Mapping the DNA Binding Domain of the Zap1 Zinc-responsive Transcriptional Activator*

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The Zap1 transcriptional activator of Saccharomyces cerevisiae plays a major role in zinc homeostasis by inducing the expression of several genes under zinc-limited growth conditions. This activation of gene expression is mediated by binding of the protein to one or more zinc-responsive elements present in the promoters of its target genes. To better understand how Zap1 functions, we mapped its DNA binding domain using a combined in vivo and in vitro approach. Our results show that the Zap1 DNA binding domain maps to the carboxyl-terminal 194 amino acids of the protein; this region contains five of its seven potential zinc finger domains. Fusing this region to the Gal4 activation domain complemented a zap1Δ mutation for low zinc growth and also conferred high level expression on a zinc-responsive element-lacZ reporter. In vitro, the purified 194-residue fragment bound to DNA with a high affinity (dissociation constant in the low nanomolar range) similar to that of longer fragments of Zap1. Furthermore, by deletion and site-directed mutagenesis, we demonstrated that each of the five carboxyl-terminal zinc fingers are required for high affinity DNA binding.

Zinc is an essential trace element necessary for the growth of all organisms. Its nutritional importance can be illustrated by the large number of proteins that require zinc for their function. For example, there are over 300 known zinc metalloenzymes (1), and it has been estimated that as many as 1% of the genes in the human genome encode proteins with C2H2 zinc finger motifs (2). In contrast, zinc excess can be toxic to cells so mechanisms of controlling intracellular zinc levels are critical to cell viability. In Saccharomyces cerevisiae, zinc homeostasis is largely regulated at the transcriptional level. The Zap1 transcriptional activator directly controls the expression of a number of genes in response to zinc (3). For example, in zinc-limited cells, Zap1 induces the expression of a high affinity zinc transporter encoded by the ZRT1 gene and a low affinity transporter encoded by ZRT2 (4, 5). Zap1 also controls the transport of stored zinc out of the vacuole by regulating the expression of yet another transporter encoded by the ZRT3 gene. In addition to these important zinc transporters, DNA microarray analysis indicated that perhaps as many as 46 total genes in the yeast genome are directly regulated by this factor. Clearly, Zap1 is a critical determinant of zinc homeostasis and the ability of cells to thrive under zinc-limiting conditions.

Zap1 binds in a sequence-specific manner to an 11-base pair sequence called the zinc-responsive element (ZRE). This element is found in one or more copies in the promoter regions of Zap1 target genes. Experimental evidence (8) and computer-aided motif analysis of Zap1 target gene promoters have indicated that the sequence 5'-ACCTTNAAGGT-3' is the preferred recognition site for this protein. Determining how Zap1 recognizes and binds to this sequence is a critical issue in understanding how Zap1 functions as a transcriptional activator. Zap1 contains seven potential C3H2 zinc finger domains, and previous studies suggested that only a subset of these may be required for ZRE binding in vitro (8). In this study, we use a combined in vitro and in vivo analysis to map the DNA binding domain of Zap1 and determine which of its zinc fingers contribute to ZRE recognition and binding.

EXPERIMENTAL PROCEDURES

Strains and Culture Conditions—The strains used in this study are YM4271 (MATα ura3–52 his3–Δ200 ade2–10 lacY2–801 leu2–3,112 trpl–901 tyr1–501 gal4–512 gal80–Δ538 ade2–1 his3::HIS3 (CLONTECH) and DEY1538 (YM4271; zap1Δ::TRP1). Yeast cultures were grown in YP medium containing 2% glucose or in synthetic defined (SD) medium supplemented with 2% glucose and any necessary auxotrophic supplements. Where indicated, EDTA and ZnCl2 were added to SD to generate zinc-limiting growth conditions. An alternative low zinc medium, LZM, was prepared as described previously (9).

Plasmid Constructions—All plasmids generated for this study were confirmed by DNA sequencing. The ZAP1 open reading frame was PCR-amplified with primers containing added 5' and 3' SalI sites. This fragment was inserted into SalI-digested pGAD424 (CLONTECH) to create pGAD-Zap1lacZ. Other GAD-Zap1 fusions were created similarly with 5' primers containing an added EcoRI site and 3' primers containing a BamHI site. The fragment containing GAD-Zap1lacZ was generated by two-step overlapping PCR (10) and cloned into pGAD424 as described above. pGAD424 allows expression of cloned inserts from the ADH1 promoter. To allow expression from the GAL1 promoter, the fragments encoding the Gal4 activation domain-Zap1 fusions were subcloned into pRS316-GAL1 (11) by gap repair (12). Expression from the GAL1 promoter using the GEV β-estradiol-responsive system was performed as described by Gao and Pinkham. Cells were co-transformed with plasmid pGEV-HIS3, which expresses a 2 U. Lyons, A. P. Gasch, L. A. Gaither, D. Botstein, P. O. Brown, and D. J. Eide, submitted for publication.

The abbreviations used are: ZRE, zinc-responsive element; GST, glutathione S-transferase; ZnF, zinc finger domain; LZM, low zinc medium; EMSA, electrophoretic mobility shift assay; GAD, Gal4 activation domain; GBD, Gal4 DNA binding domain; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; SDS, synthetic defined; DAPI, 4,6-diamidino-2-phenylindole.

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brid activator protein, GEV, that contains the Gal4 DNA binding do-
main, the human estrogen receptor hormone response domain, and the
VP16 activation domain. The activity of the GEV protein was induced by
adding 100 mM β-estradiol. The c-Myc epitope-tagged ZAPI allele was
expressed from plasmid pYef2-mycZap1. The c-Myc epitope was induced with isopropyl-1-thio-
b-D-galactoside (IPTG).

Site-directed Mutagenesis—To create a template for site-directed
mutagenesis, a 2-kilobase pair fragment encoding Zap1 amino acids
157–880 was isolated from pGEX4T-1 (Amersham Pharmacia
Biotech) using EcoRI and SalI sites to generate NH2-terminal glutathione S-transferase (GST) fusions.

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Determination of Zn2+ Stoichiometry—Amino acid analysis (Beck-
mann 6300) was used to determine protein concentration following hy-
drolysis in 5 M HCl for 24 h in vacuo at 110°C. Simultaneous readings
of iron and zinc were obtained by inductively coupled plasma
emission spectroscopy using a Perkin-Elmer Optima 3100XL instru-
ment. Zn2+ levels were also measured by atomic absorption spec-
trometry (Perkin Elmer AAAnalyst 100). Protein samples in sonication buffer
were diluted in deionized water as needed prior to analysis.

Electrophoretic Mobility Shift Assay (EMSA)—The oligonucleo-
ides ZRE-1 (5'-CCAAAGATACCTCTCAAGGTTCTACCTGTTG-3') and
ZRE-2 (5'-CCACAGTAGGATCTTGATTTTCCG-3'), containing
the consensus sequence for the ZRE recognized by Zap1, were annealed and used in both EMSA and affinity chromatography. Mutant
oligonucleotides M2a (5'-GCCCCAGATCAAAGGTTCTACCTGTTG-3') and M2b (5'-CCACAGTAGGATCTTGATTTTCCG-3'), containing
a mutated ZRE known to be inactive in vivo with respect to Zap1
function (8), were used to verify the specificity of Zap1 binding to the
ZRE site.

The standard 15-μM EMSA reaction contained hybridization mix (65
mM KCl, 0.2 mg ml⁻¹ bovine serum albumin, 20 μM Tris-HCl, pH 7,
with 20% glycerol and 0.04% IGEPAL CA-630; Sigma) with end-labeled oligonucleotides and protein in sonication buffer. Following incubation
at room temperature for 15 min, the samples were applied to a 6%
polyacrylamide nondenaturing gel and electrophoresed for 1.5 h at 30
mA. Gels and running buffer contained 1X Tris borate buffer, pH 8,
and they were pre-electrophoresed for 1 h at 30 mA. A reaction lacking
protein was used as a “free probe” control. Dried gels were viewed by
autoradiography. Results from electrophoretic mobility shifts assays
were shown to be identical following incubation of binding reactions for
1 h, 3 h, 30 min, and 5 min. Therefore, these reactions were judged to be at
equilibrium.

Measurement of Apparent Dissociation Constants (Kd)—A series
of 15-μM reactions were prepared containing different concentrations of protein in hybridization mix and 32P-radioabeled oligonucleotides
maintained at a constant DNA concentration. Reactions were allowed
to reach equilibrium by incubation at room temperature for 15 min. The
protein-DNA complex was then separated from free DNA by EMSA.
Phosphorimages of dried gels were obtained and quantified using QUANTITATION ONE software. The percentage of complex formation (as
determined by the loss of free DNA) was plotted against concentration
on a logarithmic scale to determine the apparent Kd.

RESULTS
In Vivo Mapping of the Zap1 DNA Binding Domain—The functional
and structural domains of Zap1 that were predicted from its primary amino acid sequence (3) are diagrammed in Fig. 1. Based on the distribution of acidic residues, this protein was predicted to have two potential activation domains designated AD1 and AD2. Zap1 also contains seven zinc finger motifs that fit the minimal consensus sequence C-X₉₋₁₄-C-X₁₂₋₁₅-H-X₃₋₅-H. Our previous results suggested that the region containing the five COOH-terminal zinc fingers was sufficient for sequence-specific Zap1 DNA binding (8). A Zap1 fragment including only amino acids 657–880, i.e. zinc fingers ZnF3–ZnF7, produced in vitro by coupled transcription and translation reactions
behaved in EMSA and DNase I footprinting assays in a similar fashion to the wild type protein. It was unclear from these studies, however, whether this domain contained the full DNA binding activity of the intact protein. As a first step, the in vitro test of this hypothesis, the Zap1630–880 region was fused to the Gal4 activation domain (GAD) and tested for its ability to complement a zap1Δ mutation. The GAD fusion approach allows assessment of DNA binding ability without requiring Zap1 activation
domain function. Furthermore, the GAD fragment used contains the VP16 nuclear localization sequence to facilitate trafficking of the fusions to the nucleus should the still un-

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mapped Zap1 localization signal be deleted.

Wild type and zap1Δ mutant cells grow equally well on zinc-supplemented media (Fig. 2), but the zap1Δ mutant is unable to grow under zinc-limiting conditions. When the full-length Zap1 protein (in this experiment, a functional allele with six NH2-terminal c-Myc epitope tags) was expressed in the zap1Δ mutant from the GAL1 promoter, wild type growth in low zinc was restored. Expression of the Gal4 activation domain Zap1687–880 fusion (GAD-Zap1687–880) also strongly complemented the mutant defect. These results supported the in vitro data suggesting that ZnF3–ZnF7 were sufficient for Zap1 DNA binding.

We noted that the zap1Δ strain expressing GAD-Zap1687–880 grew slightly slower than the same strain expressing full-length Zap1 (data not shown). Therefore, as an independent test of these complementation results, we compared the ability of the GAD-Zap1687–880 and other GAD-Zap1 fusions to activate expression of a ZRE-lacZ reporter gene in a zap1Δ mutant (Fig. 3). These assays were conducted using cells grown in low zinc to avoid the repressive effect of zinc on Zap1 activity. In all cases, these fusions were found to complement the zinc-limited growth defect of the zap1Δ mutant (data not shown). Although wild type cells showed high level lacZ reporter expression, the zap1Δ mutant transformed with the vector alone showed only low levels of β-galactosidase activity. The zap1Δ mutant expressing either the full-length Zap1 protein or amino acids 552–880 fused to the Gal4 activation domain showed similarly high levels of expression, i.e. ~80% of wild type. To test if ZnF1 and/or ZnF2 are important for DNA binding, the GAD-Zap1642–880 fusion was constructed in which these two fingers are deleted. Although expression is somewhat diminished relative to the larger fusion proteins, this allele retained high levels of expression suggesting that these fingers are not necessary for DNA binding. The small reduction in expression is most likely caused by the removal of a Zap1 activation domain located within the 592–642 region. Thus, deletions eliminating the NH2-terminal 640 amino acids of Zap1 can bind DNA in vivo. In contrast, the GAD-Zap1687–880 fusion produced only about 20% of wild type expression levels. These results are consistent with at least two models. First, the ZRE binding activity of this Zap1 fragment may be partially impaired by deletion of the region from amino acids 642–686. Alternatively, fusion of the Gal4 activation domain close to the Zap1 DNA binding domain may inhibit the function of either domain perhaps through steric interference. An additional fusion, GAD-Zap16553–686 in which the 642–686 region in question was deleted from the full-length protein, was constructed to address these models. This fusion showed wild type lacZ expression, indicating that the region of Zap1 from 642 to 686 is not required for DNA binding activity. Thus, the reduced ability of GAD-Zap1687–880 to complement the growth defect of the zap1Δ mutant is due to reasons other than deletion of important DNA.
Assessing the Role of Zap1 Zinc Fingers in DNA Binding by Site-directed Mutagenesis—To determine which of the Zap1 zinc fingers are required for specific ZRE binding, we used site-directed mutagenesis to convert the two conserved histidine Zn\(^{2+}\) ligands in each finger to glutamines (Fig. 1). These mutations impair Zn\(^{2+}\) binding in these sites and therefore interfere with proper formation of the zinc finger structures. The mutations were introduced into a fusion allele in which the amino acid 552–880 region of Zap1 was fused at its amino terminus to the Gal4 DNA binding domain (GBD). This domain was included to provide both an epitope tag and a nuclear localization signal for these proteins, and for future studies of Zap1 zinc responsiveness. When expressed in zinc-limited zap1\(\Delta\) mutant cells, GBD-Zap1\(_{552-880}\) induced a high level of expression from the ZRE-lacZ reporter (Fig. 4A). Mutation of zinc fingers 1 (mZnF1) or 2 (mZnF2) individually or both ZnF1 and 2 together (mZnF1/2) resulted in no loss of expression, supporting the hypothesis that these domains are not required for DNA binding. In fact, lacZ expression in each of these mutants was increased by 50–100% relative to the corresponding wild type protein fragment, suggesting that these mutations may increase activation domain function under these conditions. Similar mutations in ZnF3, ZnF4, ZnF5, ZnF6, or ZnF7 resulted in complete loss of ZRE-lacZ expression.

Although the simplest interpretation of these results is that each of these zinc fingers is required for DNA binding, a number of other explanations were also possible. For example, these mutant proteins may be unstable and fail to accumulate. Immunoblot analysis of total protein samples prepared from these strains demonstrated that this is not the case (Fig. 4B). All of the GBD-Zap1 proteins accumulated to similar levels. We did note that the nonfunctional GBD-Zap1 fusions also accumulated as lower mobility forms that may represent homo- or

![Diagram](image)

**Fig. 4. In vivo analysis of the Zap1 zinc finger mutants.** Strain DEY1538 (zap1\(\Delta\)) expressing the vector (pMA424), GBD-Zap1\(_{552-880}\), or its mutant derivatives containing histidine to glutamine substitutions in the indicated zinc finger domains were assayed for their ability to activate expression of a ZRE-lacZ reporter (A), fusion protein accumulation by immunoblotting (B), and subcellular location of GBD-Zap1 proteins (C). In panels B and C, V = vector-transformed cells, WT = GBD-Zap1\(_{552-880}\), and mZnF1, mZnF2, etc., are shown. In panel B, the arrow indicates the expected GBD-Zap1 fusion and the asterisks mark the lower mobility forms. The positions of molecular mass markers and a Vph1 loading control blot are also shown. In panel C, cells expressing the indicated proteins were viewed by Nomarski optics or epifluorescence. DAPI was used to stain the nucleus and the GBD-Zap1 protein was detected by immunofluorescence. The blue fluorescence of DAPI staining was changed to red, and the DAPI and GBD-Zap1 images were overlaid using ADOBE PHOTOSHOP (Merge). Yellow color in the merged images indicates colocalization of the markers. Cells were grown to exponential phase in LZM supplemented with 100 nM β-estradiol and 5 μM ZnCl\(_2\) (panel A) or 1000 μM ZnCl\(_2\) (panels B and C) prior to analysis. The higher zinc concentration greatly facilitated the immunoblot and immunofluorescence experiments owing to the poor growth of some of the strains in low zinc.
TABLE I
Zinc stoichiometry and ZRE binding affinities of purified Zap1 fusions and zinc finger mutants mZnF1/2-mZnF7 constructed in GST-Zap1538–880 Fusions

| Protein                  | Zinc | $K_d$  |
|--------------------------|------|--------|
| mZnF1                   | 5.8  | 3.5 ± 0.7 |
| mZnF2                   | 5.8  | 3.5 ± 0.7 |
| mZnF3                   | 4.3   | 500 |
| mZnF4                   | 4.1   | 500 |
| mZnF5                   | 4.2   | >500 |
| mZnF6                   | 5.1   | ND |
| mZnF7                   | 5.7   | >500 |
| GST-Zap1538–880         | 4.8   | 2.1 ± 2.5 |
| GST-Zap1552–880         | 5.8   | 4.9 |
| GST-Zap1611–880         | NM*  | 1.96 |
| GST-Zap1657–880         | 3.9   | 1.2 ± 3.0 |
| Zap538–880              | 4.3   | 500 |
| mZnF1/2                 | 4.3   | 500 |

* NM, not measured; ND, no binding detected.

heteromultimeric complexes of the protein that are not disrupted by SDS-PAGE. Another explanation for the inactivity of these mutant proteins is that they fail to accumulate in the nucleus despite the presence of the Gal4 nuclear localization signal. To test this hypothesis, we examined their intracellular distribution by indirect immunofluorescence microscopy using an anti-GBD antibody. Little cell-associated fluorescence was observed in untransformed cells or vector-only controls, whereas the GBD-Zap1 fusions were easily detectable (Fig. 4C, data not shown). The wild type (Fig. 4C), mZnF1, mZnF2, and mZnF1/2 (data not shown) GBD fusions clearly localized in the nucleus. Mutants mZnF3 and mZnF7 were largely present in the nucleus, and fluorescence was also visible in the cytoplasm as punctate spots of apparently aggregated protein. In the mZnF4, mZnF5, and mZnF6 mutants, the proteins were mostly visible as cytoplasmic aggregates and little nuclear staining was observed. We can conclude from this analysis that ZnF3 and Zn7 are likely required for DNA binding; these proteins accumulate in the nucleus in substantial amounts but fail to activate transcription. However, it was not possible to assess if mZnF4, mZnF5, or mZnF6 could bind to a ZRE, i.e. their failure to activate in vivo could simply be due to the inability to accumulate in the nucleus. Finally, it is intriguing that all of the mutant forms of Zap1 that are seen as cytoplasmic aggregates also appear in lower mobility forms on SDS-PAGE, suggesting that their may be a link between these observations.

**In Vitro Analysis of Zap1 DNA Binding**—To complement this in vivo analysis, in vitro DNA binding studies using purified recombinant Zap1 protein were performed. Various Zap1 truncate and mutant proteins were expressed in E. coli as NH$_2$-terminal GST fusions and purified using GST-GePhose affinity chromatography. Attempts to purify full-length Zap1 were unsuccessful because of the formation of insoluble inclusion bodies. However, GST-Zap1538–880, GST-Zap1552–880, and GST-Zap1611–880 were isolated, and each contains the functional DNA-binding domain (ZnF3–ZnF7) implicated by our in vivo studies. These GST-Zap1 fusion proteins were largely soluble in E. coli. The Zn$^{2+}$ content of GST-Zap1538–880 was found to be approximately 5 mol eq (Table I), suggesting metal occupancy of only five of the seven possible zinc finger domains in this protein. No significant quantities of Cu or Fe ions were observed in the purified samples.

Binding of these purified Zap1 proteins to a functional ZRE was determined to be approximately 2 nM (Fig. 5A, Table I). Protein-DNA complex formation was not lost with addition of $\leq 0.3$ mg ml$^{-1}$ poly(dI/dC) (data not shown). GST-Zap1538–880 protein forms two complexes with DNA in EMSA (Fig. 5B), perhaps indicative of limited proteolysis that does not affect DNA binding. Alternatively, we may be observing different oligomeric states of the Zap1 protein and this is currently under investigation. Removal of the GST fragment by thrombin cleavage of the GST-Zap1 fusion did not greatly alter the affinity of Zap1 for the ZRE duplex (Table I). Furthermore, the affinity was also not altered by further purification of the fusion on a ZRE-DNA affinity column. Thus, the GST-Zap1 fusions appear to be predominantly active for DNA binding.

The two other Zap1 truncates also showed high affinity ZRE binding interactions. Both GST-Zap1538–880 (Fig. 5, C and D) and GST-Zap1611–880 (Table I) bind specifically to the ZRE duplex with affinities similar to the longer GST-Zap1538–880 protein. The complex formed with GST-Zap1538–880 was not disrupted by addition of $\leq 0.3$ mg ml$^{-1}$ poly(dI/dC).

The high affinity ZRE binding of GST-Zap1538–880 containing only ZnF3–ZnF7 suggested that mutation of fingers 1 and 2 would not disrupt DNA binding. A mutant form of GST-Zap1 was purified in which zinc fingers 1 and 2 contained the same histidine to glutamine substitutions as were analyzed in vivo. The GST-Zap1552–880 mZnF1/2 mutant protein bound DNA with a similar degree of ZRE specificity and affinity as did the wild type GST-Zap1538–880 protein (Fig. 5, E and F, Table I). Mutation of any one of zinc fingers 3–7 resulted in the loss of this specific interaction with ZREs. These mutant proteins bound the ZRE duplex with a low affinity in the high nanomolar to low micromolar concentration range indicative of only low affinity interactions. Most of these zinc finger mutant proteins bound on average one less Zn$^{2+}$ atom per molecule than the wild type protein (Table I). The reason why mZnF6 and mZnF7 may retain a 5 mol eq Zn$^{2+}$ stoichiometry is not yet clear. Perhaps mutation of these fingers allow for Zn$^{2+}$ binding to alternate ligands in these domains.

Finally, two Zap1 truncates lacking portions of the protein’s COOH terminus were purified and evaluated. These two truncates remove either 55 (GST-Zap1552–823) or 83 (GST-Zap1552–797) COOH-terminal residues. Both of these proteins exhibited low affinity for the ZRE duplex with apparent $K_d$ values in the micromolar concentration range. GST-Zap1552–823 and GST-Zap1552–797 bind ZREs only at protein concentrations $>20$ nM, and, in each case, complex formation was abolished with the addition of 0.04 mg ml$^{-1}$ poly(dI/dC) (data not shown).

**DISCUSSION**

The fully functional DNA binding domain of Zap1 maps to the carboxyl-terminal 194 amino acids of Zap1, i.e. residues 687–880. Fusion of this region to the Gal4 activation domain complemented a zap1A mutation for growth under zinc-limiting conditions and conferred expression from a ZRE-lacZ reporter gene in vivo. In vitro, a purified 687–880 fragment and a much longer fragment with amino acids 538 (or 552)-880 bound to a ZRE-containing duplex oligonucleotide with high affinity. The dissociation constant of this interaction was estimated to be in the 0.8–4 nM range.

Region 687–880 contains five of the seven zinc finger domains found in Zap1 (Fig. 1). All seven of the Zap1 fingers fit the zinc finger consensus sequence and are predicted to form the $\beta$-sheet structure found for other such domains. Zinc finger proteins bind to DNA through a generally conserved docking arrangement with each finger $\alpha$-helix fitting into the major groove of the DNA double helix (17, 18). Residues 1, 2, 3, and 6 (numbering with respect to the start of the $\alpha$-helix) typically make key base contacts that are responsible for the sequence
specificity of the interaction. Thus, each zinc finger commonly recognizes a 4-base subsite that can overlap the recognition site of an adjacent finger by 1 base.

These considerations raise a question regarding which fingers of Zap1 contact DNA. Mutagenesis studies reported here demonstrate that all five zinc fingers are required for DNA binding, yet the 11-base pair ZRE sequence predicts that at most only three or four of these are involved in site-specific interactions. For example, three fingers in transcription factor IIIA are sufficient to recognize an 11-base pair binding site (19). Structural studies of the GLI zinc finger protein may explain this paradox (18). Like Zap1, GLI contains five tandem zinc fingers that are required for DNA binding. Although GLI zinc fingers 2 through 5 bind in the major groove, finger 1 does not contact DNA but rather makes extensive protein-protein interactions with finger 2. For Zap1, a similar structure may exist with ZnF4–ZnF7 making major groove contacts and ZnF3 making protein-protein contacts analogous to GLI finger 1. Alternatively, Zap1 ZnF3 may make nonspecific DNA contacts with the phosphate backbone.

The hypothesis that Zap1 has a protein-DNA complex structure similar to that of GLI is supported by examining the
sequences and lengths of the spacer regions between the fingers. Adjacent zinc fingers known or predicted to make major groove contacts are typically separated by a linker with the consensus sequence (T/S)-G-E-(R/K)-P (6, 17, 18). Sequences that precisely match this consensus separate Zap1 ZnF5 and ZnF6, and ZnF6 and ZnF7, whereas a closely related sequence, SKYKP, separates ZnF4 and ZnF5 (Fig. 1). The presence of these conserved linker sequences suggests that ZnF4–ZnF7 make major groove contacts. A very different linker in terms of both sequence and length, LTRGKSE, separates ZnF3 and ZnF4, suggesting that ZnF3 does not bind in the major groove. The preceding discussion assumes that Zap1 binds to DNA as a monomer, as is commonly found among zinc finger proteins. However, if Zap1 binds as a dimer, as is suggested by the palindromic nature of the ZRE (7, 8), perhaps as few as two fingers of each monomer make major groove contacts. The other fingers may form a dimer interface (7) or make phosphate backbone DNA contacts.

Significant progress has been made recently in devising a protein-DNA recognition code for zinc finger proteins like Zif268 (13). Such a code, in which the identity of DNA contacting residues can be predicted from the nucleotide sequence of the recognition site, and vice versa, would be extremely useful for designing zinc finger proteins that bind specifically to target DNA sequences. Attempts to apply the current recognition codes to predict which fingers of Zap1 bind to which bases of the DNA sequences have yet to provide unambiguous results.

Surprisingly, our results demonstrate that Zap1 ZnF1 and ZnF2 are not required for high affinity ZRE binding. Either deletion of these two finger domains or site-directed mutagenesis such that they could no longer bind Zn$^{2+}$ failed to reduce the ability of Zap1 to function in vivo or bind to DNA in vitro. Furthermore, our results suggest that ZnF1 and ZnF2 bind Zn$^{2+}$ with lower affinity than do the other five Zap1 finger domains. GST-Zap1$_{538-880}$ contains 5 mol eq of Zn$^{2+}$ when purified from E. coli and mutation of ZnF1 and ZnF2 had no effect on this stoichiometry. One explanation for their apparent lower affinity for Zn$^{2+}$ is suggested by the sequence of these two fingers. Most zinc fingers have a hydrophobic residue located in a turn between $\beta$2 and the $\alpha$-helix that provides part of a stabilizing hydrophobic core at the “tip” of each finger. For Zap1 ZnF3–ZnF7, this residue is a phenylalanine (Fig. 1). However, ZnF1 has a cysteine and ZnF2 has a glycine in the corresponding position, and neither of these residues would provide this important hydrophobic interaction.

What role then do these two fingers play in Zap1 function? One potential function is as Zn$^{2+}$ sensors; Zap1 binds to DNA and activates transcription under low zinc conditions, but this activity is repressed in high zinc. Although the precise mechanism of this regulation is unknown, we postulate that Zap1 is the primary zinc sensor and that binding of the metal to lower affinity sites in the protein represses its activation function. The apparently low affinity of ZnF1 and ZnF2 for Zn$^{2+}$ suggests that these two fingers may be regulatory rather than structural components. In this regard, it is intriguing that ZnF1 and ZnF2 are found within an activation domain of Zap1; perhaps, binding of Zn$^{2+}$ to these fingers may control the activity of this domain. The observation that ZnF1 and ZnF2 mutations increase ZRE-lacZ expression (Fig. 4A) are consistent with this model. Ongoing studies are addressing this hypothesis and further characterizing the roles of the zinc fingers in Zap1 function.

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