Sko1 indirectly affects transcription of *SFA1*, *GLR1*, and *YML131W*, although the lack of Sko1 binding might be an artifact of the microarray experiments. We observed no detectable binding to the *GRE2* promoter and only weak binding to the *AHP1* promoter, both of which have been previously shown to be direct Sko1 targets in vivo (35). In general, the failure to detect Sko1 binding at a given region in these microarray experiments is less significant than the ability to detect Sko1 binding.

A number of the experimentally confirmed or group I Sko1 targets have known or likely functions in the direct relief from hyperosmotic stress. In particular, Sko1 target genes encode cell wall proteins (Sed1 and Cwp1), vacuolar or cytoplasmic transporters (Stl1, Hxt5, Put4, and Cot1), and confirmed or likely enzymatic activities (Faa1, Sor1, Ald6, and Yor246c). Most of them were previously identified by genomic profiling as being strongly induced upon salt stress. More interestingly, Sko1 targets also include known or putative regulators (Ptp3, Cin1, Rho3, Rpi1, Uth1, Prr2, and Srl1) or transcription factors (Msn2, Mot3, Mga1, Rox1, and Gat2). With the exception of Ptp3 and Msn2 (discussed later), none of these molecules has been functionally related to osmotic or salt stress adaptation, although it should be noted that subtle effects on the osmotic stress response might not have been detected by the phenotypic assays employed to date. It will be therefore of special interest to unravel their contribution to the osmotic stress response.

Sequence comparison of target promoters suggests that Sko1 binding in vivo requires additional factors beyond recognition of ATF/CREB motifs. Sko1 belongs to the family of ATF/CREB DNA binding proteins, and it associates with the T(G/T)ACGT(C/A)A consensus sequence via its bZIP domain. We therefore searched for ATF/CREB motifs, allowing one mismatch to the consensus, in the 20 confirmed Sko1 target regions, and 19 of the 20 intergenic regions contain at least one ATF/CREB motif within the upstream 1,000 nucleotides for a total of 47 sites, but only one of these motifs is identical to the consensus. The number of ATF/CREB motifs in the confirmed Sko1 target regions is expected by chance, because the yeast genome contains over 15,000 such motifs among the 6,000 intergenic regions. The average distance of these ATF/CREB motifs from the ATG initiation codon is 549 bp, a position expected by chance and considerably further upstream than the 200 to 300 bp that is typical for bona fide binding sites in *Saccharomyces cerevisiae*. Furthermore, an attempt to find common motifs by AlignAce resulted in several potential candidates of modest statistical significance, but the ATF/CREB motif was not among them.

Although many of the ATF/CREB motifs in the confirmed Sko1 target regions are of dubious functional significance, 15 of these motifs are conserved among the four *Saccharomyces* sensu stricto yeasts, *S. cerevisiae*, *S. mikatae*, *S. bayanus*, and *S. paradoxus* (Table 2). In addition, sequence comparison of these evolutionarily conserved motifs reveals additional sequence preferences in the ±4 position that immediately flanks the motif (Table 3). The preferences for A at the −4 position and T at the +4 position are remarkably similar to those of Gcn4 for the related AP-1 site (30). These observations suggest that at least some of the evolutionarily conserved ATF/CREB motifs may be relevant for Sko1 binding in vivo (4, 18). It should be noted, however, that some of the conserved ATF/CREB motifs are located in the coding region of the upstream gene, casting doubt on their functional significance.

Taken together, these results suggest that Sko1 binding to target regions in vivo can require factors in addition to direct recognition of ATF/CREB motifs. One possibility is that Sko1 interacts with other proteins that associate with the target promoter. Such additional proteins could be typical DNA-binding proteins (e.g., those binding to the potential motifs identified by AlignAce) or other proteins (e.g., Cyc8-Tup1 corepressor) that might impart some sequence preferences beyond the ATF/CREB motif. Alternatively, Sko1 might bind

TABLE 2. Alignment of CRE sites identified in 15 novel SkoI target promoters which are conserved in *Saccharomyces cerevisiae*, *Saccharomyces paradoxus*, *Saccharomyces mikatae*, and *Saccharomyces bayanus*

Sequence[a]	Gene	Position
ttgct**CCACGTCA**tgcat	*SED1*	(−522)[b]
atcat**ATACGTCA**taact	*STL1*	−851[c]
tcaac**TTGCGTCA**tttta		−615
atcca**CTACGTCA**gaaag		−422
caatt**TTACTTCA**tagct	*HXT5*	−70
ttctc**TTACGAAA**acaag	*RPI1*	−666
ttaaa**TTACGGAA**aaagg		−625
cggca**AAACGTCA**tcgga	*PTP3*	(−367)[b]
ttgta**TGACGTGA**agcga	*MGA1*	−512
acttg**TTACGTAT**atgat		−481
aaccg**TGACTTCA**ttttt	*GAT2*	−543
tctgt**CTACGTCA**tacat	*MSN2*[d]	−645
gcccc**TCACGTAA**gggca	*ALD6*	−698
ttgga**TTATGTAA**aaggt		−672
caccg**AGACGTCA**ttgtt		−534
actaa**TGACGTAT**tattg	*SOR1*	−727
cggct**TTACGTCA**ttccq	*FMP4*3	−273
aggaa**TGACGTCT**ctgcc	*SRL1*	−475
cgggg**TGACGCAA**gtatg	*ROX1*[d]	−745[c]
aagta**TGAAGTCA**tgtgt		−734[c]
agcaa**TTACGTAG**aggga		−712[c]
tcact**TTGCGTAA**tgtaa		−265
aagtt**TTACTTAA**atata		−167
tggtg**TGGCGTCA**cacag	*CPA1*[d]	−876[c]
agctt**CTACGTAA**gcgtg		−722[c]
tcgca**TTACCTCA**tgatg		−464
ggtgt**TGACGTTT**tgccg	*UTH1*	(−146)[b]

[a] Motifs that are perfectly conserved between the four *Saccharomyces* species are underlined.
[b] CRE site with only one perfect half-site and more than one mismatch in the adjacent half-site.
[c] Sequence is located in the upstream ORF.
[d] No matching *S. bayanus* sequence available for this promoter.

TABLE 3. Consensus motif for the Sko1 binding site[a]

Nucleotide	Occurrence at indicated position									
	−4	−3	−2	−1	−0	+0	+1	+2	+3	+4
A	8	0	0	21	1	0	1	8	20	4
C	2	0	1	0	22	1	0	13	0	1
G	3	0	7	1	0	22	1	1	0	1
T	3	16	10	0	1	3	23	1	3	12
Consensus	(A)	T	G/T	A	C	G	T	A/C	A	(T)

[a] Only the regions conserved among the four *Saccharomyces* species (underlined sequences in Table 2) are included.

a variety of sequences in addition to ATF/CREB sites, and in this regard, some bZIP domains can bind different DNA sequences. Lastly, the position and density of nucleosomes might play an important role in where Sko1 actually binds in vivo. In any event, it is clear that the relationship between ATF/CREB motifs and Sko1 binding in vivo is complex.

In addition to Sko1, *Saccharomyces cerevisiae* has two other ATF/CREB factors, Aca1 and Aca2 (8). These three ATF/CREB proteins bind the consensus and near-consensus ATF/CREB sites with comparable affinity, and they modulate the expression of artificial ATF/CREB-driven promoters. However, the biological functions of these proteins are different, as judged by their mutant phenotypes (8). As a consequence, it is very likely that these three ATF/CREB proteins associate with distinct subsets of promoters in vivo, although this has yet to be determined experimentally. Distinct in vivo binding patterns of the ATF/CREB proteins might reflect differential preferences for sequence variations of the CRE motif, as observed for the glucose-regulated Cat8 and Sip4 transcription factors (42). Alternatively, the ATF/CREB proteins might interact with different factors, thereby resulting in distinct promoter selectivity.

Sko1 regulates the expression of Msn2, a transcriptional activator protein that mediates the general stress response. Msn2 and Msn4 are homologous proteins that activate transcription of many defense genes in response to a wide variety of different stress conditions (25, 43). Msn2 and Msn4 accumulate in the nucleus upon these various stress conditions, whereupon they bind to promoters containing stress response elements (10), Genomic profiling experiments have revealed a subset of stress genes that depend on Msn2/4 for the response to hyperosmotic stress (38). As Sko1 binds the *MSN2* promoter region (Fig. 1) and expression of Msn2 is induced in response to osmotic stress (25), we analyzed *MSN2* RNA levels in wild-type and *sko1* and *hog1* mutant cells under normal growth conditions and after osmotic shock (Fig. 2).

As expected, *MSN2* transcription is induced sevenfold upon osmotic shock in the wild-type strain. This induction is significantly diminished but not completely abolished in the absence of Sko1 or the Hog1 kinase. In contrast, *MSN4* is not induced by osmotic shock and is unaffected in *sko1* or *hog1* mutants (Fig. 2). Thus, although Msn2 and Msn4 are closely related proteins that mediate the general stress response, Sko1 specifically regulates the expression of Msn2 in response to osmotic stress. Sko1-dependent regulation of Msn2 represents an interesting situation in which a transcription factor that mediates a specific stress response (hyperosmolarity) induces a transcription factor that mediates the general stress response. Thus, in addition to being directly activated by osmotic stress, Msn2 is indirectly activated by Sko1-dependent induction of its expression, reinforcing the general stress response that occurs upon osmotic shock. As Sko1-dependent induction of Msn2 is a two-step process for activating Msn2-responsive genes, it is likely that it plays a more important role after the initial response to osmotic stress.

Sko1 regulates the osmoinducible expression of *MOT3* and *MGA1*, genes that encode transcription factors. In addition to Msn2, Sko1 target genes also include those encoding the DNA-binding transcription factors Mot3, Rox1, and Mga1. *MOT3* and *MGA1* transcription is highly inducible by salt stress (6-fold and 15-fold, respectively), and this induction is significantly reduced in a *sko1* strain and even more strongly affected in a *hog1* strain. In contrast, expression of *ROX1* is only slightly induced upon osmotic stress, and this induction is independent of Sko1 or Hog1. Thus, Sko1 directly regulates the transcription of at least three genes encoding transcription factors, *MSN2*, *MOT3*, and *MGA1*. In these three cases, Sko1 significantly contributes to but does not fully account for osmotic induction in wild-type cells.

Mot3 and Rox1 synergistically repress the transcription of hypoxic genes (17, 24, 44). Both genes are inducible by hyperosmotic stress, which points to a physiological function of both repressors upon salt stress. This function is not known to date, although a physiological connection between anaerobicity and osmotic stress tolerance has been reported recently (19). Mot3 is also involved in the regulation of ergosterol synthesis and vacuolar function (12), which might be critical under water stress and ion imbalance during osmotic shock. Sko1 binds to the *MOT3* and *ROX1* promoters, but it appears to play a more important role in osmotically inducible expression of *MOT3*. We suggest that Sko1-dependent induction of Mot3 repressor might further repress hypoxic genes, allowing the cell to devote more of its energy to deal with hyperosmotic stress.

The biological function of Mga1 is poorly understood, although Mga1 overexpression causes the induction of pseudohyphal growth (23). Although pseudohyphal growth is a long-term phenomenon associated with nutrient deprivation, the process may also be initiated in a transient manner upon other environmental stresses. We speculate that, in response to osmotic stress, Sko1 induces Mga1 to initiate pseudohyphal formation, and that progression towards this distinct cellular state is stopped upon successful adaptation to hyperosmotic conditions.

Sko1-regulated expression of Ptp3, a tyrosine phosphatase that acts on Hog1, provides evidence for a transcriptional feedback mechanism. Sko1 binds in vivo to the *PTP3* promoter (Table 1). *PTP3* and *PTP2* encode two homologous protein tyrosine phosphatases that negatively regulate the HOG pathway by dephosphorylation and sequestration of Hog1 MAP kinase (16, 28, 48). We tested whether Sko1 was responsible for the stress-regulated expression of *PTP3* and *PTP2* (Fig. 2). *PTP3* expression is induced 3.5-fold upon osmotic shock, whereas *PTP2* expression is only mildly increased upon stress. Induction of *PTP3* transcription is completely dependent on Sko1, while the mild activation of *PTP2* seems to be independent.

Our results are strongly suggestive of a transcriptional feedback mechanism in which stress-activated Hog1 kinase directly activates Sko1 by multiple phosphorylations (33, 35) at the *PTP3* promoter, thereby inducing *PTP3* expression and hence Ptp3 phosphatase levels in response to osmotic stress. As a consequence, increased Ptp3 phosphatase levels favor dephosphorylation of tyrosine-phosphorylated Hog1 kinase, leading to inactivation of the HOG pathway at the later stages of osmotic stress adaptation. As *PTP2* expression is much less inducible by osmotic stress and is independent on Sko1, we speculate that Ptp2 might be responsible for HOG inactivation under other environmental stresses recently reported to activate Hog1, such as heat, oxidative or citric acid stress (2, 21, 47).

DISCUSSION

To date, there are seven transcription factors with a specific function in salt stress induction of defense genes: Sko1 (34),

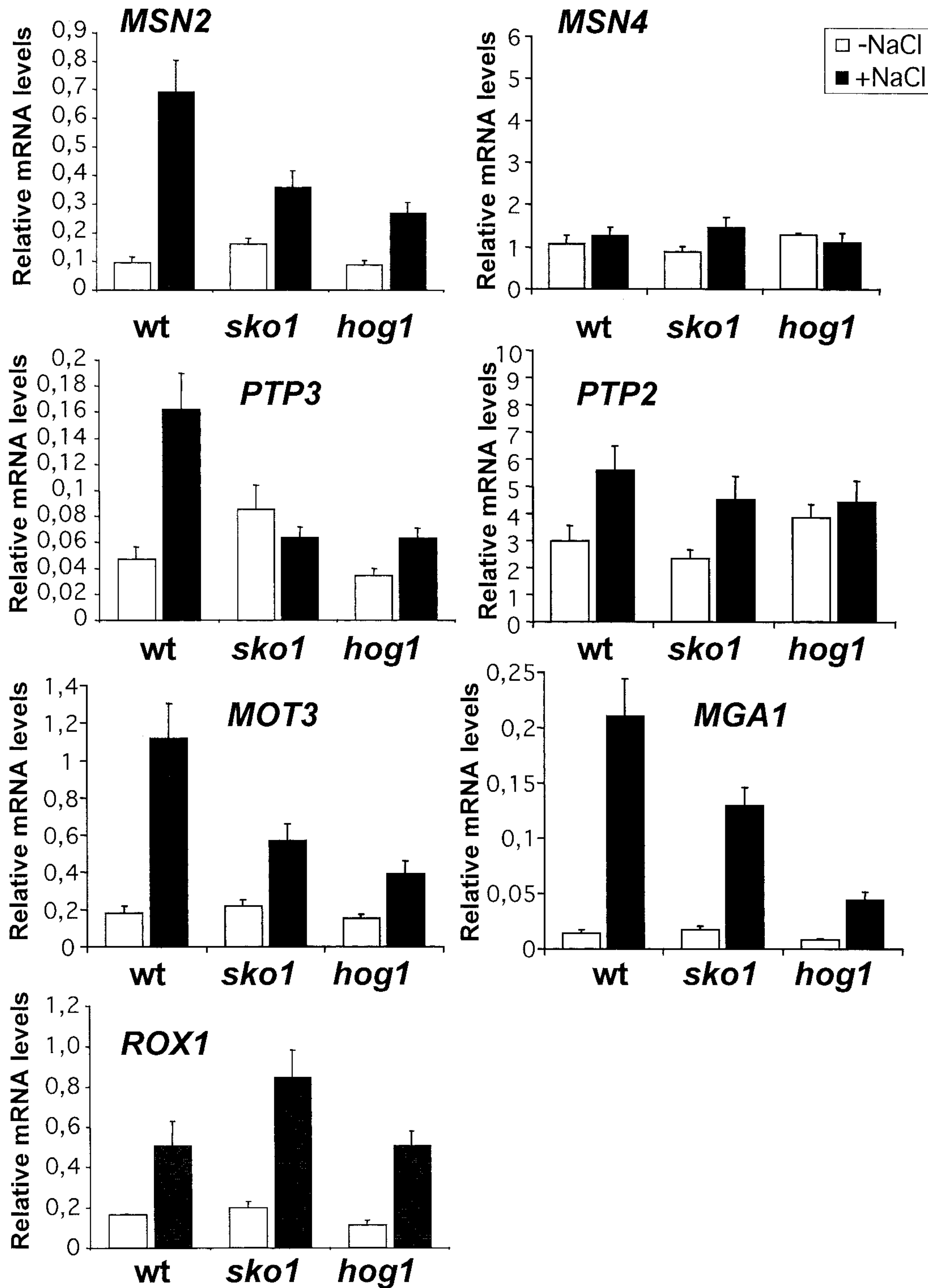


FIG. 2. Sko1 regulates the osmoinducible expression of *MSN2*, *MOT3*, *MGA1*, and *PTP3*. Reverse transcriptaion analysis of the indicated mRNA levels under nonstress (−NaCl) and osmotic stress (+NaCl; 0.4 M NaCl for 10 min) conditions in wild-type (W303-1A) and *sko1* (MAP19) and *hog1* (MAP32) deletion strains. DNA regions were quantified by quantitative PCR in real time. Transcript levels are given relative to the *TBP1* control.

Msn1 and Hot1 (40), Msn2 and Msn4 (25), Smp1 (6), and Crz1 (27, 46). Multiple transcription factors act at highly inducible genes such as *CTT1* and *GPD1* (37). Such functional redundancy makes it difficult to identify direct Sko1 (or other transcription factor) targets by transcriptional profiling. Although previous genomewide transcriptional profiling only identified two genes whose transcription levels were significantly altered in a *sko1* mutant (39), the results presented here suggest that Sko1 has approximately 40 targets with diverse biological functions (Fig. 3).

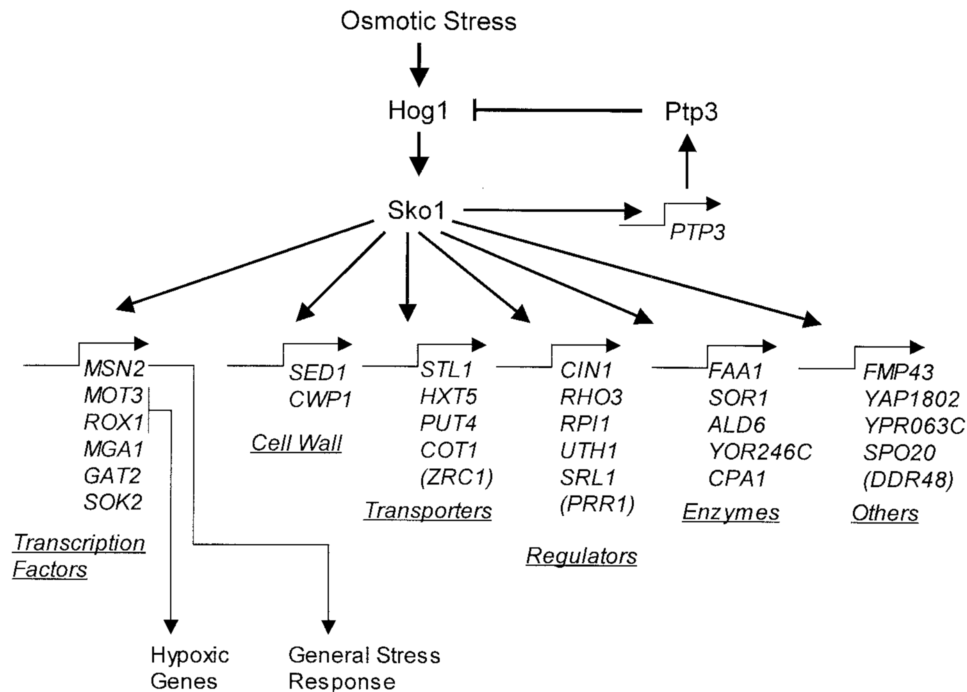


FIG. 3. Schematic overview of Sko1-bound genes and their biological functions. Only experimentally confirmed Sko1 targets are included with the exception of *ZRC1*, *PRR1*, and *DDR48*, which are bound with a probability *P* of $<10^{-2}$, but are not confirmed by standard chromatin immunoprecipitation.

The relative contribution of Sko1 varies considerably for the osmotic-inducible expression of the transcription factor encoding genes *MSN2*, *MOT3*, *MGA1*, and *ROX1*. All four promoters are bound by Sko1 and robustly activated by osmotic shock, but Sko1 contributed >50% to *MSN2* and *MOT3* transcription, <50% to the *MGA1* transcription, and not at all to the *ROX1* transcription. The modest Sko1-dependent transcriptional effects strongly suggest that these other factors contribute to regulation of these promoters. In addition, Sko1 associates with the *STL1* promoter, even though its high inducibility upon osmotic stress was previously attributed solely to the Hot1 activator (38). It is likely that the expression of most osmoinducible genes will be determined by the combination of various specific transcription factors acting at the same promoter. The combination of genomic location analysis and transcriptional profiling of more transcription factors will further reveal the complexity and redundancy of the transcriptional program operated upon osmotic stress.

Transcription factor networks have been identified as an important component of complex transcriptional programs such as the cell cycle (45), but also at a general level (11, 22). Our results indicate that Sko1 activates a regulatory network in addition to its induction of genes that encode proteins that directly relieve osmotic stress. First, Sko1 activates transcription of *MSN2*, permitting the initial response to a specific stress to reinforce the more general stress response. Second, Sko1-mediated activation of *MOT3* permits the response to a specific stress to down-regulate genes that mediate a response to a different stress, allowing the cell to focus its energy on responding to the actual environmental insult. Third, Sko1-mediated activation of *MGA1* might contribute to sending cells down a developmental program associated with more stressful conditions.

In a different vein, Sko1 activates *PTP3*, which encodes a tyrosine phosphatase that dephosphorylates Hog1, the ultimate signaling component that mediates the response to osmotic stress. In this regard, the response to osmotic stress is transient, and increased expression of Ptp3 phosphatase should be appropriately timed to contribute to the down-regulation of the response. Taken together, the transcriptional regulatory network activated by Sko1 is likely to be important for fine-tuning the response to osmotic stress (Fig. 3). Comparable regulatory networks are likely to be mediated by the other activator proteins that participate in the osmotic stress response.

Our results contribute to the emerging view that the relationships between protein binding in vivo, DNA sequence motifs, and transcriptional function are complex. Although many Sko1 target promoters confer induced transcription upon osmotic stress, others show minimal or no regulation. Although ATF/CREB motifs are likely to be important for Sko1 binding, these motifs are not overrepresented among Sko1 targets, indicating that the motifs per se are not sufficient to account for Sko1 binding in vivo. The complex relationships between protein binding in vivo, DNA sequence motifs, and transcriptional activity are not specific to Sko1, and indeed have been observed in many experiments in yeast and human cells (3, 11, 26). In fact, it has been difficult to identify DNA sequence motifs for in vivo binding by many of the yeast DNA-binding proteins that have been analyzed by chromatin immunoprecipitation on a genomewide basis (11). Taken together, these observations indicate that biological specificity and regulatory

complexity depend on many parameters and are not easily reduced to simple relationships.

ACKNOWLEDGMENTS

We thank Francesc Posas and Stefan Hohmann for providing datasets of the genomic profiling experiments under osmotic stress.

This work was supported by an EMBO Long Term Fellowship and the "Ramón y Cajal" program of the Spanish Ministry of Science and Technology to M.P. and a grant to K.S. from the National Institutes of Health (GM30186).

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