Regulation of Zinc Homeostasis in Yeast by Binding of the ZAP1 Transcriptional Activator to Zinc-responsive Promoter Elements*  

Hui Zhao‡, Edward Butler‡, Jacquelyn Rodgers‡, Thomas Spizzø, Sara Duesterhoeft§, and David Eide¶  

From the ‡Nutritional Sciences Program, University of Missouri, Columbia, Missouri 65211 and the §Department of Biochemistry and Molecular Biology School of Medicine, University of Minnesota, Duluth, Minnesota 55812  

Zinc homeostasis in yeast is controlled primarily through the regulation of zinc uptake. Transcription of the ZRT1 and ZRT2 zinc transporters increases in zinc-limited cells, and this induction is dependent on the ZAP1 gene. We hypothesized previously that ZAP1 encodes a zinc-responsive transcriptional activator. Expression of ZAP1 itself increases in zinc-limited cells. This response is also dependent on ZAP1 function through a potential positive autoregulatory mechanism. In this report, we describe the characterization of zinc-responsive elements (ZREs) in the promoters of the ZRT1, ZRT2, and ZAP1 genes. A ZRE consensus sequence, 5'-ACCCYNAAGGT-3', was identified and found to be both necessary and sufficient for zinc-responsive transcriptional regulation. We also demonstrate that ZREs are DNA binding sites for ZAP1. First, a dominant ZAP1 mutation, ZAP1-1⁰, which causes increased expression of ZAP1-regulated genes in zinc-replete cells, exerted its effects specifically through the ZREs. Second, electrophoretic mobility shift assays and in vitro DNase I footprint analyses indicated that ZAP1 binds to ZREs in a sequence-specific fashion. These studies demonstrate that ZAP1 plays a direct role in controlling zinc-responsive gene expression in yeast by binding to zinc-responsive elements in the promoters of genes that it regulates.

The variety of roles that zinc plays in cellular processes is a prime example of the utility of metal ions in biology. Zinc is required for the activity of more than 300 enzymes, including alcohol dehydrogenase, Cu/Zn superoxide dismutase, carbonic anhydrase, and many proteases (1). Zinc is also important for the correct folding of specific domains in many proteins. The largest class of proteins that require zinc as a structural cofactor are transcription factors that contain zinc-dependent DNA binding motifs, such as zinc fingers and zinc clusters (2). For example, almost 2% of the genes in the genome of the yeast Saccharomyces cerevisiae contain zinc-dependent DNA binding domains (3, 4). When zinc-dependent enzymes are also considered, perhaps as many as 5% of all yeast proteins require zinc for their function.

Although an essential nutrient, zinc can be toxic if excess amounts are accumulated. The precise cause of zinc toxicity is unknown, but the metal may bind to inappropriate intracellular ligands or compete with other metal ions for enzyme active sites, transporter proteins, and so forth. Therefore, in the face of fluctuating extracellular zinc availability, cells must maintain an adequate intracellular zinc level to meet cellular requirements while preventing metal ion overaccumulation. Mechanisms of regulating the amount or availability of intracellular zinc include binding of the metal by metallothioneins (5), storage in intracellular compartments (6), and transport of zinc out of the cell (7). In S. cerevisiae, the regulation of zinc homeostasis is mediated primarily through the control of zinc uptake across the plasma membrane (8–10). S. cerevisiae has two separate uptake systems to obtain zinc from its environment. One system has a high affinity for zinc, and the ZRT1 gene encodes the transporter protein of this system (9). The second system is encoded by the ZRT2 gene and has lower affinity for zinc (8). Both ZRT1 and ZRT2 are regulated by zinc availability; zinc limitation induces ZRT1 and ZRT2 transcription, whereas growth under zinc-replete conditions represses their expression (9, 10). This zinc-responsive transcriptional regulation requires the activity of the ZAP1 gene (10). From our initial characterization of ZAP1, we proposed that this gene encodes a transcriptional activator, the function of which is repressed by zinc. This hypothesis was based on several observations. First, deletion of the ZAP1 gene resulted in zinc-deficient phenotypes and abolished zinc-responsive expression of the ZRT1 and ZRT2 genes. Second, a semidominant mutation in ZAP1, called ZAP1-1⁰, caused expression of these target genes in both zinc-limited and zinc-replete cells. Third, sequence analysis of ZAP1 indicated that the encoded protein shares similarity with many transcriptional activators; the C-terminal region contains five C2H2 zinc finger domains and the N terminus contains two acidic regions that could act as transcriptional activation domains. Finally, we found that ZAP1 was required to increase its own transcription in response to zinc, potentially through a positive autoregulatory mechanism.

These observations established that ZAP1 plays a central role in zinc ion homeostasis by regulating transcription of the zinc uptake systems. As compelling as these data were, however, they failed to show whether ZAP1 plays a direct or an indirect role in the response. For example, an equally plausible alternative model was that ZAP1 is part of a signal transduction pathway that communicates the cellular zinc status to another protein, which then regulates transcription. In this report, we demonstrate that ZAP1 is in fact the transcriptional activator that directly controls zinc-responsive gene expression in yeast and identify its binding sites in the promoters of the ZRT1, ZRT2, and ZAP1 genes.

MATERIALS AND METHODS

Yeast Strains and Growth Conditions—Strains used were DY1457 (MATa ade6 can1 his3 leu2 trp1 ura3) and ZHY7 (MATa ade6 can1 his3

* Funding for this research was provided by National Institutes of Health Grants GM48139 and GM58265 (to D. E.) and by the Howard Hughes Medical Institute (to J. R.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed. Tel.: 573-882-9686; Fax: 573-882-0185; E-mail: eided@missouri.edu.
leu2 trp1 ura3 ZAP1–1) (10). Yeast were grown in standard culture media (synthetic defined and yeast extract-peptone media) (11) supplemented with 2% glucose and auxotrophic supplements. A low zinc medium (LZM)1 was prepared in a similar manner as LIM (12) and had the following composition: 0.17% yeast nitrogen base without amino acids (NH, Nippon Shokuhin Kako Co., LTD); 0.4% malt extract; 10 mM 2-mercaptoethanol; pH 4.2; 2% glucose; 1 mM Na₃EDTA; and 0.01% each adenine, histidine, leucine, and tryptophan. The MnCl₂ and FeCl₃ concentrations in LZM were adjusted to final concentrations of 25 and 10 μM, respectively. Cell number in liquid cultures was determined by measuring the optical density of cell suspension at 600 nm (A₆₀₀) and converting to cell number with a standard curve.

β-Galactosidase Assays and Atomic Absorption Spectroscopy—Cells were grown for 15–20 h to mid-exponential phase (A₆₀₀ = 0.5–1) in LZM or synthetic defined medium supplemented with different amounts of ZnCl₂. β-Galactosidase activity was assayed by described by Guarente (13) and is expressed in Miller units calculated as follows: ([A₆₀₀ × 1000]/min × ml of culture used × absorbance of the culture at 600 nm). Control plasmids for β-galactosidase assays were pPH54-UAS (14) and pLG3312 (15). Measurement of cell-associated zinc levels was performed by atomic absorption spectroscopy. Cells were washed twice with an equal volume of distilled deionized water, twice with an equal volume of 1 mM EDTA, resuspended in one-fifth volume 6 M nitric acid, and incubated at 95 °C for 24 h. The acid-digested samples were then analyzed for zinc content on a Varian Spectra AA-30 atomic absorption spectrometer.

Plasmids and DNA Manipulations—Escherichia coli and yeast transformations were performed using standard methods (16, 17). The fusion and deletion junctions for all plasmids constructed were confirmed using the TaqTrack DNA sequencing method (Promega). Insert DNA fragments used for the 5' deletion analysis of the ZRT1 promoter were generated by the polymerase chain reaction using primers containing either a BamHI site (upstream primer) or an EcoRI site (downstream primer) added to their 5'-ends. These fragments were digested with BamHI and EcoRI and inserted into similarly digested integrating vector YEp353 (18) to fuse the ZRT1 promoter region and translation initiation codon to the lacZ open reading frame. The numbering system used throughout this report to describe subcloned promoter regions is relative to the first base of the translation initiation codon, which is used throughout this report to describe subcloned promoter regions is relative to the first base of the translation initiation codon, which is designated as +1. The resulting plasmids were linearized with NcoI to allow integration of the plasmid at the chromosomal URA3 locus after transformation (19).

Insert fragments for the plasmids containing internal deletions were generated by two-step overlapping polymerase chain reaction (20), such that the 5'-overhangs the zinc-responsive element (ZRE) sequences were precisely removed. The resulting fragments were inserted into either YEp353 (for ZRT1 analysis) or YEp353 (18) (for ZRT2 and ZAP1 analyses). Cloning of oligonucleotide inserts was performed using the synthetic oligonucleotides listed in Table I. Each single-stranded oligonucleotide was dissolved in water (100 μM) and phosphorylated using T4 DNA kinase (16). To anneal complementary oligonucleotides, 200 pmol of each fragment was mixed and incubated at 55 °C overnight. The annealed products, which contain 5'-overhangs suitable for cloning into EcoRI, were end-labeled with [α-32P]dCTP. Subsequent digestion of the plasmid with BamHI generated a 360-bp fragment that was used as a probe for in vitro DNAase I footprint analysis. Probes for the ZRT2 and ZAP1 promoters were generated in a similar fashion from pSK-ZRT2 (8) and pKO-ZAP1 (10), respectively. These plasmids were linearized with EcoRI, end-labeled with [α-32P]dATP, and then digested with PstI. Five μl of in vitro transcription/translation product was mixed with the labeled probe in the same binding reaction mixture used for EMSAs. Two μl of DNAse I (1U/μl) was then added to each binding reaction and incubated for 1 min at room temperature. Reactions were terminated by adding 30 μl of stop buffer (20 mM EDTA pH 8.0, 1% SDS, 0.2 μM NaCl, and 200 μg/ml yeast tRNA). Samples were extracted once with phenol/chloroform/isoamyl alcohol (25/24/1), precipitated with ethanol, resuspended in formamide loading buffer (80% formamide, 1 mg/ml xylene cyanol FF, 1 mg/ml bromophenol blue, 10 mM EDTA), and resolved on a 6% denaturing polyacrylamide gel prior to autoradiography.

RESULTS

Identification of ZREs in the ZRT1 Promoter—Our previous studies indicated that the promoter of ZRT1 was completely contained within the upstream region extending from −706 to the translation initiation codon (+1) (9). To map the ZREs that control expression of the ZRT1 gene, a progressive series of 5' deletions were generated such that increasingly smaller fragments of the ZRT1 promoter were fused to a lacZ reporter gene on an integrating plasmid vector (Fig. 1A). These plasmids were transformed into a wild type strain and assayed for expression in cells grown under zinc-limiting and zinc-replete conditions. Deletion of 5'-flanking sequences from −706 to −521 had no effect on the zinc responsiveness of the reporter gene (data not shown). Deletion of the region from −521 to −361 reduced β-galactosidase activity in zinc-replete cells by approximately 20% with little effect on expression in zinc-replete cells. When sequences from −332 to −305 were deleted, an additional loss of zinc-limited expression was observed, again with no change in the zinc-replete expression. Finally, deletion from −221 to −201 completely eliminated expression in both media conditions. Initiation of transcription of ZRT1 was found by primer extension analysis to occur at a single site, position −45 (data not shown), indicating that the ZRT1 TATA box is unlikely to be affected by these deletions.

The results obtained with the 5' deletion series described in Fig. 1A are consistent with there being at least three different ZREs in the ZRT1 promoter. Analysis of the DNA sequence of this promoter revealed the presence of a conserved 11-bp sequence (5'–ACC(CT)/CAT/ATG/ACC–3') in each of the three regions implicated by the deletion series. These sequences are located at positions −319 to −309, −204 to −194, and, in the opposite orientation, −454 to −444, and we refer to them as ZRE1, ZRE2, and ZRE3, respectively. Deletion of any single ZRE from the full-length promoter had little or no effect on zinc-responsive gene expression. However deletion of two elements (ZRE1 and ZRE2) led to greatly reduced expression. Deletion of all three elements completely eliminated expression. These data demonstrate that ZRE1, ZRE2, and ZRE3 are necessary to confer zinc-responsive gene expression on the ZRT1 promoter. Moreover, the results show that these elements are required for the low level of ZRT1 expression observed in the zinc-replete conditions used in these experiments. We suggest

References

1 The abbreviations used are: LZM, low zinc medium; bp, base pair(s); ZRE, zinc-responsive element; EMSA, electrophoretic mobility shift assay.

In Vitro DNAse I Footprint Analysis—Plasmid pSK-FFP was linearized with PstI and end-labeled with Klenow DNA polymerase and [α-32P]dCTP. Electro-
that this expression is due to incomplete repression by zinc under these growth conditions. Consistent with this hypothesis, higher concentrations of zinc (2 mM) completely repressed a full-length \textit{ZRT1-lacZ} reporter gene (data not shown).

To determine whether ZRE1, ZRE2, and/or ZRE3 are sufficient to confer this regulation, oligonucleotides containing these elements or portions thereof (Table I) were inserted into the promoter of a \textit{lacZ} reporter gene bearing the \textit{CYC1} TATA boxes but lacking an upstream activation sequence (i.e., a “minimal” \textit{CYC1} promoter). Each value represents the average of three independent assays, and the error bars indicate 1 S.D. A, deletion analysis of the \textit{ZRT1} promoter. The internal deletions span bases −318 to −308 (ZRE1, filled box), −203 to −193 (ZRE2, hatched box), and −454 to −444 (ZRE3, open box). B, deletion analysis of the \textit{ZRT2} promoter. The deletions span bases −310 to −300 (ZRE1, filled box) and −361 to −251 (ZRE2, hatched box). C, deletion analysis of the \textit{ZAP1} promoter. The deletion spans bases −143 to −133 (filled box).
ZAP1 Binds to Zinc-responsive Promoter Elements

Two single-stranded oligonucleotides were annealed to yield the following double-stranded fragments with flanking four base overhangs (not shown) for cloning into EagI and SacI sites. The bases of the ZREs are underlined and the mutated bases are in lowercase letters. Dg2, Dg0, and Dg3 contain all or portions of ZRE1 from the ZRT1 promoter. M1, M2, and M3 are mutant variants derived from the Dg2 oligonucleotide. These oligonucleotides were inserted into pNB404 and transformed into wild type (DY1457) and ZAP1–1up (ZHY7) cells. Zinc responsiveness was assayed in wild type cells grown in low (–Zn) or high (+Zn) media as described in Fig. 1 and the induction is the ratio of the two values. ZAP1–1up allele effects were determined in ZHY7 (Up) and DY1457 wild type (WT) strains grown in SD glucose medium. U/W is the ratio of these two values. Each value is the average of three independent assays ± 1 S.D. ND, not determined.

| Oligonucleotide | Sequence | Zinc responsiveness | ZAP1–1up allele effect |
|-----------------|----------|---------------------|------------------------|
| Vector          |          | −Zn | +Zn | Induction | Up | WT | U/W |
| ZRT1 Dg0        | CACTGAAAACCTCGAAGGACCAAAAGATAC | −317 | 42 ± 2 | 17 ± 1 | 2 | ND | ND | 1 |
| Dg3             | CCTCAAGGTTTCATGCTGCGTATTCTTT    | −287 | 28 ± 2 | 10 ± 1 | 3 | ND | ND | 1 |
| Dg2             | CCAAAGATACCTCAAGGTTCTCATGCTG    | −297 | 1174 ± 15 | 9 ± 1 | 130 | 91 ± 3 | 3 ± 1 | 30 |
| M1              | GaaactcgcACCTCAGAAGTCTCATGCTGG  | −296 | 1145 ± 76 | 4 ± 1 | 286 | ND | ND | 1 |
| M2              | GCCAAAAGTCAaaagaccttgCTCATGCTGG | −296 | 7 ± 1 | 2 ± 1 | 4 | ND | ND | 1 |
| M3              | GCCAAAAGTACCTCAAGGTTCTCATGCTG   | −296 | 778 ± 29 | 2 ± 1 | 389 | ND | ND | 1 |
| ZRE2 Dg2        | GCATATTGACCTCGAAGGTCAGGAAG      | −184 | 1314 ± 84 | 89 ± 2 | 15 | 280 ± 7 | 20 ± 1 | 14 |
| ZRE3             | AGAGTTGACCTCGAAGGTCATGCTGG     | −433 | 1816 ± 1 | 10 ± 1 | 182 | 294 ± 4 | 19 ± 1 | 15 |
| ZRT2 Dg0        | GCCAAACATACCTCAAGGTTCTCATGCTG   | −289 | 1388 ± 30 | 33 ± 1 | 42 | 348 ± 19 | 13 ± 1 | 27 |
| ZRE2             | GCCAAATACCTCAAGGTTCTCATGCTG    | −240 | 1583 ± 22 | 93 ± 2 | 12 | 166 ± 2 | 39 ± 2 | 4 |
| ZAP1             | AGTCATCATCCCTAGGCTGATGAAAC      | −122 | 1119 ± 21 | 47 ± 2 | 24 | 230 ± 5 | 4 ± 1 | 58 |
| pUAS-HIS4        |          | ND | ND |          | 289 ± 23 | 269 ± 20 | 1 |
| pLG3312          |          | ND | ND |          | 406 ± 9 | 405 ± 5 | 1 |

| 5′-ACCYYNAAGGT-3′ | CONSENSUS |
|------------------|-----------|
| ZRE1             | -323 asagATACCTCAAGGttctca  |
| ZRE2             | -208 atttgACCTCGAAGGTCatg   |
| ZRE3             | -439 tgggtACCCCAAAGGtccaa   |
| ZRT1             | -135 atcataACCTCAAAGGttatat |
| ZRT2             | -246 atctgACCTCAAAGGttgta  |
| ZAP1             | -148 cctcATACCTCAAGGTCatg  |

**FIG. 2.** Derivation of a ZRE consensus sequence. The sequences of the ZREs identified in the ZRT1, ZRT2, and ZAP1 genes are aligned from 5′ to 3′. The numbers refer to the first and last nucleotides of the displayed sequences. The ZREs are indicated by uppercase letters, and flanking sequences are indicated by lowercase letters. A consensus ZRE was derived from the identity of bases common to all of the sequences. The position of ZAP1 is correct, the increased expression caused by the ZAP1–1up allele in zinc-replete cells should be exerted through the ZRE sequences. Therefore, we measured the effect of the ZAP1–1up allele on several of the ZRE-lacZ fusion constructs described in Table I. Prior to assay for β-galactosidase activity, cells were grown in a zinc-replete medium in which the ZAP1–1up allele effects are observed. In each case, expression of the ZRE lacZ reporter was elevated in the ZAP1–1up mutant relative to wild type cells (Table I). We also examined the effect of the ZAP1–1up allele on the vector alone, on a HIS4-lacZ fusion (pUAS-HIS4), and on a fusion containing the entire CYC1 promoter fused to lacZ (pLG3312). ZAP1–1up had no effect on expression of any of these promoters (Table I), indicating that this allele does not cause global changes in gene expression. Thus, the effects of the ZAP1–1up allele are mediated specifically through the ZRE sequences.

**ZAP1 Binds to ZREs in a Sequence-specific Manner—**An additional prediction of the hypothesis that ZAP1 is the direct regulator of zinc-responsive gene expression is that the ZAP1 protein binds directly to ZREs. To test this prediction, the full-length ZAP1 protein or a truncated ZAP1 polypeptide containing the five C-terminal zinc fingers (ZnF1-5) (Fig. 3A) was produced with an in vitro transcription/translation system and
used in EMSAs. The ZnF$_{1-5}$ polypeptide comprises amino acids 687–880 of the full-length protein, and the probe used contained the ZRE1 sequence of ZRT1 (i.e. oligonucleotide Dg2, Table I). Protein/DNA complexes were not observed in reactions containing either no protein or increasing amounts of vector-programmed transcription/translation product (Fig. 3B, lanes 1–4). Protein/DNA complexes were observed when either the full-length ZAP1 (Fig. 3B, lanes 5–7) or the ZnF$_{1-5}$ fragment (Fig. 3B, lanes 8–10) was used in the assay. Whereas ZnF$_{1-5}$ formed only a single complex, the full-length protein formed three distinct complexes. The multiple bands detected using full-length ZAP1 may be due to the formation of homo-
regions was observed between the full-length and truncated ZREs. Moreover, no difference in the length of the protected region was observed between the no DNA synthesis and the vector-only control (data not shown). However, the DNase I digestion products was observed between the no DNA synthesis and the vector-only control (data not shown). However, the DNase I digestion products was observed between the no DNA synthesis and the vector-only control (data not shown).

ZAP1 Binds to Zinc-responsive Promoter Elements

Fig. 5. Assessment of ZAP1 DNA binding specificity by in vitro DNase I footprint analysis. Four-μl volumes of a transcription/translation lysate programmed for synthesis of the vector-only control (lane 1), full-length ZAP1 (lane 2), or the ZnF_{1-5} polypeptide (lane 3) were incubated with 32P-labeled ZRT1, ZRT2, and ZAP1 promoter fragments and subjected to partial DNase I digestion. The boxes indicate the position of ZREs, and the numbers refer to the first and the last nucleotide (relative to translation initiation codon) protected from DNase I cleavage. Fragment sizes were determined relative to a DNA sequencing ladder (not shown).

An independent test of the sequence-specificity of ZAP1 DNA binding was provided by in vitro DNase I footprint analysis of the ZRT1, ZRT2, and ZAP1 promoters (Fig. 5). No difference in the DNase I digestion products was observed between the no protein and vector-only controls (data not shown). However, full-length ZAP1 and the ZnF_{1-5} polypeptide gave clear protection from DNase I cleavage in the regions of the ZRT1, ZRT2, and ZAP1 promoters that correspond to the ZREs. These data further demonstrate that the ZAP1 protein binds specifically to ZREs. Moreover, no difference in the length of the protected regions was observed between the full-length and truncated ZAP1 proteins. This observation strongly supports the hypothesis that truncated DNA binding domain. Only weak binding of ZAP1 was observed in similar experiments using the ZRE3 region of the ZRT1 promoter as the probe, suggesting that this site has lower affinity for ZAP1 binding (data not shown).

Differential Regulation of the ZRT1, ZRT2, and ZAP1 Promoters—Our previous studies of zinc uptake in yeast (8, 9) indicated that although the ZRT1 high affinity transporter was completely repressed in zinc-replete cells, the low affinity ZRT2 transporter remained active. Surprisingly, the analyses described in this and a previous report (10) demonstrated that expression of both ZRT1 and ZRT2 is zinc-responsive and regulated by ZAP1. These observations presented a paradox; how can ZRT2 be active in zinc-replete cells yet still be regulated by ZAP1? To address this question, we examined expression of the ZRT1, ZRT2, and ZAP1 promoters during growth in media containing a range of zinc concentrations. Previous results suggested that repression of these genes occurred in response to an intracellular pool of the metal (9). Because no methods are currently available to directly measure the concentration of this regulatory zinc pool, we estimated its level by measuring total cell-associated zinc levels. As zinc concentrations in the medium rose, so did the cell-associated zinc levels (Fig. 6). The ZAP1 and ZRT1 promoters were extremely zinc responsive. For example, approximately 50% repression of both promoters was observed at 5 μM zinc in the medium, corresponding to a cell-associated zinc level of 75–100 pmol/10^6 cells. In contrast, the ZRT2 promoter was repressed to a similar degree only at a zinc concentration in the medium that was 50-fold higher, i.e. 250 μM. This medium zinc concentration corresponded to a cell-associated zinc level of 175 pmol/10^6 cells. Therefore, although ZRT1, ZRT2, and ZAP1 are all ZAP1 target genes, the ZRT2 gene requires higher zinc levels to repress its expression.

**DISCUSSION**

The ZAP1 gene is required for zinc-responsive gene expression in yeast (10). Target genes regulated by ZAP1 include the ZRT1 and ZRT2 zinc transporter genes and the ZAP1 gene itself. We proposed previously that ZAP1 encodes a transcriptional activator that binds to the promoters of these genes and it would be helpful for the reader if this information was clearly stated.
activates their transcription when intracellular zinc levels are low. The experiments described in this report directly support this hypothesis, i.e., ZAP1 binds to zinc-responsive elements in the promoters of these genes in a sequence-specific fashion. Moreover, the identification of a ZAP1 binding site within its own promoter further supports the hypothesis that ZAP1 regulates its own expression in response to zinc via a positive autoregulatory mechanism. Presumably, this autoregulation provides a rapid and amplified response to changes in ZAP1 activity in response to zinc.

Characterization of zinc-responsive elements in ZAP1-regulated promoters has identified an 11-bp consensus sequence. This sequence appears to contain all of the information required for sequence-specific ZAP1 binding and this conclusion is based on two observations. First, sequence conservation among the different ZREs was found only within this 11-bp element and not in flanking sequences. Second, mutation of the flanking nucleotides had no effect on the zinc responsiveness of the ZRT1 ZRE1 element. Our studies have also illuminated several characteristics of ZREs. First and foremost, ZREs are both necessary and sufficient for zinc-responsive gene expression. Second, ZREs can function in either orientation relative to the start site of transcription. We also found that single ZREs are active; multiple copies are not required for function. Moreover, when these elements are present in multiple copies in a promoter, they are additive rather than cooperative in their effects (Fig. 1).

Surprisingly, there was no clear correlation between the zinc responsiveness of a given ZRE-lacZ fusion and its responsiveness to the ZAP1–1up allele. For example, the ZRE1 and ZRE2 elements identified in the ZRT1 promoter showed relatively similar responsiveness to the ZAP1–1up allele, whereas ZRE1 had 9-fold higher zinc responsiveness than the ZRE2-containing promoter (Table 1). Likewise, the ZRE from the ZAP1 promoter showed only moderate zinc responsiveness (24-fold), whereas it showed the greatest response (58-fold) to the ZAP1–1up allele. These inconsistencies may reflect differences in the affinity of ZAP1 for different ZREs, or the ZAP1–1up allele may alter the DNA binding specificity of the ZAP1 protein. We view this latter hypothesis as unlikely given that the ZAP1–1up mutation, a cysteine-to-serine substitution at amino acid 203, is far removed from the DNA binding domain.

The additive effects of multiple ZREs in a promoter can explain the different levels of expression among ZAP1 target genes. On Northern blots of mRNA from zinc-limited cells, we observed previously that ZRT1 is expressed at the highest level, ZRT2 is expressed at an intermediate level, and ZAP1 is expressed at the lowest level (10). This pattern of relative expression levels correlates with the number of ZREs found in these promoters (three, two, and one, respectively) and fits well with our understanding of the different functions of these genes. ZRT1 is needed at high levels under zinc limitation because of its critical role in supplying zinc to the cell under these extreme conditions. ZRT2, the low affinity transporter, plays more of a housekeeping function, supplying zinc to the cell under zinc-replete conditions, and ZAP1 is expressed only at low levels because of its role as a transcriptional regulator.

Our studies have also demonstrated that there is differential zinc responsiveness among the ZRT1, ZRT2, and ZAP1 genes; i.e., significantly more zinc is required to repress the ZRT2 promoter than is required to repress the ZRT1 or ZAP1 promoters (Fig. 6). This differential sensitivity to zinc is also consistent with the different functions of these proteins and leads us to propose the following scenario: basal (i.e., ZAP1-independent) expression of the ZRT2 low affinity transporter is sufficient to supply zinc to cells under zinc-replete conditions (8). As cells first become zinc-limited, their initial response is to increase the activity of the ZRT2 transporter. If zinc limitation becomes more severe, the ZRT1 high affinity transporter is induced to provide high affinity uptake activity for zinc accumulation. Increased expression of the ZAP1 gene, which would allow maximum expression of its target genes, would only be needed under conditions of extreme zinc limitation. The mechanism underlying the differential regulation of these ZAP1 target genes is not yet known. If zinc controls the affinity of ZAP1 for its ZRE binding sites, one possible model is that other proteins bind to the ZRT2 promoter and help stabilize binding of ZAP1 to the ZREs, thus increasing the affinity of ZAP1 for these sites. This and other possible models will be addressed in future studies.

Given the size of the ZRE sequence, we propose that this site is bound by a single ZAP1 polypeptide. Our current understanding of how zinc fingers bind to DNA comes largely from x-ray crystal structures of protein-DNA complexes (22–26). In all of these structures, there are contiguous zinc finger interactions with base pairs in the major groove. In Zif268, for example, each of three fingers binds to a 4-bp site that overlaps the site of the adjacent finger by a single bp. We predict that three consecutive zinc fingers of ZAP1 would bind to a 10-bp sequence, similar in size to the 11-bp ZRE. Given that there are five potential zinc fingers in the DNA-binding, C-terminal 194 amino acids of ZAP1, and only three may be required for site-specific binding, we propose that the two additional fingers play roles in nonspecific DNA binding interactions and/or in protein-protein interactions. We have also recently learned of the presence of two additional zinc finger motifs at amino acids 581–604 and 618–641 (4), and our data indicate that these upstream fingers are not required for DNA binding. The functions of each of the zinc finger domains in ZAP1 are currently under investigation. It should be noted that our ZRE consensus contains a near 2-fold dyad symmetry, suggesting the alternative possibility that ZAP1 binds as a dimer rather than a monomer.

An intriguing question that remains to be answered is precisely how zinc regulates ZAP1 activity. The characterization of the ZAP1 binding site in its target promoters is a critical step toward understanding this regulation. For example, in vitro and in vivo studies are now possible to determine whether zinc alters ZAP1 DNA binding. The characterization of a ZRE consensus sequence also provides us with a powerful tool to identify other zinc-responsive genes in the yeast genome, made possible by the recent completion of the Saccharomyces genome sequence. Using the consensus ZRE sequence derived in this study, sequence data base analysis (PatMatch software; http://genome-www.stanford.edu/Saccharomyces/) identified a total of 20 genes in the yeast genome that contain one or more ZRE-like sequences in their promoters. This list of potential ZAP1 target genes is an exciting resource for the future analysis of how eukaryotic cells respond to zinc limitation and maintain zinc homeostasis.

Acknowledgments—We thank Jim Browning for technical assistance during the course of this work. We also thank Susan Henry for supplying us with pNB404, Seigfried Bohm for pointing out the location of the two additional zinc fingers in ZAP1, Geoff Gadd for advice on atomic absorption spectroscopy, and Amanda Bird, Raad Gitan, and Ann Ther for critical reading of the manuscript.

REFERENCES
1. Valle, B. L., and Auld, D. S. (1990) Biochemistry 29, 5647–5659
2. Schwabe, J. W., and Klug, A. (1994) Nat. Struct. Biol. 1, 345–349
3. Schjørring, J., and Heimberg, S. (1996) Nucleic Acids Res. 24, 4599–4607
4. Bohm, S., Frishman, D., and Mewes, H. W. (1997) Nucleic Acids Res. 25, 2464–2469
5. Hamer, D. H. (1986) Ann. Rev. Biochem. 55, 913–951
