Genome-wide characterization of the Zap1p zinc-responsive regulon in yeast

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The Zap1p transcription factor senses cellular zinc status and increases expression of its target genes in response to zinc deficiency. Previously known Zap1p-regulated genes encode the Zrt1p, Zrt2p, and Zrt3p zinc transporter genes and Zap1p itself. To allow the characterization of additional genes in yeast important for zinc homeostasis, a systematic study of gene expression on the genome-wide scale was used to identify other Zap1p target genes. Using a combination of DNA microarrays and a computer-assisted analysis of shared motifs in the promoters of similarly regulated genes, we identified 46 genes that are potentially regulated by Zap1p. Zap1p-regulated expression of seven of these newly identified target genes was confirmed independently by using *lacZ* reporter fusions, suggesting that many of the remaining candidate genes are also Zap1p targets. Our studies demonstrate the efficacy of this combined approach to define the regulon of a specific eukaryotic transcription factor.

DNA microarray analysis allows assessment of the regulation of a large number of genes in response to a given stimulus or genetic alteration (1–3). The completed *Saccharomyces cerevisiae* genome sequence enables us to apply this technique to examine the expression of every known or predicted gene in this organism. In this report, we describe the use of DNA microarrays to advance our understanding of zinc homeostasis in yeast.

Zinc is an essential nutrient. Being oxidatively inert, it is less likely than other metals to damage sensitive cellular components (4). This property may explain why zinc is such a ubiquitous structural or catalytic cofactor. For example, almost 2% of *S. cerevisiae* proteins contain zinc-dependent DNA binding domains (5, 6). After iron, zinc is the most abundant trace element in the human body (4). Although severe zinc deficiency is uncommon among humans, it has been estimated that 4 million people in the United States alone suffer from mild zinc deficiency (7). In contrast, excess zinc can be toxic to cells and has been postulated to play a role in ischemic injury (8). These observations highlight the importance of zinc homeostasis.

Despite a wealth of information on the functions of zinc, little is known about how this metal is taken up, stored, and used by cells. We are using the yeast *S. cerevisiae* as a model system to address these questions. In yeast, zinc uptake across the plasma membrane occurs via two transporters, Zrt1p and Zrt2p (9, 10). Moreover, mobilization of zinc stored in the vacuole is mediated by the Zrt3p transporter (11). Expression of *ZRT1*, *ZRT2*, and *ZRT3* increases in zinc-limited cells. This induction is mediated by the Zap1p transcriptional activator, which also controls its own expression (12). Zap1p binds to a conserved sequence, called the zinc-responsive element (ZRE), present in the *ZRT1*, *ZRT2*, *ZRT3*, and *ZAP1* promoters. A consensus ZRE sequence derived from the elements in these promoters is 5′-ACCYYNAAGGT-3′ (11, 13). A single ZRE is sufficient to confer Zap1p-dependent expression.

Even with this recent progress, many aspects of zinc homeostasis remain unknown. To address these issues, we hypothesize that additional Zap1p target genes in the genome will play important roles in zinc homeostasis. In this study, we identify 46 genes that are potential Zap1p targets. This collection of genes is an exciting resource for understanding zinc metabolism at the molecular level. Moreover, the approach used in this study serves as a useful example of how DNA microarray analysis can be used to define the regulons and binding sites of specific transcription factors.

Materials and Methods

Yeast Methods. Strains used were DY1457 (*Mata ade6 can1 his3 leu2 trp1 ura3*), ZHY6 (DY1457 *zap1*::*TRP1*), and ZHY7 (DY1457 *ZAP1-1*^{up}) (12). Chelex-treated synthetic defined medium (CSD) was used to limit zinc availability. CSD was prepared by dissolving of 20 g of dextrose, 5.1 g of yeast nitrogen base without divalent cations or potassium phosphate (Bio101, Vista, CA), and 0.1 g each of adenine, histidine, tryptophan, and leucine into 1 liter of distilled H_2O. Chelex-100 ion exchange resin (25 g; Sigma) was added, and the mixture was stirred overnight at 4°C. After removal of the resin, the pH was adjusted to 4.0 with HCl, and the following were added to recommended (Bio101) final concentrations: $MnSO_4$, $FeCl_3$, $CuSO_4$, $CaCl_2$, $MgSO_4$, and KPO_4 monobasic. The solution was then filter-sterilized into polycarbonate flasks. All plasticware used for media preparation and cell culturing was washed with Acationox detergent (Baxter Scientific Products, McGaw Park, IL) before use. CSD contained less than 100 nM zinc as measured by atomic absorption spectroscopy. Cells were inoculated from overnight cultures into CSD at an initial $OD_{600} = 0.01$ and grown to mid-log phase ($OD_{600} \approx 0.5$) before RNA isolation or β-galactosidase assays.

Microarray Analysis. Wild-type and *zap1* cells were grown in 100 ml of CSD containing three different concentrations of zinc: deficient (<100 nM), replete (10 μM), and excess (3 mM). Cells were collected by centrifugation (for 5 min at 1,000 × *g*) at room temperature. Total RNA was extracted with hot phenol, and poly(A)$^+$ RNA was purified by oligo(dT) chromatography (Qiagen, Chatsworth, CA). Cy3-dUTP and Cy5-dUTP were incorporated during reverse transcription of the mRNA, and microarray analysis was performed as described (2) with SCANALYZE software (available at http://rana.stanford.edu/software/) for data extraction.

Five microarray experiments were performed, each in duplicate. Experiment 1 compared expression in wild-type cells grown in zinc-deficient conditions with wild-type cells grown in excess zinc. Experiment 2 compared zinc-replete wild-type cells to wild-type cells grown in excess zinc. By subtracting the data (in log 2 space) for each measurement in experiment 1 from the data in experiment 2, we obtained a comparison of expression in zinc-deficient versus replete cells. Experiments 3 and 4 compared zinc-deficient *zap1* cells to zinc-replete and to excess-zinc conditions. Experiment 5 compared wild-type cells with *zap1* cells grown under zinc deficiency. A gene was considered to have consistently altered expression if it averaged ≥2-fold change in duplicate arrays. This threshold

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Abbreviations: ZRE, zinc-responsive element; CSD, Chelex-treated synthetic defined medium; MEME, multiple expectation-maximization for motif elicitation; ADH, alcohol dehydrogenase.

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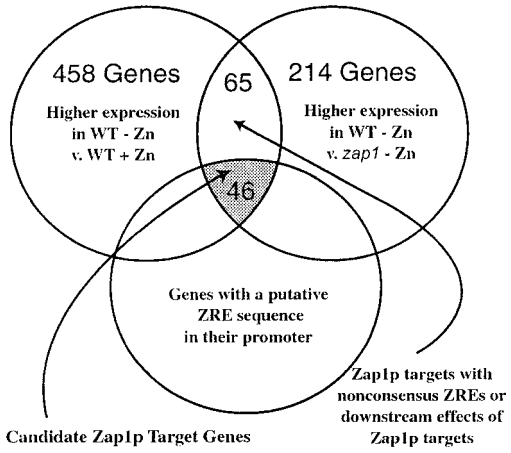

Fig. 1. A diagrammatic representation of our approach to identify Zap1p target genes. WT, wild type.

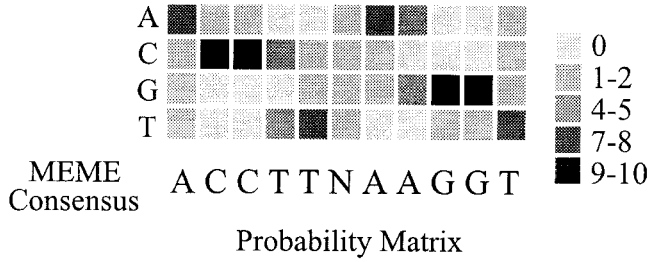

Fig. 2. The probability-based ZRE motif derived from MEME analysis. The plot shows the letter-probability matrix (17) of the ZRE based on the elements found within the 46 potential Zap1p target genes. The scale indicates the degree of shading that corresponds to the probability of each possible base occurring at each position of the motif multiplied by 10 and rounded to the nearest integer. The most probable form of the ZRE (i.e., the MEME consensus) derived from the probability matrix is shown.

was chosen arbitrarily, and some consistently affected genes may not have been included in our analysis. The complete data set is available on the internet at http://genome-www.stanford.edu/zinc.

DNA Manipulations. Plasmid pGI1 contains the *ZRT1* promoter fused to *lacZ* (10). Other *lacZ* fusions were constructed in YEp353 (14) by gap repair (15). PCR products were generated from genomic DNA that contained 1,000 bp of the target promoter sequence flanked by regions of vector homology. These fragments were gel-purified (Promega) and cotransformed into DY1457 with *Eco*RI–*Bam*HI-digested YEp353; transformants were selected for Ura^+ prototrophy. Plasmids were then transferred to *Escherichia coli* and confirmed by sequencing. The *ADH4* ORF in the *Saccharomyces* Genome Database (http://genome-www.stanford.edu/Saccharomyces) reports an incorrect start codon, and the correct site (16) was used for that fusion. The translational start site for YNR039c may also be assigned incorrectly in the database. Based on sequence alignment with a similar protein from *Schizosaccharomyces pombe* (accession no. CAB16906), we predicted that the fifth in-frame ATG in the ORF would be the functional translation start site, and a construct that fused this ATG to the *lacZ* ORF resulted in expression. Insertion of ZREs into the pNB404 *lacZ* reporter plasmid was performed as reported (13). The *FET4* ZRE plasmid contains a tandem repeat of the element. Multiple expectation-maximization for motif elicitation (MEME) was used to identify motifs shared in the promoters of similarly regulated genes (17). Additional information and a version of MEME running on a parallel supercomputer is available at http://www.sdsc.edu/MEME.

β-Galactosidase Assays. β-Galactosidase activity was measured as described (18), and activity units were calculated as follows: $(\Delta A_{420} \times 1{,}000)/(\text{min} \times \text{ml of culture used} \times \text{culture } A_{600})$.

Results

Effects of Zinc and *ZAP1* on the Yeast Transcriptome. Zinc-dependent proteins play a large number of biochemical roles. Therefore, it was likely that expression of many genes would be altered in zinc-deficient cells. To identify Zap1p target genes specifically among all of the genes likely to be affected, we designed an approach based on the following predictions (Fig. 1). First, Zap1p-regulated genes would be expressed at higher levels in zinc-limited wild-type cells than they would in zinc-replete cells. Second, these genes would be expressed at higher levels in zinc-limited wild-type cells vs. zinc-limited *zap1* mutants. Finally, we predicted that Zap1p target genes would contain one or more ZREs in their promoters. For this study, we arbitrarily chose the region between the predicted ATG start codon and 1,000 bp upstream as containing potential promoter elements.

As predicted, expression of a large number of genes (934 or ≈15% of the genome) was altered in zinc-limited wild-type cells. Of these, 458 genes were expressed at higher levels (i.e., averaging ≥2-fold) in zinc-limited cells, and 476 genes were more highly expressed in zinc-replete cells. Expression of 571 genes was altered in wild-type vs. *zap1* mutant cells grown in zinc-limiting medium. Of these, 214 genes were expressed at higher levels in wild-type cells. The overlap of the two sets of array results, 111 genes total, is likely to include Zap1p target genes.

Of these 111 genes, 11 initially met our third criteria of containing one or more copies of the experimentally derived ZRE consensus sequence within their promoters (Table 1). We recognized that this consensus may not define the requirements of a ZRE completely, and nonconsensus sequences may also be functional Zap1p binding sites. To identify genes containing ZREs that diverge significantly from the original consensus, the promoters of the 111 genes showing Zap1p-dependent expression were analyzed by using MEME (17). MEME allows the unbiased detection of shared motifs in a set of input sequences with a probability-based algorithm called expectation maximization. This method can identify potential regulatory elements shared among the promoters of similarly regulated genes, in some cases, identifying previously undiscovered promoter elements and, in others, redefining the consensus sequence for previously identified binding sites. MEME analysis of the promoters of potential Zap1 targets resulted in the identification of ZRE-related sequences in many of the promoters. MEME successfully identified the consensus ZRE in the 11 genes originally identified, thus validating the usefulness of this approach and independently confirming our prior knowledge of the ZRE consensus. Moreover, related sequences divergent from the original consensus were found in the promoters of 35 additional genes (Table 1). Based on these results, we propose that the all 46 genes are regulated directly by Zap1p.

In addition to identifying common sequence motifs, MEME analysis provides a probability matrix for each position of the sequence. This matrix provides a theoretical measure of the information content at each position of the ZRE that may represent more fully the sequence preference of Zap1p DNA binding. The probability matrix shown in Fig. 2 represents the likelihood that a particular base will be found in any given position in a ZRE. The "MEME consensus" shows the most probable form of the motif as identified by the probability matrix. It is intriguing that this sequence, ACCTTNAAGGT, is a perfect palindrome suggesting that Zap1p may bind to DNA as a dimer. Experimental tests of this hypothesis are currently underway.

The experimental results describing the zinc- and Zap1p-dependent regulation of the 46 candidate target genes are shown in

Table 1. Potential Zap1p target genes

Gene	Description	Start*	Score†	ZRE sequence	Average ratio of expression‡ WT −Zn/+Zn	WT/*zap1* −Zn	*zap1* −Zn/+Zn
Genes with consensus ZREs (ACCYYNAAGGT)							
YNL254C	Unknown	431	15.10	ACCTTCAAGGT	9.7	12.0	0.9
YOL084W	Major facilitator superfamily	765	15.10	ACCTTCAAGGT	9.7	2.1	6.5
ZRT1	**High-affinity Zn uptake transporter**	**203**	**15.05**	**ACCTTGAAGGT**	**24.2**	**18.9**	**3.4**
		318	**15.02**	**ACCCTCAAGGT**			
		445	**12.84**	**ACCTTTGGGGT**			
		337	**10.78**	**ACCTCGAAGGA**			
YNR039C	**Putative transporter**	**527**	**15.05**	**ACCTTGAAGGT**	**3.2**	**3.5**	**0.9**
YOL154W	**Zn-metalloprotease-like protein**	**328**	**15.05**	**ACCTTCAGGGT**	**13.9**	**10.1**	**2.1**
		313	**14.97**	**ACCCTGAAGGT**			
YLL020C	Unknown	932	15.02	ACCCTCAAGGT	5.1	3.1	1.5
ZRT2	**Low-affinity Zn uptake transporter**	**261**	**14.61**	**ACCTTTAGGGT**	**5.6**	**12.3**	**0.9**
		310	**14.50**	**ACCCTAAAGGT**			
YDR492W	Putative transporter	416	14.61	ACCTTTAGGGT	2.8	4.4	0.5
MCD4	Major facilitator superfamily	103	14.58	ACCTTAAAGGT	4.1	2.5	1.7
ZRT3	**Vacuolar Zn efflux**	**154**	**14.53**	**ACCTTAAGGGT**	**7.9**	**8.7**	**1.2**
ZAP1	**Zn-responsive transcription factor**	**143**	**14.50**	**ACCCTAAAGGT**	**7.5**	**16.9**	**1.5**
Genes with ZRE-like sequences							
YGL258W	Unknown–similar to YOR387C	231	12.91	ACCCTGCGGGT	16.3	4.0	1.7
YOR387C	Unknown–similar to YGL258W	230	12.91	ACCCTGCGGGT	23.6	19.3	1.3
RAD27	Endonuclease	996	12.79	ACCCGGAGGGT	2.2	4.7	0.5
YJR061W	Unknown–similar to MNN4	277	12.73	TCCTTGAAGGT	4.6	2.1	1.6
DPP1	**DAGPP phosphatase**	**451**	**12.64**	**ACCTTTCAGGT**	**5.5**	**3.6**	**0.8**
ZIP1	Synaptonemal complex component	574	12.45	ACCTGAAAGGT	4.5	2.0	1.6
YMR086W	Unknown	144	12.11	ACCTTAAAGGA	2.0	2.4	0.7
ADH4	**ADH**	**268**	**12.09**	**ACCTTCACGGT**	**26.2**	**5.6**	**0.7**
ZRC1	**Vacuolar Zn influx**	**173**	**12.00**	**GCCTTGAAGGT**	**2.1**	**4.7**	**0.8**
GRE2	Stress response protein	919	11.95	GCCTTGAGGGT	2.5	2.6	1.5
MNT2	Mannosyltransferase	493	11.93	ACCGTGAAGGT	2.3	4.4	0.5
NRG2	Negative regulator of glucose control	919	11.56	ACCCTCAAGTT	5.1	2.2	1.2
YOL131W	Unknown	999	11.47	AACTTCAGGGT	6.6	2.3	2.5
		457	10.45	ACCTGGAAGGA			
YGL121C	Unknown	207	11.45	ACCGTAAAGGT	10.8	2.7	10.7
		286	10.85	CCCTTCGAGGT			
YKL174C	Putative amino acid permease	492	11.10	ACCATAAGGGT	3.2	2.0	1.8
PRC1	Vacuolar carboxypeptidase Y	181	11.07	ACCCGCGGGGT	3.0	3.5	1.0
ADE17	Adenine biosynthesis	97	10.85	ACCTTTAGTGT	5.2	3.7	1.3
TKL2	Transketolase II	843	10.84	ACCTTATGGGT	5.4	2.0	2.6
COS4	Unknown	308	10.82	ACCTTAAATGT	2.6	2.0	1.4
COS6	Unknown	308	10.82	ACCTTAAATGT	2.7	2.2	1.4
COS8	Unknown	313	10.82	ACCTTAAATGT	2.2	2.1	1.5
COS1	Unknown	313	10.82	ACCTTAAATGT	2.1	2.0	1.7
COS2	Unknown	313	10.74	ACCCTAAATGT	2.1	2.0	1.7
COS3	Unknown	313	10.74	ACCCTAAATGT	2.7	2.2	1.4
FET4	**Low-affinity Fe uptake transporter**	**383**	**10.63**	**ACCCGTGGGGT**	**2.3**	**2.4**	**0.8**
YJL132W	Similar to phospholipase D	153	10.61	ACCCAAAGGGT	5.2	3.8	1.7
YPL250C	Unknown	755	10.57	CCCTTCCGGGT	2.1	2.3	0.9
		523	10.44	TCCCTTGGGGT			
BAG7	Putative GTPase-activating protein	324	10.49	CCCCTGCAGGT	5.9	2.0	4.0
YOL002C	**Similar to YDR492W**	**225**	**10.48**	**TCCTCTAGGGT**	**2.0**	**2.0**	**0.8**
PST1	Unknown	404	10.21	TCCTTGAGGGA	5.0	2.7	2.5
		885	10.18	ACCCCAAGGGA			
URA10	Pyrimidine biosynthesis	142	10.13	ACCTTTCGGGA	6.1	2.5	2.6
YBL048W	Unknown	857	10.06	CCCTTGAGGGA	7.7	2.0	5.8
YBL049W	Unknown	386	10.06	CCCTTGAGGGA	10.7	2.2	6.3
PEP4	Vacuolar proteinase A	395	9.99	GCCTTCCGGGT	2.6	2.1	1.2
YNL234W	Globin-like heme binding protein	67	9.95	CCCCTCAAGGG	2.6	2.1	2.3

Shown are genes showing Zap1p-dependent regulation pattern whose promoters contain sequences that match the previously published consensus sequence or sequences related to the MEME-derived ZRE consensus. Zap1p-dependent regulation of genes in bold has been confirmed independently by *lacZ* fusions either in this study or in other reports. ADH, alcohol dehydrogenase; WT, wild type.

*Refers to the distance from the first base of the motif to the putative ATG initiation codon.

†Calculated for each sequence with the MEME-generated scoring matrix.

‡Expression ratios were determined by microarray analysis. Each value is the average of two independent arrays.

Table 1. Most of the genes showed similar ratios of expression in both experimental regimens, i.e., their induction by low zinc in the wild-type strain was comparable to their increased expression in zinc-limited wild-type vs. *zap1* cells. This observation suggests that Zap1p activity alone plays the major role in controlling their expression under these conditions. However, the regulatory profile of a few genes was more complex. For example, YOL084w and YGL121c were induced to a high degree in zinc-limited vs. zinc-replete wild-type cells (10- and 11-fold respectively) but only 2- to 3-fold when comparing zinc-limited wild-type cells vs. limited *zap1* mutant cells. To investigate this discrepancy, additional experiments comparing expression of these genes in the *zap1* mutant with or without zinc were performed (Table 1). A gene regulated solely by Zap1p would not be expected to show greatly increased expression in zinc-deficient *zap1* cells, a prediction that proved true for most of the candidate genes. However, genes such as YOL084w and YGL121c showed significant induction under zinc limitation in the *zap1* mutant. Thus, although these and similarly regulated genes may indeed be bona fide Zap1p targets (i.e., compare wild type vs. *zap1* −Zn), their increased expression in low zinc must be mediated by other factors as well.

Finally, of the 111 genes that showed the regulatory pattern expected of a Zap1p target, 65 do not contain sequences within their promoters that match the original ZRE consensus or the sequences identified with MEME. These genes may exhibit Zap1p-dependent expression, because (*i*) they are direct Zap1p targets that contain unrecognizable ZREs or (*ii*) their regulation is affected as a downstream consequence of the activity of a Zap1p target.

Confirming Zap1p-Dependent Regulation of Selected Genes. To assess whether our approach was successful in identifying Zap1p target genes, we selected seven genes from the list in Table 1 and fused their promoters to the *E. coli lacZ* gene. Two candidate genes whose promoters contain the original ZRE consensus (YNR039c and YOL154w) and five genes with more divergent ZRE-like sequences identified by MEME (*DPP1*, *ADH4*, *ZRC1*, *FET4*, and YOL002c) were chosen for this analysis. The results obtained with these reporters demonstrated that all seven of these genes are Zap1p-regulated. First, each promoter showed zinc-responsive gene expression over a range of zinc concentrations similar to that observed with a *ZRT1–lacZ* fusion (Fig. 3 *Left*). Second, the zinc-limited induction of each depended on Zap1p; no zinc-responsive expression was observed in a *zap1* mutant (Fig. 3 *Center*). The increased expression of YNR039c, *DPP1*, *ZRC1*, *FET4*, and YOL002c in zinc-treated *zap1* mutants is probably the result of an overall improvement in cellular metabolic processes; *zap1* mutants grown in low zinc are severely zinc-deficient and grow very slowly. Furthermore, similar effects have been observed previously with other reporter fusions such as *HIS4–lacZ* (10).

As an additional test, the effects of the *ZAP1-1*^{up}, a dominant allele of *ZAP1* that increases expression of *ZRT1*, *ZRT2*, *ZRT3*, and *ZAP1* in zinc-replete cells (11, 12), were also assessed. The *ZAP1-1*^{up} allele caused increased expression of *ZRT1*, YOL154w, *DPP1*, *ADH4*, and *FET4* (Fig. 3 *Right*). YNR039c, *ZRC1*, and YOL002c did not show increased expression in the *ZAP1-1*^{up} strain, perhaps because of other factors affecting their transcription. However, the zinc-responsive and Zap1p-dependent expression of these genes indicates that they are Zap1p targets, thus expanding the list of established Zap1p-regulated genes to 11 (Table 1). These data strongly support the hypothesis that many of the remaining candidates are also direct Zap1p targets.

The potential ZREs in the promoters of YOL154w and YNR039c match the experimentally derived consensus sequence and are therefore likely to be functional. To test whether the more divergent ZRE-like sequences in the promoters of *DPP1*, *ADH4*, *ZRC1*, *FET4*, and YOL002c are functional ZREs, oligonucleotides containing these elements were inserted into the promoter of a *lacZ* reporter gene bearing the *CYC1* TATA boxes but lacking any upstream activation sequences. β-Galactosidase activity was assayed in wild-type and *zap1* mutant cells containing these reporter plasmids and grown under zinc-replete and limiting conditions. Similarly to a bona fide ZRE from the *ZRT1* promoter, the elements from these promoters conferred Zap1p-dependent zinc responsiveness to *lacZ* expression (Table 2). Induction in wild-type

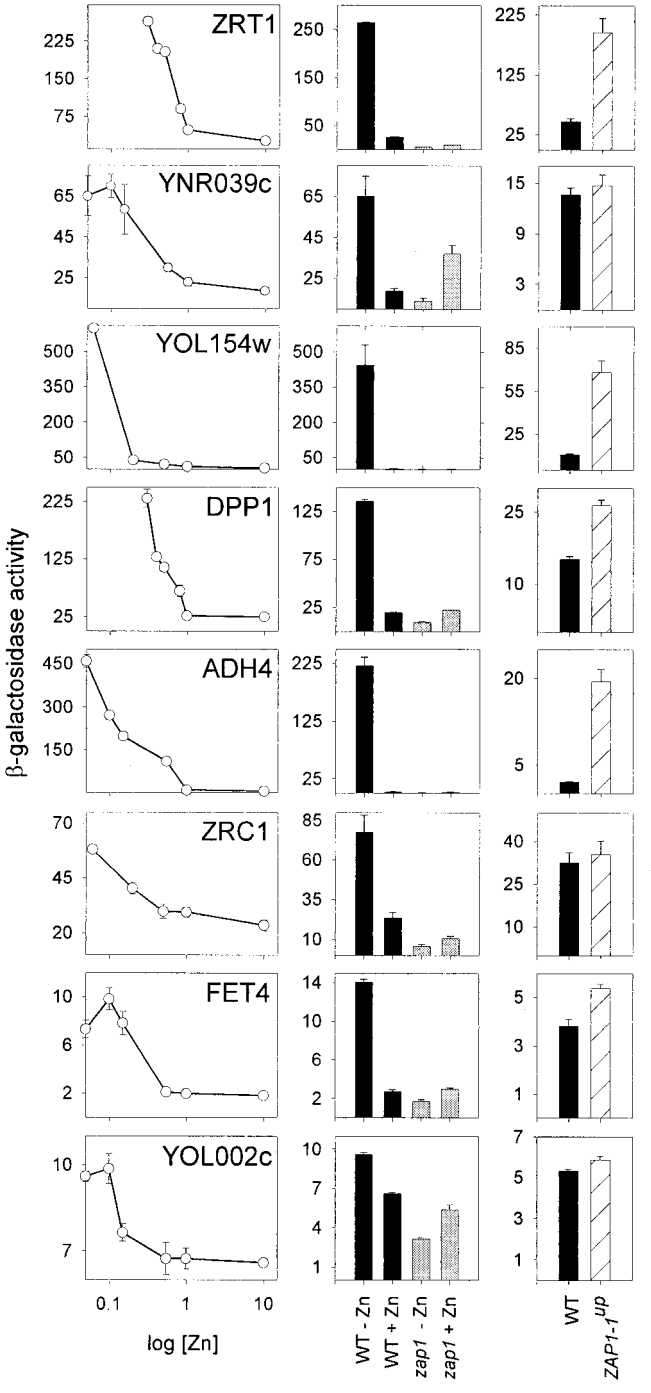

Fig. 3. Zinc responsiveness of intact promoter regions fused to the *lacZ* gene. (*Left*) β-Galactosidase activity in wild-type cells (DY1457) in response to zinc in CSD. (*Center*) The same fusions assayed in wild-type (DY1457) and *zap1* mutant (ZHY6) cells grown in CSD with (+Zn) or without (−Zn) 10 μM $ZnCl_2$ added. (*Right*) The activity of the same fusions assayed in the wild-type (DY1457) and *ZAP1-1*^{up} (ZHY7) strains grown in SD. Each graph is a representative experiment, and error bars represent 1 SD. WT, wild type.

Table 2. Zinc regulation of isolated ZREs inserted into the CYC1 promoter lacking the upstream activation sequences

ZRE	β-Galactosidase activity, Miller units			
	WT −Zn	WT +Zn	*zap1* −Zn	*zap1* +Zn
None*	0.5 ± 0.1	0.5 ± 0.1	1.2 ± 0.1	0.5 ± 0.1
ZRT1†	854.8 ± 58.0	6.3 ± 0.9	5.6 ± 1.4	3.3 ± 0.4
DPP1	15.7 ± 0.9	1.1 ± 0.1	2.1 ± 0.5	1.0 ± 0.1
ADH4	153.1 ± 7.7	3.2 ± 0.3	5.2 ± 0.6	2.8 ± 0.3
ZRC1	452.3 ± 42.3	4.8 ± 2.9	5.8 ± 0.6	3.4 ± 0.4
FET4	301.6 ± 5.3	7.1 ± 0.9	4.3 ± 1.1	8.2 ± 0.3
YOL002c	54.2 ± 2.0	4.1 ± 0.5	5.4 ± 0.1	4.0 ± 0.1

WT, wild type.
*Nonfunctional mutant M2 of ZRT1 ZRE1 (13).
†ZRE1 in the ZRT1 promoter, ACCCTCAAGGT (13).

cells ranged from 13- to 140-fold in response to zinc limitation with little change (≤2-fold) occurring in *zap1* mutants. These results demonstrated that these ZRE-like sequences are indeed functional ZREs.

Discussion

This study demonstrates the power of combining microarray expression profiling and motif analysis to identify the target genes of a specific transcription factor. The value of microarray analysis is abundantly clear from previous studies that used this technique (e.g., refs. 1–3). The addition of MEME makes microarray analysis even more powerful by allowing the identification of potential regulatory elements in the promoters of similarly regulated genes. This method was extremely useful in this study, because most of the potential Zap1 target genes were not identified by using only the experimentally derived consensus sequence. In a broader context, this study demonstrates the efficacy of our approach in characterizing the regulon of unknown transcription factors. Such a tool is clearly necessary as microarray analyses continue to identify large collections of similarly regulated genes. The fact that MEME could identify the ZRE without the benefit of prior experimental results demonstrates that this combined approach will be an extraordinarily useful tool for future studies of yeast and other organisms.

Zinc deficiency causes pervasive changes in the gene expression pattern of yeast. More than 15% of the genes had significantly altered expression in response to this condition. A large number of these genes (46 total) seem to be regulated directly by Zap1p, but most are not. We also examined the array data to determine whether any genes whose promoters contain potential ZREs are repressed rather than activated by Zap1p. No such genes were identified, suggesting that Zap1p does not serve a direct negative regulatory role in addition to its positive regulatory functions. An indirect role in negatively regulating expression of certain genes, however, is still possible (see below).

The list of potential Zap1 target genes generated in this study provides a unique resource for probing cellular zinc homeostasis at a molecular level. More than half of the genes identified have no known function. Their regulation by zinc and by Zap1 provides one of the first clues about the function of these genes. Moreover, with this list in hand, we can better outline potential strategies that yeast employs to thrive under zinc-deficient conditions. First, by increasing Zap1p expression in zinc-limited cells (12), yeast amplifies the transcriptional response of the entire Zap1p regulon. A second strategy is the induction of the Zrt1p and Zrt2p plasma membrane transporters, which allows more efficient scavenging of zinc from the medium (9). In this regard, it is intriguing that *FET4*, encoding the low-affinity iron transporter (19), is a Zap1p target gene. Overexpression of Fet4p makes cells hypersensitive to zinc, suggesting that this protein can transport zinc (20). Moreover, Fet4p has been shown recently to be a low-affinity copper transporter (R. Hassett, D. Kosman, and D.J.E., unpublished work), and its regulation by Zap1p suggests that it may play a similar role for zinc.

Another Zap1p target gene identified in this study, YOL154w, may also participate in zinc uptake. This gene encodes a potentially secreted protein similar in sequence to zinc metalloproteases (21). Given that yeast normally grows on decaying vegetation, increased expression of a protease that degrades extracellular proteins, thus making protein-bound zinc available for uptake, may be an adaptive response to low-zinc conditions. Similar proteins in *Aspergillus* are also zinc-regulated and are the dominant antigens in aspergillosis infections (22). Because sequestration of micronutrients, such as zinc and iron, is an important part of the host response to infection (23), the induction of genes like YOL154w may be a reciprocal response of pathogenic fungi to overcome this host defense.

A third strategy is to mobilize intracellular zinc stores. This conclusion comes from our recent characterization of another Zap1p target gene, *ZRT3* (11). Zrt3p is a member of the same family of metal transporters that includes Zrt1p and Zrt2p and is responsible for transporting stored zinc out of the vacuole during the transition from zinc-replete to zinc-limiting conditions. Zinc uptake into the vacuole in zinc-replete cells is apparently mediated by two other transporters, Zrc1p and Cot1p (11, 24). Surprisingly, we found in this study that the *ZRC1* gene is also a Zap1p target gene. Thus, zinc-limited cells increase expression of both vacuole zinc influx (Zrc1p) and efflux (Zrt3p) transporters. These seemingly paradoxical observations suggest that it may be important to maintain a zinc flux through the vacuole under zinc deficiency, perhaps to provide a sufficient level of the metal for the activity of zinc-dependent vacuolar proteins.

Once zinc has been transported into the cytoplasm of a zinc-limited yeast cell, it needs to be partitioned to those proteins that require the metal for function. For cytoplasmic proteins, diffusion

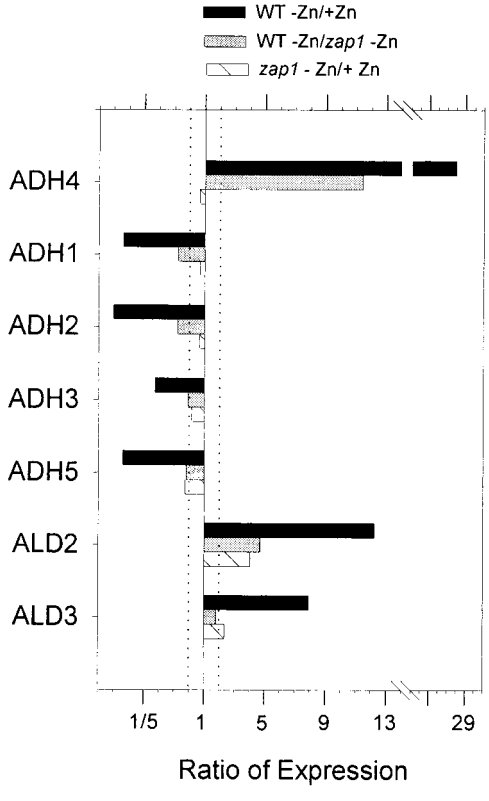

Fig. 4. Average expression ratios, as determined by microarray analysis, for several genes involved in fermentation and alcohol use. The dotted line denotes 2-fold changes. WT, wild type.

of zinc to the appropriate binding sites may be sufficient. However, copper ions require specific chaperone proteins to guide them properly to copper-dependent proteins (25). There is no evidence that any of our putative Zap1p targets act as zinc chaperones, but it is possible that one or more may function as such. In this regard, microarrays made up of ORFs identified from the genome sequence may be of limited usefulness. These ORFs were identified, because they encoded proteins greater than 100 amino acids in length. Copper chaperone proteins such as Atx1p are smaller and would not have been identified as genes without prior information. Thus, other methods, e.g., serial analysis of gene expression (26), may be needed to identify smaller, but nonetheless important, Zap1p targets.

Many zinc-dependent proteins are located inside organelles, e.g., mitochondria, endoplasmic reticulum, etc.; thus, it is important that zinc be transported into these organelles efficiently. As described above, Zrc1p may fulfill this role in the vacuole. Several other genes in our list of Zap1p targets also encode potential zinc transporter proteins including YNR039c and YOL002c. Future studies will address the potential role of these proteins in transporting zinc in and out of intracellular organelles.

It is also conceivable that some Zap1p targets are involved in coping with rather than alleviating zinc deficiency. Zinc-dependent enzymes are likely to be impaired by zinc limitation. Therefore, an inventive adaptation to this condition would be to activate isozymes of zinc-binding proteins that do not rely on this metal as a cofactor. This strategy is suggested by the induction of *ADH4*. Most of the ADH isozymes in *S. cerevisiae* (Adh1p, Adh2p, Adh3p, and Adh5p) use zinc as a cofactor. In contrast, Adh4p may use iron instead of zinc. This protein is remarkably related (53% identical) to an iron-dependent ADH from *Zymomonas mobilis* (16). Although there is controversy over whether Adh4p uses iron or zinc as its cofactor (27), we postulate that this protein is up-regulated to compensate for loss of zinc-dependent ADH activity. This hypothesis is supported further by the observation that all four of the zinc-dependent ADH isozymes are expressed at lower levels in limiting zinc conditions (Fig. 4). At least some component of this reduced expression seems to be Zap1p-dependent (i.e., compare wild type vs. *zap1* −Zn). Thus, there may be a concerted effort to shut off expression of these isozymes in low zinc and to replace their activities with Adh4p. In addition, several other enzymes involved in ethanol metabolism (e.g., the aldehyde dehydrogenases encoded by *ALD2* and *ALD3*; Fig. 4) are also affected in a Zap1p-dependent manner, suggesting that this biochemical pathway is particularly sensitive to zinc deficiency. (It should be noted that the four zinc-dependent ADH genes are closely related, as are ALD2 and ALD3. Thus, some of their apparent coregulation may in fact be due to cross-hybridization of the cDNA probes.) *Z. mobilis* regulates the activity of its zinc- and iron-dependent ADH isozymes in response to the availability of their respective nutrients (28). Thus, this strategy may be a conserved adaptive response to zinc-limiting conditions.

The promoters of many apparently Zap1p-responsive genes do not contain potential ZREs. These include genes induced by zinc-deficiency as well as genes like *ADH1*, etc., that are repressed in low zinc. The lack of ZREs in the promoters of these genes suggests that some genes directly controlled by Zap1p serve as intermediate factors regulating expression of downstream pathways. A potential regulatory protein among the Zap1p targets is the product of the *DPP1* gene. *DPP1* encodes diacylglycerol pyrophosphate phosphatase (29). Although the function of diacylglycerol pyrophosphate in yeast is unknown, its relatively low abundance in membranes suggested a potential role as a second messenger in some signal transduction process (30). *DPP1* is highly zinc-regulated (6-fold higher in zinc-limited cells), suggesting that major changes in diacylglycerol pyrophosphate levels occur in zinc-limited cells.

Finally, the large number of genes that change under zinc deficiency indicates that this state has pervasive effects on cell physiology. Approximately half of the genes affected in wild-type cells with or without zinc were altered similarly in the *zap1* mutant. This observation indicates that these changes in expression result from indirect effects of zinc deficiency on a variety of metabolic processes. A detailed consideration of these effects would provide insight into the various functions of zinc in eukaryotic cells and the metabolic defects that result from zinc deficiency.

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