Roles of Two Activation Domains in Zap1 in the Response to Zinc Deficiency in Saccharomyces cerevisiae*

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Eukaryotic transcription factors are generally modular in structure having separable domains for DNA binding and transcriptional activation. The DNA-binding domains of these factors contain protein structures such as zinc fingers or bZip domains that allow for their sequence-specific interaction with target promoters. Activation domains are structurally less defined and are therefore identified functionally by their ability to increase transcription of a gene when bound to a promoter. Mapping of activation domains has been traditionally done by generating deletions in the transcription factor or by fusing subdomains to heterologous DNA-binding domains. At a more mechanistic level, activation domains induce transcription through their interaction with various proteins, termed coactivators, which enhance the process of transcription initiation. Some coactivators function by physically moving nucleosomes in the promoter region to expose protein-binding sites. Other coactivators modify histones in a variety of ways to decrease the degree to which DNA is packaged within chromatin. Coactivators can also recruit RNA polymerase or general transcription factors by direct protein-protein interactions with those factors.

Although most transcriptional activators contain only single activation domains, several of these proteins have been found to have two or more such domains. These include the yeast activators Gal4, Gcn4, Hap4, and Hsf1 (1–3). Among higher eukaryotes, members of the steroid hormone receptor family (e.g. glucocorticoid receptor), Oct-2a, and MTF-1 all contain two or more functionally mapped activation domains (4–6). The importance of multiple activation domains to the function of these different transcription factors remains controversial. In some proteins such as Gal4 and Gcn4, one of the mapped activation domains may be cryptic and not functional in the context of the full-length protein (7, 8). In other proteins such as Hap4, it was proposed that having multiple activation domains allows for the recruitment of different coactivators thereby improving the efficiency of transcription initiation (3). For glucocorticoid receptor, Rogatsky et al. (9) showed that the two activation domains of this protein play different roles on some promoters. Some target promoters required the activity of both domains for their expression, whereas other promoters primarily required one or the other domain. A similar conclusion arose from a recent analysis of the yeast heat shock factor, Hsf1 (10). Thus, although several transcriptional activators contain multiple activation domains, the importance of those domains to target gene regulation remains largely unexplored.

Another transcriptional activator with more than one activation domain is the Zap1 protein of yeast. Zap1 plays a central role in the homeostatic regulation of this essential yet potentially toxic metal ion. Under conditions where zinc is limiting, Zap1 activates expression of ~80 target genes in the yeast genome (11, 12). When cells are zinc-replete, Zap1 function is shut off (13). Genes regulated by Zap1 encode such proteins as the plasma membrane zinc uptake transporters Zrt1, Zrt2, and Fet4, the vacuolar zinc transporters Zrc1 and Zrt3, and many other proteins involved in zinc homeostasis and the adaptation of cellular processes to conditions of zinc limitation (14).

Zap1 is a zinc finger (Znf) protein containing a DNA-binding domain that is responsible for specific recognition of zinc-
response elements (ZREs), found in one or more copies in the promoters of Zap1 target genes (15). Zap1 also contains two activation domains, AD1 and AD2 (16), that turn on target gene expression in response to low zinc. Both AD1 and AD2 are likely to be regulated by zinc binding directly to regions of the protein, termed zinc-responsive domains, which encompass the activation domains (17–19).

A remarkable feature of the Zap1 activation domains is that they are regulated independently of each other by zinc (16, 17). Considered more broadly, Zap1 is distinctive among all transcription factors that have been studied in having multiple activation domains that are independently regulated by the same physiological signal. Interestingly, both AD1 and AD2 and their regulatory ligand residues are conserved among Zap1 orthologs found in other fungal species (17, 20). In addition, previous studies demonstrated that AD1 and AD2 from Ashbya gossypii Zap1 were both able to independently confer zinc-responsive regulation on Zap1 targets in a zap1Δ strain of Saccharomyces cerevisiae, indicating that AD1 and AD2 function and regulation are conserved in the A. gossypii ortholog (17, 20). Evolutionary divergence of S. cerevisiae and A. gossypii occurred before the genome duplication event in Hemiascomycetes thought to have occurred ~100 million years ago (21). The evolutionary conservation of both AD1 and AD2 over millions of years suggests that both domains are critical for Zap1 function and each plays distinct roles so as to be retained through natural selection. In this study, we characterized the roles of AD1 and AD2 in Zap1 function. We found that although AD1 plays the primary role in zinc deficiency, AD2 may be important when zinc-limited cells experience other environmental stresses.

EXPERIMENTAL PROCEDURES

Yeast Strains and Growth Conditions—Media were used as synthetic defined (SD), and low zinc medium (LZM), as described previously (22). LZM is zinc-limiting because it contains 1 mM EDTA and 20 mM citrate to buffer metal availability. Therefore, only a small fraction of the total zinc in LZM is available for uptake by cells. In all experiments, 2% glucose was used as the carbon source. Zinc was supplied as ZnCl2. To induce heat stress, cells were grown at 37 °C rather than 30 °C. DY1457 (MATα ade6 can1 his3 leu2 trp1 ura3) was used as a wild type strain for normalizing expression from the promoter by the addition of β-estradiol. VEL1-lacZ was constructed by amplifying the 1000-bp region upstream of the translational start site of the VEL1 gene using primers with 40 bp of homology to YEp353. The resulting fragment was then inserted into BamHI-, EcoRI-digested YEp353 using homologous recombination. SSA3-lacZ was a kind gift from Dr. Elizabeth Craig (University of Wisconsin-Madison). All newly constructed plasmids were verified by sequencing.

Protein Analyses—Cells were grown to exponential phase in zinc-limiting media (LZM + 3 μM ZnCl2). Following one wash with 1 × PBS, protein extracts were generated by lysis in the presence of 10% trichloroacetic acid and protease inhibitor mixture. Lysates were resolved on a 6.5% SDS-polyacrylamide gel before immunoblotting. Primary antibodies were anti-Myc, anti-Zap1-DBD, anti-Pgk1, and horseradish peroxidase-conjugated secondary antibodies. For preparation of crude nuclei, cells were grown to mid-log phase in 200 ml of LZM supplemented with 3 μM ZnCl2. Cells were washed twice with buffer A (100 mM KH2PO4, 1.2 mM sorbitol), and spheroplasts were generated by incubating the cells in 50 units/ml zymolyase for 2 h at 30 °C. Following two additional washes with buffer A, the spheroplasts were resuspended in 18% Ficoll buffer and broken in a Dounce homogenizer. The homogenate was centrifuged at 5,000 × g for 10 min to remove cell debris, and the resulting supernatant was centrifuged at 25,000 × g for 30 min to isolate crude nuclei.

β-Galactosidase Assays—Cells were grown to mid-log phase in LZM supplemented with the indicated amount of ZnCl2. Assays were performed using the method of Guarente (28). β-Galactosidase activity was normalized to cell density, as determined by absorbance at 595 nm.

S1 Nuclease Protection Assays—RNA was extracted from cells grown to mid-log phase using hot acid phenol extraction. S1 analysis was performed as described previously (29). Ten μg of total RNA was hybridized to a 32P-end-labeled oligonucleotide probe before digestion by S1 nuclease and separation on an 8% polyacrylamide gel. Probes used are listed in supplemental Table S1. Band intensities were quantified by phosphorimager analysis using OptiQuant Image Analysis software.

Microarray Analyses—Cells were grown to an A600 of ~1.0 in LZM supplemented with 3 μM ZnCl2. Two paired conditions were used as follows: ABY9 cells expressing Zap1 WT versus Zap1 AD1 and ABY9 cells expressing Zap1 WT versus Zap1 AD1 was generated by amplifying Znf1/2 from pCMV-Gli1 using primers with 40 bp of homology to regions immediately upstream of cysteine 1 of Znf1 and downstream of histidine 2 in Znf2. This fragment was inserted into BstXI-linearized pYef2-L-Zap1–6x-myc by homologous recombination. The URA3 marker on the resulting plasmid was changed to LEU2 using Smal-digested pUL9 (24). Other deletion alleles were made using overlap PCR and inserting these fragments via homologous recombination into BstXI-linearized pYef2-L-Zap1–6x-myc. Reporters pDG2 (ZRE-lacZ) (13), ZRT1-lacZ, DPP1-lacZ, ZPS1-lacZ, ZRC1-lacZ (12), TSA1-lacZ (25), MET30-lacZ (26), and ZRT3-lacZ (27) were all constructed as described previously. pGEV-HIS3 (23) was used to control expression of Zap1 from the GAL1 promoter by the addition of β-estradiol. VEL1-lacZ was constructed by amplifying the 1000-bp region upstream of the translational start site of the VEL1 gene using primers with 40 bp of homology to YEp353. The resulting fragment was then inserted into BamHI-, EcoRI-digested YEp353 using homologous recombination. SSA3-lacZ was a kind gift from Dr. Elizabeth Craig (University of Wisconsin-Madison). All newly constructed plasmids were verified by sequencing.

The abbreviations used are: ZRE, zinc-response element; AD, activation domain; TES, 2-[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]ethane-sulfonic acid; LZM, low zinc medium.
Zap1AD2. Each experiment was performed in quadruplicate using independent cultures. Total RNA was isolated using hot acid phenol extraction, and mRNA was then isolated using a PolyATract™ mRNA isolation kit (Promega, Madison). Cy3-dUTP or Cy5-dUTP was incorporated during reverse transcription of the mRNA. The fluorescently labeled products were hybridized to yeast whole genome arrays (University of Utah Health Science Center Microarray Facility), washed, and scanned as described previously (30). To remove intensity-dependent artifacts, we normalized the log Cy3/Cy5 fold changes within each microarray slide using the locally weighted scatterplot smoothing (LOWESS) algorithm (31). Differences in activation domain contribution were assessed using a two-sided Student’s t test where significant differences in contribution were defined as p < 0.05 relative to the paired Zap1WT control.

Chromatin Immunoprecipitation—Chromatin immunoprecipitation was performed as described previously (32). Cells were grown to an A600nm of ~0.5, after which they were cross-linked using 1% formaldehyde. The cross-linking reaction was quenched by adding 2.5 ml of 2M glycine. Following two 25-ml washes with ice-cold PBS, the cells were resuspended in lysis buffer containing complete protease inhibitor mixture (Roche Applied Science), 1 mM PMSF, and 2 mM benzamidine. Cells were lysed by vortexing the cell suspension for 45 min in the presence of glass beads. The lysates were centrifuged at 16,000 × g for 10 min to remove cell debris and glass beads. The supernatants were then incubated in the presence of anti-Myc antibody overnight at 4 °C. Following the isolation of DNA–protein complexes using protein A-Sepharose, cross-linking was reversed in TES, and immunoprecipitated DNA was amplified using primers that flanked the ZRE of the indicated gene by 100 bp (supplemental Table S1). Primers specific to the CMD1 promoter were used as a negative control. The quantitative nature of the analysis was confirmed by using serial dilutions of the samples in separate PCRs (data not shown).

RESULTS

Construction of Alleles to Assess AD1 and AD2 Contributions to Zap1 Function—A diagram depicting the functional domains of Zap1 is shown in Fig. 1A. The DNA-binding domain maps to residues 705–880 and consists of five C2H2-type zinc fingers designated Zn3–Zn7. Our previous results had mapped AD1 to amino acids 332–402 (supplemental Fig. S1). This region is found within a zinc-responsive domain, ZRDAD1, which spans residues 182–502. AD2 was previously mapped to residues 611–640 and is found within a second zinc-responsive domain, ZRDAD2, defined by two other zinc fingers, Znf1 and Znf2 (residues 579–641). Zinc binding to residues in ZRDAD1 and ZRDAD2 appears to control the activity of AD1 and AD2, respectively.

To assess the function of each of these activation domains, alleles of Zap1 were constructed in which the protein contained both ADs or either AD1 or AD2 alone. These alleles were constructed in a GAL1 promoter expression vector to allow precise control of expression levels using the GEV hybrid activator and graded levels of its inducer, β-estradiol. The GEV protein contains the Gal4 DNA-binding domain, the VP16 activation domain, and the hormone-response domain of the human estrogen receptor. In addition, each allele was tagged with six N-terminal Myc epitopes and expressed from its native promoter, zap1Δ cells expressing either Zap1WT (Zap1WT) or an empty vector (zap1Δ), and the isogenic wild type strain (end. Zap 1) were grown in low zinc (LZM + 3 μM ZnCl2), and immunoblot analysis was performed using an anti-Myc antibody. Zap1WT migrates slower than endogenous Zap1 due to the Myc tags. Pgk1 was used as a loading control. C, to verify all Zap1 alleles accumulated to similar levels, zap1Δ cells expressing either Zap1WT, Zap1AD1, or Zap1AD2 were grown in LZM + 3 μM ZnCl2, and whole cell extracts (WCE) were immuno-blotted with an anti-Myc antibody (left panel). Crude nuclear extracts were also prepared from those cells and immuno-blotted with anti-Myc (right panel). Coomassie (Coom) staining was used to verify equal protein loading of the nuclear fractions. vec, vector.

FIGURE 1. Zap1 domains and alleles used in this study. A, schematic representation of the Zap1 protein. Zap1 contains seven zinc fingers (filled boxes numbered 1–7), and fingers 3–7 compose the DNA-binding domain (DBD). The two activation domains, AD1 and AD2 (black boxes), are embedded within larger zinc-responsive domains (ZRDs, orange boxes), which are responsible for regulating AD activity in response to zinc status. Zap1WT(1–880), Zap1AD1 (ΔZnf1/2;GliZnf1/2), and Zap1AD2 (Δ6–551) were used to assess AD contribution to Zap1 activity. Gli zinc fingers Znf1/2 are marked in red. All of these alleles were tagged with six N-terminal Myc epitopes and expressed from the GAL1 promoter with the β-estradiol-responsive GEV activator to equalize expression levels. B, to verify equal accumulation of Zap1WT under the control of the GAL1 promoter with chromosomal Zap1 expressed from its native promoter, zap1Δ cells expressing either Zap1WT (Zap1WT) or an empty vector (zap1Δ), and the isogenic wild type strain (end. Zap 1) were grown in low zinc (LZM + 3 μM ZnCl2), and immunoblot analysis was performed using an anti-Myc antibody. Zap1WT migrates slower than endogenous Zap1 due to the Myc tags. Pgk1 was used as a loading control. C, to verify all Zap1 alleles accumulated to similar levels, zap1Δ cells expressing either Zap1WT, Zap1AD1, or Zap1AD2 were grown in LZM + 3 μM ZnCl2, and whole cell extracts (WCE) were immuno-blotted with an anti-Myc antibody (left panel). Crude nuclear extracts were also prepared from those cells and immuno-blotted with anti-Myc (right panel). Coomassie (Coom) staining was used to verify equal protein loading of the nuclear fractions. vec, vector.
known and we know of no structural ortholog of this domain, we were unable to generate an allele analogous to the Gli substitution in Zap1AD1. Nonetheless, given the extent of the deletion, Zap1AD2 lacks all AD1 function.

We next established protein expression levels of the Myc-tagged Zap1 proteins similar to that of endogenous wild type Zap1. Preliminary experiments were performed to determine the concentration of the β-estradiol inducer needed to generate levels of Zap1WT similar to that of endogenous Zap1 in zinc-limited cells. This was assessed using an anti-Zap1 antibody (data not shown; Fig. 1B). We then confirmed the expression of Zap1AD1 and Zap1AD2 equal to that of Zap1WT by immunoblotting using an anti-Myc antibody (Fig. 1C, left panel). Immunoblot analysis was also performed on crude nuclei preparations to verify equal nuclear accumulation of these proteins (Fig. 1C, right panel). No myc-Zap1 was detectable in whole cell extracts at exposure conditions comparable with the immunoblot of the nuclear preparations indicating that the majority of each protein is localized correctly to the nucleus of cells (data not shown). Thus, any phenotypic effects of the mutant alleles that we observed were not due to over- or underexpression or mislocalization of the protein.

**AD1 and AD2 Both Contribute to Growth under Zinc-limiting Conditions**—As a first assessment of AD function, we determined the ability of these alleles to support growth under low zinc conditions. Previous studies have shown that a zap1Δ strain is unable to grow when zinc is limiting in the media (13). As expected, zap1Δ mutants transformed with the vector grew very poorly under low zinc conditions (Fig. 2A). When low zinc growth was assessed over time, Zap1AD1 and AD2 complementation was indistinguishable from that of Zap1WT until the cells reached late log phase (Fig. 2A). In contrast, Zap1AD2 complemented poorly during all phases of growth. We also assayed these strains for growth yield after 16 h in media containing zinc over a range of concentrations. As expected, zap1Δ vector transformants grew poorly under all conditions except when supplemented with high concentrations of zinc (Fig. 2B). Zap1AD1 cells showed a zinc dose response indistinguishable from wild type. In contrast, cells expressing Zap1AD2 showed a clear growth defect relative to Zap1WT-containing cells in the presence of 1 μM ZnCl₂, and this growth defect was not completely rescued until zinc supplementation exceeded 100 μM ZnCl₂ (p < 0.05). These results indicate that AD1 and AD2 do not functionally redundant in supporting zinc-limited growth. Although AD1 clearly plays the major role in zinc-limited cells during exponential growth, AD2 is important after prolonged zinc deficiency or depletion of other nutrients. No defect was observed for any of these strains in high zinc conditions.

**AD1 and AD2 Contributions to Zap1 Target Gene Induction Are Promoter-specific**—To assess the contribution of AD1 and AD2 to Zap1 target gene induction, we first used Zap1 target promoter-lacZ fusions. In these reporter constructs, the promoter region of each gene was fused to the *Escherichia coli lacZ* gene. For some promoters tested, i.e. MET30, ZRC1, and DPP1, Zap1AD1 and Zap1AD2 contributed equally to lacZ induction (Fig. 3). Although AD1 and AD2 were functionally redundant for maximal expression of MET30-lacZ and ZRC1-lacZ, both ADs were required for full expression of DPP1-lacZ. For other promoters, AD1 played a clearly dominant role, and AD2 was much less active. Although AD2 could drive ~50% of maximal expression of ZRT1-lacZ and ZRT3-lacZ, AD2 was almost totally incapable of activating VEL1-lacZ and ZPS1-lacZ. The reciprocal effect was seen with the TSA1-lacZ reporter. For this reporter, AD2 was capable of activating full expression, whereas AD1 was inactive. These results suggested that the relative contributions of AD1 and AD2 to driving maximal expression in low zinc are promoter-specific. Although some promoters respond better to AD1, others may respond better to AD2. These results also support the conclusion of the growth experiments indicating that these activation domains are not functionally redundant at all promoters.

To verify these relative contributions of AD1 and AD2 on expression of chromosomal Zap1 target genes, we performed microarray analyses under similar conditions as were used for
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FIGURE 3. Assessing AD contribution to Zap1 target gene induction using promoter-lacZ fusions. lacZ fusions under the control of various Zap1 target gene promoters were used to assess AD activity. zap1Δ cells expressing the indicated promoter-lacZ reporter and either Zap1WT, Zap1AD1, Zap1AD2, or the empty vector were grown to mid-log phase in LYM supplemented with either 1 μM (filled bars) or 1000 μM (open bars) ZnCl2. Cells were then harvested, and β-galactosidase assays were performed. The values shown are the means of three independent cultures, and the error bars indicate ± 1 S.D. vec, vector.

the lacZ reporter assays. Whole genome microarrays were used for these experiments to allow simultaneous assessment of the entire Zap1 regulon. In addition, these arrays were also useful in assessing the effects of other transcription factors on Zap1 target genes regulated by combinatorial control (see below). In these experiments, we performed two pairwise comparisons each done in quadruplicate with independent cultures. In the first pairwise comparison, target gene expression in zap1Δ mutant cells expressing Zap1WT was compared with cells expressing Zap1AD1. In the second analysis, expression in Zap1WT-expressing cells was compared with cells bearing Zap1AD2. All cells were grown under zinc-limiting conditions to induce Zap1 activity. The results for several representative Zap1 target genes are provided in Fig. 4, and the data for the full Zap1 regulon are provided in supplemental Table S2.

From these experiments, three general classes of Zap1 target genes emerged based on the relative contribution of AD1 and AD2 to their induction. Genes in target class I (Fig. 4A) were defined as those genes where AD1 and AD2 contributed equally to their maximal induction in low zinc. Zap1AD1 and Zap1AD2 alone were capable of driving expression of all class I targets to levels similar to those conferred by Zap1WT with the exception of ADE17 and IZH1 (p < 0.05). Both AD1 and AD2 were required for maximum expression of these promoters. All other expression levels were not statistically different from wild type Zap1 indicating that AD1 and AD2 are functionally redundant for most of the class I target genes. Class I contained 24 total genes (supplemental Table S2).

Class II contained 21 targets and was defined as those genes where Zap1AD1 induced target expression to levels significantly higher than Zap1AD2 (Fig. 4B and supplemental Table S2). For some genes such as ZRT3, ZRG17, and ZRT1, AD2 was still capable of driving substantial expression although not as high as AD1. For other genes, such as ADH4, ZPS1, VEL1, and YOR387C, AD1 was absolutely required for expression, and Zap1AD2 was only able to induce expression to levels ≤10% of Zap1WT. One explanation for these effects is that the Zap1AD2 protein is unable to bind to the ZREs in the promoter of these specific targets for some unknown reason. However, chromatin immunoprecipitation experiments indicated that Zap1AD2 still occupied two selected class II promoters (ZPS1 and YOR387C) in zinc-limited cells (Fig. 5). The TSA1 gene was also found in class II, which is inconsistent with the TSA1-lacZ results (see “Discussion”). With that lone exception, all of the promoters characterized using lacZ fusions showed the same AD specificity when the chromosomal genes were assayed by microarray analysis.

In the class III group, Zap1AD2 appeared to induce expression of Zap1 targets stronger than Zap1AD1 (Fig. 4C and supplemental Table S2). Surprisingly, however, we noted that Zap1AD1 retained the ability to drive induction to wild type levels or higher for all class III targets. This result indicated that AD1 is fully functional on these promoters as well, and the higher target gene expression observed in Zap1AD2-expressing cells was due to hyper-induction of these genes.

To confirm these microarray results, two members of each class were selected for analysis of mRNA levels by S1 nuclease protection assays (Fig. 4). With independent cultures grown under the same conditions that were used for the microarray experiments, these results were confirmed for class I (DPP1 and ZRC1), class II (ZRT1 and ZPS1), and class III (HSP26 and SED1) targets.

One Mechanism Explaining Zap1AD2 Hyper-induction of class III Genes, Activation of the Heat Shock Response by Severe Zinc Deficiency—We were intrigued by the unexpected characteristic of class III targets, where expression was induced to higher than normal levels in cells expressing Zap1AD2 (Fig. 4C). One possible explanation for this hyper-induction is that the large deletion made to generate the Zap1AD2 allele rendered AD2 a more potent activator of class III promoters, possibly by exposing a cryptic activation domain that is normally masked by other regions of Zap1. To test this hypothesis, we generated Zap1Δ182–502 (designated Zap1AD2), which has a much smaller deletion than Zap1AD2 (i.e. Zap1Δ6–551) and retains more of the Zap1 N terminus.
However, hyper-induction of the class III targets SED1 and HSP26 was still observed in cells expressing Zap1\(^{AD2}\) (supplemental Fig. S3), suggesting this effect was not due to a structural artifact of the original Zap1\(^{AD2}\) allele.

We noted that many of the class III targets (e.g. HSP26, TKL2, and SED1) are also regulated by stress-induced transcriptional activators such as the heat shock factor Hsf1, and we therefore investigated the hypothesis that stress responses play some role in class III hyper-induction. From our microarray data, we noted that ZRT1 expression in cells expressing Zap1\(^{AD2}\) was ~30% of that observed in cells expressing either Zap1\(^{WT}\) or Zap1\(^{AD1}\). Because Zrt1 is the major zinc uptake transporter in zinc-deficient cells, we reasoned that Zap1\(^{AD2}\)-expressing cells would be more severely zinc-limited than Zap1\(^{WT}\) or Zap1\(^{AD1}\) under the same low zinc growth conditions. Because increased cellular stress is a hallmark of severe zinc deficiency (25, 33), it was possible that stress-induced activators are responsible for hyper-induction of class III targets in Zap1\(^{AD2}\)-containing cells, as opposed to increased activity of AD2 per se. Consistent with this model, we noted in our microarray data that several Hsf1 target genes were induced in Zap1\(^{AD2}\)-expressing cells relative to Zap1\(^{WT}\) and Zap1\(^{AD1}\)-expressing cells and similar to the effect seen for class III targets (Fig. 6A). For example, Hsf1 targets such as SSA3, SSA4, and HSP42 were induced to levels ~2-fold higher in Zap1\(^{AD2}\)-expressing cells relative to control cells. Because
these promoters are not regulated by Zap1, these results suggested that Hsf1 may be responsible for the increased expression of at least some class III target genes in cells expressing Zap1<sup>AD2</sup>.

Increased activity of Hsf1 in Zap1<sup>AD2</sup>-containing cells was verified using a lacZ reporter under the control of the SSA3 promoter, which contains heat shock-response elements but does not contain any ZREs. Zap1<sup>AD2</sup>-expressing cells induced this reporter 2-fold higher than Zap1<sup>WT</sup> and Zap1<sup>AD1</sup> cells (Fig. 6B). Vector-transformed cells not expressing any Zap1 showed 6-fold higher levels of induction from the SSA3 promoter than the Zap1<sup>WT</sup> control, indicating that severe zinc deficiency induces the heat shock response. These results are consistent with the hypothesis that increased activity of stress-induced factors such as Hsf1 are responsible for hyperinduction of class III targets in cells expressing Zap1<sup>AD2</sup>.

**Assessing the Role of AD2 in Modulating Zap1 Transcriptional Activation**—Although class III targets are hyperinduced in Zap1<sup>AD2</sup> cells, Zap1<sup>AD1</sup> was capable of driving maximal expression of these target genes in zinc-limited cells. In addition, for the large majority of class I and class II Zap1 target genes, AD1 alone was sufficient to fully induce their expression in response to zinc deficiency. The role of AD2, although needed for maximal expression of two Zap1 targets (ADE17 and IZH1), was still unclear. One hypothesis we considered was that AD1 and AD2 have different zinc dose responses and that having both ADs present may alter the overall zinc dose responsiveness of Zap1. To test this hypothesis, we first examined the zinc response profiles of lacZ reporters in cells grown to mid-log phase over a range of zinc concentrations (Fig. 7, A and B). For both the DPP1-lacZ and ZRT1-lacZ reporters, the zinc response profiles of Zap1<sup>WT</sup> and Zap1<sup>AD1</sup>-expressing cells were similar indicating that AD2 did not modulate the zinc dose response of these promoters as conferred by wild type Zap1. This conclusion was confirmed when we examined the zinc dose response of a chromosomal target gene, ZRT1 (Fig. 7, C and D). In addition, when we examined the zinc response of Zap1<sup>AD2</sup>-expressing cells, zinc dose responses similar to Zap1<sup>WT</sup> and Zap1<sup>AD1</sup> were observed, although the maximal level of ZRT1-lacZ and ZRT1 mRNA expression was lower. These observations suggest that AD1 and AD2 have similar zinc response profiles and are also consistent with our conclusion that AD2 does not alter the overall zinc responsiveness of Zap1.

A second hypothesis we considered was that AD2 might alter the kinetics of induction of Zap1 target genes. For example, if AD2 responded faster to zinc deficiency than did AD1, a Zap1 protein with both activation domains would induce expression more quickly following transfer from zinc-replete to zinc-limiting media than would a Zap1 allele containing

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**FIGURE 5. High AD1 dependence of class II targets is not due to disruption of promoter binding in Zap1<sup>AD2</sup> mutants in vivo.** Zap1Δ cells expressing either Zap1<sup>WT</sup>, Zap1<sup>AD1</sup>, Zap1<sup>AD2</sup>, or the empty vector (vec) were grown to exponential phase in low zinc (LZM + 3 μM ZnCl<sub>2</sub>). These cells were then cross-linked with formaldehyde and harvested, and chromatin immunoprecipitation (IP) assays were performed using an anti-Myc antibody to immunoprecipitate Myc-tagged Zap1. Coprecipitation of specific DNA fragments was then assessed by PCR using primers flanking the ZREs of ZRT1, YOR387C, and ZPS1. Primers specific for the promoter region of CMD1 were used as a negative control. Inputs shown represent 1000-fold dilutions of whole cell extracts.

**FIGURE 6. Hsf1 activity is increased in cells expressing Zap1<sup>AD2</sup>.** A, data for representative gene targets of the Hsf1 transcription factor from the microarray experiments described in Fig. 4. The asterisks indicate a significant difference (p < 0.05) of Zap1<sup>AD2</sup>-containing cells relative to Zap1<sup>AD1</sup>- and Zap1<sup>WT</sup>-expressing cells as determined by two-sided Student’s t test. B, to assay Hsf1 activity, an SSA3-lacZ reporter was used. Zap1 cells carrying this reporter and also expressing Zap1<sup>WT</sup>, Zap1<sup>AD1</sup>, Zap1<sup>AD2</sup>, or an empty vector were grown to mid-log phase in low zinc (+, LZM + 1 μM ZnCl<sub>2</sub>), and β-galactosidase assays were performed. To verify reporter response, controls were included where zinc-replete (+, LZM + 100 μM ZnCl<sub>2</sub>) cells were incubated at 30 or 37 °C for 1 h prior to assay. The values shown are the means of three independent cultures, and the error bars indicate ± 1 S.D. The asterisks indicate significant differences (p < 0.05) relative to Zap1<sup>WT</sup>-expressing cells as determined by two-sided Student’s t test. vec, vector.
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**FIGURE 7.** Contributions of AD1 and AD2 to the zinc dose response of Zap1. zap1Δ cells bearing DPP1-lacZ (A) or ZRT1-lacZ (B) reporter plasmids and expressing Zap1WT, Zap1AD1, Zap1AD2, or an empty vector were grown to mid-log phase in LZM supplemented with the indicated concentration of ZnCl2. Cells were then harvested, and β-galactosidase assays were performed. The values shown are the means of three independent cultures, and the error bars equal ± 1 S.D. C, zap1Δ cells expressing either Zap1WT, Zap1AD1, Zap1AD2, or an empty vector were grown to mid-log phase in LZM supplemented with the indicated concentration of ZnCl2. Total RNA was then isolated and analyzed for ZRT1 and CMD1 mRNA levels by S1 nuclease protection assays. Shown is a representative of two independent experiments. The graph shows the ZRT1 results obtained from C normalized to CMD1 levels. vec, vector.

**FIGURE 8.** Contributions of AD1 and AD2 to Zap1-mediated induction during zinc withdrawal. zap1Δ cells expressing Zap1WT, Zap1AD1, Zap1AD2, or an empty vector were grown to mid-log phase in high zinc (LZM 1000 μM ZnCl2) and then transferred to low zinc (LZM + 1 μM ZnCl2). Cells were harvested at the indicated times; total RNA was isolated, and S1 nuclease protection assays were performed to determine the mRNA levels of ZRT1 and CMD1. Shown is a representative of two independent experiments. The graph shows the ZRT1 results normalized to CMD1 levels. vec, vector.

only AD1. To test this hypothesis, cells expressing Zap1WT, Zap1AD1, or Zap1AD2 were grown to mid-log phase under zinc-replete conditions and then transferred to zinc-limiting media. Zap1WT cells induced ZRT1 expression to a maximal level after 20 h of zinc withdrawal, and mRNA levels then decreased to an intermediate level (Fig. 8). This decrease in expression is likely due to the reduced rate of cell growth (Fig. 2) and therefore lower zinc demand of cells grown to late log phase. Zap1AD1-expressing cells showed a response to zinc withdrawal that was very similar to wild type indicating that AD2 does not greatly alter the kinetics of Zap1 activation. Consistent with these results, induction of ZRT1 in Zap1AD2 cells was much slower than that observed in Zap1WT- or Zap1AD1-expressing cells. These results indicate that AD2 is kinetically much less responsive than AD1 and does not play a major role in dictating the kinetics of Zap1 induction of these promoters.

AD2 Is Important for Zap1 Activity under Heat Stress Conditions—These studies demonstrated that AD1 is largely sufficient to induce expression of Zap1 targets when cells are faced solely with zinc limitation. One condition we found where AD1 was not sufficient for Zap1 function was when zinc-limited cells were grown to late log phase (Fig. 2). This result suggested that AD2 might be important for gene expression when zinc deficiency was combined with other stressors. One such stress we considered was heat stress. When grown in zinc-limiting media at the standard growth temperature of 30 °C, Zap1WT- and Zap1AD1-expressing cells showed similar growth yields, whereas Zap1AD2-expressing cells showed a reduced growth yield as observed previously (Figs. 2 and 9A). When these cells were grown under zinc-limiting conditions at the elevated temperature of 37 °C, growth of both Zap1AD1 and Zap1AD2 cells lagged behind the cells expressing wild type Zap1. This suggested that AD1 may be less able to activate transcription when cells are under heat stress as well as zinc limitation. This hypothesis was confirmed when mRNA levels of ZRT1 were examined. At 30 °C, Zap1AD1 and Zap1WT conferred similar levels of ZRT1 expression, whereas Zap1AD2-activated expression was lower (Fig. 9B). At 37 °C, however, both AD1 and AD2 were unable to activate transcription to wild type levels. Immunoblot analysis indicated that Zap1WT and Zap1AD1 accumulated to similar levels at 37 °C (data not shown) indicating the diminished activity of Zap1AD1 was not due to degradation of the mutant protein at the elevated temperature. These results indicate that both AD1 and AD2 are required for zinc-limited cells exposed to heat stress and suggest that AD2 plays a larger role in Zap1 function under conditions of cellular stress.

**DISCUSSION**

Zap1 is remarkable in having two activation domains that are independently regulated by zinc. The mechanism of regulation for both appears to be direct zinc binding to residues located within and flanking the activation domain. The evolutionary conservation of these activation domains strongly sug-
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FIGURE 9. Both AD1 and AD2 are required for wild type Zap1 function when cells are simultaneously exposed to heat stress and zinc limitation. A, zap1Δ cells expressing Zap1WT, Zap1AD1, Zap1AD2, or an empty vector were inoculated at a starting A600 of 0.03 in L2M supplemented with 3 μM ZnCl2. Cells were grown for either 18 h at 30 °C or 48 h at 37 °C, after which the A600 values of the cultures were recorded. The indicated values are the means of three independent cultures, and the error bars indicate ± S.D. B, S1 nuclease protection assay of ZRT1 and CMD1 mRNA extracted from cells grown as described in A. Shown is a representative of two independent experiments. vec, vector.

suggests that both are essential for full Zap1 function. What those contributions might be was previously unclear.

To assess AD1 and AD2 function, alleles were used in which either activation domain was deleted. Zap1AD1 and Zap1AD2 were analyzed for their ability to complement the growth defect of zap1Δ mutants in low zinc. AD1 was sufficient to provide full Zap1 function in exponential phase but was not fully active during late log phase when other nutrients are also limiting. This result is consistent with a role for AD2 in cells experiencing other stresses in addition to zinc limitation (see below). In contrast, Zap1AD2 complemented poorly during all phases of growth and over a broad range of zinc. Our analysis of Zap1 target gene expression indicated that Zap1AD2 was incapable of fully activating ZRT1 expression. Zrt1 is the primary zinc uptake transporter in zinc-limited cells, and impaired expression of this gene alone is sufficient to cause poor growth in low zinc. Consistent with this hypothesis, we found that expression of the ZRT1 gene from the constitutive PGK1 promoter restored growth of Zap1AD2 cells in low zinc to the wild type rate (data not shown).

We assessed the contributions of AD1 and AD2 to Zap1 target gene expression using both lacZ reporters and microarray analysis of mRNA levels. In almost all cases, these methods gave results that were in close agreement with each other. The lone exception was the TSA1 promoter. Although the TSA1-lacZ reporter was highly dependent on AD2 for induction, expression of chromosomal TSA1 relied more on AD1. The cause of this discrepancy is unclear. One possibility is that nucleosomal positioning on the plasmid-borne TSA1-lacZ reporter differs from that on the chromosomal TSA1 promoter such that AD2 can now drive expression of the TSA1-lacZ reporter although AD1 cannot.

By examining regulation of the entire Zap1 regulon, we identified three distinct classes of Zap1 target genes. Class I genes were those in which AD1 and AD2 contribute equally. In most cases, these domains were functionally redundant. Class II genes were those in which AD1 contributed more to expression than AD2. In some cases (e.g. VEL1 and YOR387C), the dependence on AD1 was absolute with no detectable contribution of AD2. The observation that Zap1AD2 was still capable of binding to these promoters raised the following question, Why, when AD1 is sufficient to induce full expression of almost all Zap1 targets, does AD2 only contribute to expression of some Zap1 targets but not others? One explanation is that AD1 and AD2 recruit different coactivator complexes. We propose that at class I promoters where AD1 and AD2 contribute equally to target induction, the coactivators they recruit are functionally redundant. On class II promoters, however, we propose that the specific coactivators recruited by AD2 are less effective in activating transcription than those recruited by AD1. We are currently characterizing the coactivator interactions of AD1 and AD2 to address this hypothesis. An alternative explanation for these effects is that AD2 is a weak activation domain and only contributes to target gene expression when its activity is combined with that of another transcription factor bound elsewhere in the promoter. Arguing against this hypothesis, we have previously shown that AD2 could strongly activate transcription of a lacZ reporter in which a single ZRE had been inserted into the CYC1 promoter from which other regulatory binding sites had been deleted (16). AD1 and AD2 were similar in their abilities to activate this highly inducible ZRE-lacZ reporter suggesting that AD2 is not intrinsically weaker than AD1.

The third class of genes highlighted an important message about the Zap1 regulon. Specifically, many of these genes are coregulated by other stress response factors that may also be activated by zinc deficiency. Class III genes were expressed at near wild type levels in Zap1AD1-expressing cells and hyperinduced to even higher levels in Zap1AD2-expressing cells. Several class III genes are also known targets of other stress response factors such as Hsf1, the yeast heat shock factor. This observation, combined with our prediction that cells relying solely on AD2 for Zap1 target gene expression are more zinc deficient than wild type or Zap1AD2 cells grown under the same conditions, suggested that Hsf1 activity may
be elevated in Zap1AD2-expressing cells. This hypothesis was supported by analysis of other Hsf1 target genes in the microarray data as well as by direct analysis of the promoter activity of an SSA3-lacZ reporter. The observation that SSA3-lacZ expression is induced to even higher levels in zap1Δ mutant cells transformed with the vector alone suggests that Hsf1 activity is induced even higher by more severe zinc deficiency. To our knowledge, this is the first demonstration of the heat shock response being induced by zinc deficiency. Increased Hsf1 activity in low zinc may be due to the accumulation of unmetallated zinc-binding proteins or the decreased activity of zinc-dependent components of the protein folding machinery itself, e.g. the Ydj1 cochaperone, that may function suboptimally in zinc-limited cells (34). Finally, we hypothesize that other stress-response factors may also contribute to hyper-induction of class III genes. For example, the class III genes TKL2 and SED1 are regulated by the Msn2/Msn4 transcriptional activator. Work of Gauci et al. (35) has previously suggested that Msn2/Msn4 activity is also induced in low zinc.

Our analysis has now focused our attention on a single question. If AD1 is sufficient for most aspects of Zap1 function, what is the function of AD2? We found that AD1 was capable of fully activating all Zap1 targets with the exception of ADE17 and IZHI. Is providing full expression of these two target genes the sole contribution of AD2 or does this domain play other roles? We determined that AD2 does not affect the overall zinc dose response of Zap1 target gene expression nor does it influence the kinetics of Zap1 induction of at least one target promoter, ZRT1. Although these results do not preclude different effects occurring on other target promoters, they do suggest that AD2 plays some other role. One possibility is that having both AD1 and AD2 present alters the cell-to-cell variability in expression levels. Studies by others have shown that there can be large differences in gene expression among individual cells in a population, and this variability may benefit adaptation to a changing environment (36, 37).

An alternative hypothesis was that AD2 might be of greater importance when cells experience other environmental stresses. This was originally suggested by our finding that Zap1AD1-expressing cells grew less well than Zap1WT cells in late log phase when zinc deficiency was combined with deficiency of other nutrients. Consistent with this hypothesis, we found that AD2 was more important for Zap1 function when cells experienced both zinc deficiency and heat stress. One explanation for this increase in the importance of AD2 is that coactivator complexes are present in limiting concentrations in the cell and would limit the capacity of Zap1 to drive expression of its targets if other stress-induced regulons, such as that of Hsf1, are activated. The limiting nature of coactivator complexes in transcription was demonstrated in vivo when activator proteins were expressed at high levels. For example, overexpression of Gal4 increased expression from Gal4-regulated promoters, whereas expression from promoters activated by other activators was reduced (38). This observation, termed “transcriptional squelching,” was attributed to an abundant activator sequestering coactivator complexes required by other activators (39). Other studies directly measuring coactivator abundance confirmed the relatively low abundance of these complexes within the cell. For example, the Swi/Snf complex, a transcriptional coactivator that promotes expression through ATP-dependent chromatin remodeling, is only present in 100–200 copies per cell (40, 41). It seems possible that by heat-treating cells and activating other stress pathways, coactivator complexes may become less available to Zap1. Having two activation domains would increase the efficiency with which Zap1 is able to recruit these coactivators to the promoters of its target genes. It will be interesting to determine whether other stress conditions (e.g. oxidative stress, UV irradiation) also place a greater demand on AD2 as was found for heat stress.

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