Adaptability at the protein–DNA interface is an important aspect of sequence recognition by bZIP proteins

(DNA-binding protein/yeast GCN4/transcription factor/gene regulation/leucine zipper)

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ABSTRACT The related AP-1 and ATF/CREB families of transcriptional regulatory proteins bind as dimers to overlapping or adjacent DNA half-sites by using a bZIP structural motif. Using genetic selections, we isolate derivatives of yeast GCN4 that affect DNA-binding specificity at particular positions of the AP-1 target sequence. In general, altered DNA-binding specificity results from the substitution of larger hydrophobic amino acids for GCN4 residues that contact base pairs. However, in several cases, DNA binding by the mutant proteins cannot be simply explained in terms of the GCN4–AP-1 structure; movement of the protein and/or DNA structural changes are required to accommodate the amino acid substitutions. The quintet of GCN4 residues that make base-pair contacts do not entirely determine DNA-binding specificity because these residues are highly conserved in the bZIP family, yet many of the bZIP proteins bind to distinct DNA sites. The α-helical fork between the GCN4 DNA-binding and dimerization surfaces is important for half-site spacing preferences, because mutations in the fork alter the relative affinity for AP-1 and ATF/CREB sites. The basic region in the protein–DNA complex is a long isolated α-helix, with no constraints from other parts of a folded domain. From all of these considerations, we suggest that small shifts in position and orientation or local deformations in the α-helical backbone distinguish one bZIP complex from another.

The DNA-binding domains of most eukaryotic transcriptional regulatory proteins can be classified into a relatively small number of distinct structural classes. The bZIP motif (50–60 amino acid residues) consists of two distinct segments, the leucine zipper and the basic region (1). The C-terminal 30 residues form a two-stranded parallel coiled coil (the leucine zipper), which mediates dimerization (2). This leucine zipper symmetrically positions a divergent pair of basic-region α-helices, which pass through the major groove of each DNA half-site (3–6). Upon specific DNA-complex formation, the bZIP segment undergoes a folding transition. The previously unfolded basic region becomes α-helical (7–9), and a quintet of conserved basic-region residues are positioned to make contacts with the DNA (6).

Yeast GCN4 belongs to the AP-1 family of transcription factors that includes the Jun and Fos oncoproteins. The optimal AP-1–GCN4 recognition sequence, ATGA(C/G)TCAT, consists of overlapping half-sites, which are nonequivalent because of the asymmetry imposed by the central C·G base pair (defined as position 0) (6, 10–12). GCN4 also binds with only slightly reduced affinity to the ATF/CREB sequence (ATGA CGTCAT), in which the half-sites abut rather than overlap (13). In contrast, the structurally and

immunologically related ATF/CREB transcription factors bind much more efficiently to ATF/CREB sites than to AP-1 sites (14). We have therefore proposed that AP-1 and ATF/CREB proteins make similar DNA sequence-specific contacts but differ in their half-site spacing requirements (13).

We previously isolated a specificity mutant of GCN4 by a genetic selection for derivatives activating transcription from promoters containing mutant binding sites (15). The mutant protein contains a tryptophan in place of the invariant basic-region asparagine (Asn-235), and it affects specificity at the ±4 position of the AP-1 site. Here, we address the basis of DNA-binding specificity at the more critical positions of the AP-1 site by isolating additional GCN4 specificity mutants. Furthermore, we address the specificity of half-site spacing by generating GCN4 derivatives with altered preferences for the AP-1 and ATF/CREB sites. The resulting changes in DNA-binding specificity are interpreted in terms of the x-ray structure of the GCN4–DNA complex (6), and the implications of these results for the DNA-binding specificities of other bZIP proteins are discussed.

MATERIALS AND METHODS

The methods for degenerate oligonucleotide mutagenesis, genetic selections for GCN4 specificity mutants, phenotypic analysis, and DNA-binding specificity determinations have been described (15).

RESULTS

Isolation of GCN4 Mutants That Functionally Interact with Altered DNA Sites. GCN4 proteins that activate transcription from altered AP-1 target sites were isolated by the genetic selection described (15). A library of 10^4 GCN4 derivatives averaging 2-bp substitutions in the basic region (residues 236–246) was introduced into yeast strains containing symmetrically mutated AP-1 sites (A<u>G</u>GACTC<u>C</u>T; AT<u>T</u>AC<u>T</u>AAT) upstream of the *his3* TATA element and structural gene (Fig. 1). Yeast transformants with increased levels of *his3* expression were selected by their growth in the presence of aminotriazole. GCN4 plasmids were recovered from these strains, sequenced, and analyzed for their ability to stimulate transcription from a variety of mutant AP-1 sites (Fig. 2).

Upon selection for proteins that could activate transcription from A<u>C</u>GACTC<u>G</u>T, we isolated a derivative in which Ala-238 is changed to tyrosine. This Tyr-238 protein also stimulates transcription from a *his3* promoter containing A<u>G</u>GACTC<u>C</u>T, but it is unable to function on A<u>A</u>GAC-TC<u>T</u>T. Although the Tyr-238 protein only weakly activates transcription from the mutant target sites, it is clearly more effective than wild-type GCN4. The Tyr-238 protein retains the ability to activate transcription efficiently from the opti-

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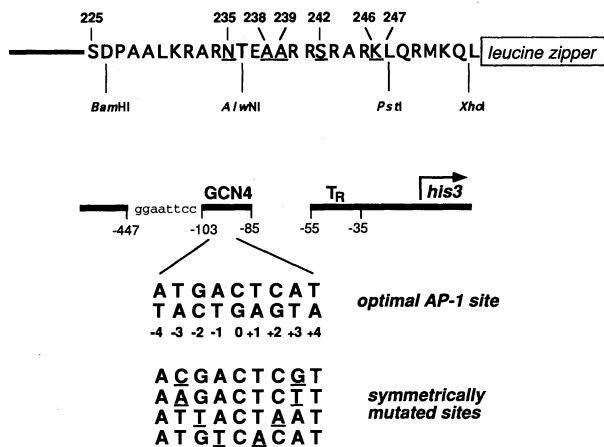
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FIG. 1. Isolation of GCN4 specificity mutants. (*Upper*) Amino acid sequence of the GCN4 basic region adjacent to the leucine zipper is shown with residues identified by specificity mutants underlined. The library of mutant GCN4 proteins subjected to genetic selection was generated by replacing the region between the *Alw*NI and *Pst* I sites with a degenerate oligonucleotide containing an average of 2-bp substitutions. (*Lower*) Target promoters used for genetic selection are derived from a molecule containing the optimal GCN4 binding site upstream of the *his3* T_R element and structural gene. The central C·G base pair of the optimal binding site is defined as position 0, base pairs to the right are defined as +1 to +4, and base pairs to the left are defined as −1 to −4 (12). Symmetrically mutated derivatives of the GCN4 binding site that respond to the various specificity mutants are shown below with nonoptimal bases underlined.

mal AP-1 site. Thus, the Tyr-238 substitution in the GCN4 basic region broadens the specificity at the ±3 position.

Two other derivatives were selected for their ability to activate transcription strongly from a promoter containing ATTACTAAT. Both of these derivatives stimulate transcription from the optimal AP-1 site, but they are inactive on sites with other symmetric substitutions at the ±2 position. Both of these ±2 specificity mutants contain two amino acid substitutions. In one case, Ala-239 and Ser-242 are changed to Val-239 and Leu-242. Examination of the individual substitutions indicates that the Val-239 protein functions at ATTACTAAT, although less efficiently than the double-mutant protein, whereas the Leu-242 protein does not. In the other case, Ser-242 and Lys-246 are changed to Cys-242 and Gln-246; both of these substitutions contribute to transcriptional activity on ATTACTAAT.

DNA-Binding Specificities of the Mutant GCN4 Proteins. The various proteins were synthesized *in vitro* and incubated with 13 target DNA sequences representing all possible symmetric mutations at positions ±1, ±2, ±3, and ±4 (Figs. 3 and 4; Table 1). In general, the mutant proteins have gained the ability to bind specific sites to which wild-type GCN4 does not bind. Otherwise, the mutant proteins retain normal GCN4 sequence recognition properties including binding the optimal site with near wild-type affinity. In all cases, the DNA-binding properties of the mutant proteins are in excellent agreement with their transcriptional activation properties *in vivo*.

The Tyr-238 protein behaves similarly to GCN4 except that it binds detectably (although weakly) to AGGACTCCT and ACGACTCGT, confirming that it only affects sequence recognition at the ±3 position. The Cys-242/Gln-246 double-mutant protein also behaves similarly to GCN4, except that it binds to ATTACTAAT with an affinity comparable to that of the optimal site. Both amino acid substitutions are important for high-affinity binding to ATTACTAAT, because the Cys-242 and Gln-246 single-mutant proteins bind this site
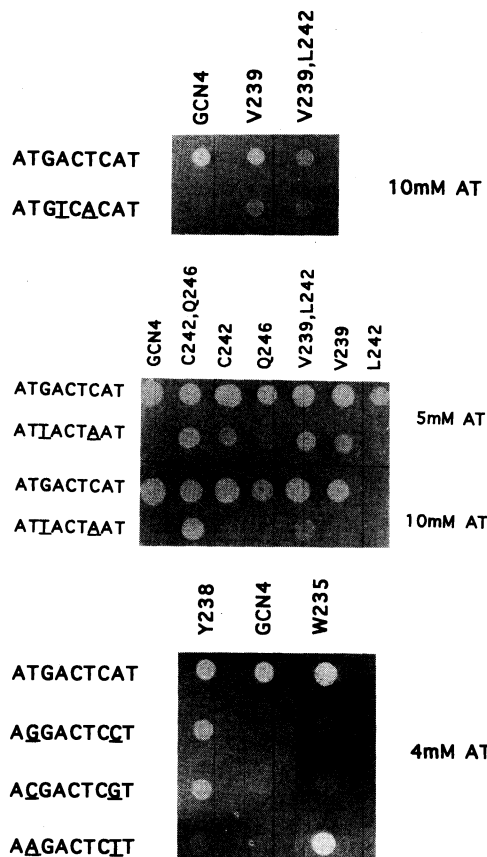

FIG. 2. Phenotypic analysis. Strains containing the indicated GCN4 and *his3* promoter derivatives were plated on medium containing various concentrations of aminotriazole (AT). From top to bottom are shown analyses of specificity at positions ±1, ±2, and ±3.

very weakly. Thus, the dual DNA-binding specificity of the Cys-242/Gln-246 protein is restricted to the ±2 position.

In accord with their genetic properties, the Val-239 and Val-239/Leu-242 proteins have a slightly reduced affinity for the optimal site, and they efficiently bind ATTACTAAT; the double-mutant protein is more active. Although selected by a change at ±2, the Val-239 and the double-mutant protein also bind to ATGTCACAT as well as they do to that of the
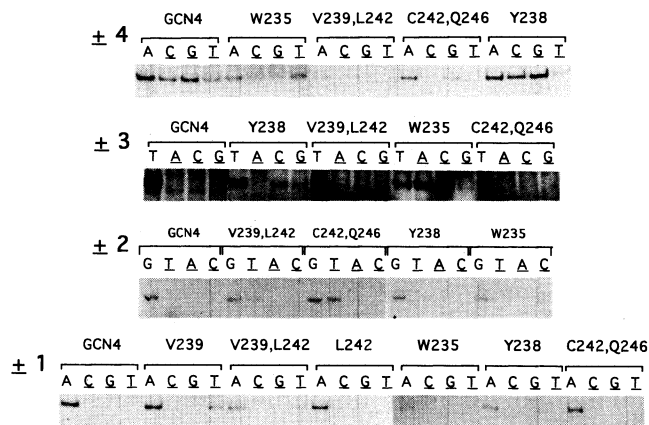

FIG. 3. DNA-binding specificities of GCN4 and the mutant proteins. Protein–DNA complexes formed by incubating equivalent amounts of *in vitro* synthesized ^{35}S-labeled proteins (determined by SDS/PAGE) with the indicated target sequences (mutated residues are underlined). From top to bottom are indicated DNA-binding specificities at positions ±4, ±3, ±2, and ±1.
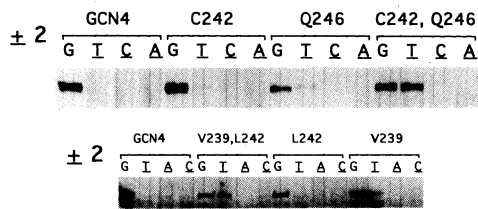
FIG. 4. DNA-binding specificity at the ±2 position of single- and double-mutant proteins. Protein–DNA complexes formed by incubating equivalent amounts of *in vitro* synthesized ^{35}S-labeled proteins (determined by SDS/PAGE) with the target sequences containing mutated residues (underlined) at position ±2.

FIG. 5. GCN4 derivatives that alter half-site spacing specificity. Protein–DNA complexes formed by incubating equivalent amounts of *in vitro* synthesized ^{35}S-labeled proteins (determined by SDS/PAGE) with the individual or a mixture of DNA fragments containing the ATF/CREB or AP-1 target sequences. Differences in electrophoretic mobility between complexes with the ATF/CREB and AP-1 binding sites are due to the length differences of the two DNA fragments.

optimal site. Consistent with this observation, these proteins activate transcription from a promoter containing ATGTCACAT upstream of the *his3* TATA element. Thus, substitution of Ala-239 with valine affects DNA-binding specificity at both the ±1 and ±2 positions.

We also carried out detailed DNA-binding specificity experiments on the Trp-235 protein that had previously been shown to affect recognition at the ±4 position (15). The Trp-235 protein binds extremely strongly to AAGACTCTT but not to AGGACTCCT, AAGACTCTT, or to any sequence variants at the ±1 or ±2 positions. Indeed, the affinity for AAGACTCTT is higher than for the optimal site, indicating that the Trp-235 substitution alters sequence recognition at both positions ±3 and ±4, with the more pronounced effect being at ±3.

Mutations of GCN4 That Affect Half-Site Spacing Specificity. We previously suggested that AP-1 and ATF/CREB proteins make similar DNA contacts but differ in half-site spacing preferences, and we predicted that the connection between the leucine zipper and basic region (residues 244–250) determines the flexibility and specificity of half-site spacing (13). In this regard, there is a consistent difference at the position corresponding to GCN4 residue 247: ATF/CREB proteins have a positively charged residue (nearly always lysine), whereas AP-1 proteins do not (GCN4 contains a leucine) (16).

We therefore analyzed Lys-247 and Arg-247 derivatives of GCN4 for their relative binding to AP-1 and ATF/CREB sites (Fig. 5). Unlike GCN4, which prefers the AP-1 site over the ATF/CREB site by a factor of 5 (13), the Lys-247 and
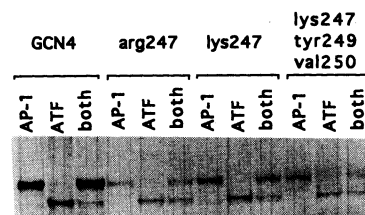
Arg-247 proteins bind the AP-1 and ATF/CREB sites with comparable affinities. In comparison to GCN4, these proteins bind with reduced affinity to the AP-1 site but with wild-type affinity to the ATF/CREB site. A GCN4 derivative in which residues 247, 249, and 250 are replaced by the corresponding residues in CREB appears to show a further reduction in AP-1 binding activity such that the ATF/CREB site is preferred by a factor of 2. Thus, these substitutions alter half-site spacing specificity, but they do not fully convert GCN4 into a protein with typical ATF/CREB DNA-binding properties. Nevertheless, the results indicate that the region between the leucine zipper and DNA-binding surface is critical for half-site spacing specificity, with position 247 playing an important but not fully determinative role.

Modeling of the Mutant Protein–DNA Complexes. The crystal structure of the GCN4 bZIP–AP-1 DNA complex (6) demonstrates that Asn-235, Ala-238, Ala-239, Ser-242, and Arg-243 are in contact with the central 7 bp of the binding site. In addition, numerous basic residues anchor GCN4 to its binding site by hydrogen bonds and electrostatic interactions with the phosphodiester backbone. This structure provides a framework for interpreting the functional consequences of amino acid substitutions in the GCN4 mutant proteins. Because these proteins function well on the optimal AP-1 site present in the crystallized protein–DNA complex, we have tried to configure the mutant side chains in orientations that minimally disrupted the wild-type structure. As described below, some of the substituted residues cannot be accommodated by the wild-type orientation of the GCN4 basic region on DNA.

Tyr-238. In the GCN4 complex, the thymine methyl group at ±3 interacts with the methyl group of Ala-238. The Tyr-238 substitution creates a steric clash with the phosphates of bases 4 and 5. This clash can be relieved by a local adjustment of the DNA backbone conformation—for example, as observed in complexes of the bacteriophage 434 repressor with different operators (17). The tyrosine hydroxyl group might then donate a hydrogen bond to the phosphate of the ±5 pyrimidine residue. However, the tyrosine ring would still crowd the DNA at position ±4, requiring further adjustment. A distributed set of small, local structural changes, relative to wild-type, may contribute to broadened specificity at position ±3.

Trp-235. Asn-235 interacts directly with both strands of the optimal target site through hydrogen bonds with the ±3 thymine and the ±2 cytosine, and it may also communicate with position ±4 through a hydrogen bond to an intervening water molecule. If Trp-235 is oriented with the long axis of its indole ring pointing away from the DNA, it can be accommodated in the wild-type structure without interfering with DNA contacts made by other residues. The crystal structure is consistent with the possibility that in a complex of the Trp-235 protein with AAGACTCTT, the tryptophanyl side

Table 1. Binding of GCN4 mutant proteins to various target sequences

| Target site | GCN4 derivative | | | | | |
	Wild type	Trp-235	Tyr-238	Val-239	Val-239/Leu-242	Cys-242/Gln-246
ATGACTCAT	+++	++	+++	++	,++	+++
CTGACTCAG	++	+	++	−	−	+
GTGACTCAC	+++	+	+++	+	+	++
TTGACTCAA	++	++	+	−	−	+
AAGACTCTT	−	+++	−	−	−	−
ACGACTCGT	−	−	+	−	−	−
AGGACTCCT	−	−	+	−	−	−
ATAACTTAT	−	−	−	−	−	−
ATCACTGAT	−	−	−	−	−	−
ATTACTAAT	−	−	−	+	++	+++
ATGCCGCAT	−	−	−	−	−	−
ATGGCCCAT	−	−	−	−	−	−
ATGTCACAT	−	−	−	++	+	−

Relative DNA-binding abilities (based on data in Figs. 3 and 4 and in additional experiments) are indicated as follows: +++, wild-type affinity; ++, somewhat weaker than wild-type affinity; +, weak binding; −, not detectable. When tested, transcriptional activation of the GCN4 derivatives on the indicated target sites *in vivo* was in excellent accord with the DNA-binding properties *in vitro*.

chains might stack against the ±3 thymine methyl groups in the mutant site. The Trp-235 residue is relatively close to ±4, but the basis of the observed specificity change at this position is unclear. The Trp-235 substitution eliminates two hydrogen bonds, including the only direct contact to ±2. It is therefore surprising that the Trp-235 protein binds the optimal GCN4 site with only slightly diminished affinity and that it shows the wild-type preferences at ±2.

Cys-242/Gln-246. In GCN4, Lys-246 is not in close contact with DNA, but Ser-242 directly interacts with the ±3 thymine methyl. Thus, the Gln-246 substitution must affect DNA-binding specificity indirectly, probably by altering the position of the basic region to accommodate new bases at position ±2. It is unclear why Cys-242 is more effective than Ser-242 in allowing dual specificity at position ±2.

Val-239 and Val-239/Leu-242. Ala-239 contacts the ±1 thymine methyl group in the wild-type GCN4 complex. Substitution of the larger Val-239 residue would not affect the thymine contact, but it would crowd the Arg-243 side chain that contacts the central base pair. Arg-243 is invariant in the set of known bZIP proteins, and its contact to the central guanine is energetically significant because GCN4 will bind to an ATGAC half-site but not to ATGAG (13). Crowding of Arg-243 by Val-239 requires some conformational adjustment, which might account for the reduction in affinity of the Val-239 and Val-239/Leu-242 proteins for an optimal AP-1 site. Although Val-239 is located near the ±1 base pair, it is unknown how it tolerates the T·A but not the G·C or the C·G substitution.

Lys-247 and Arg-247. Residue 247 of each monomer lies within the "fork" region where the basic regions diverge from the leucine zipper. If we assume that the protein contacts AP-1 and ATF/CREB half-sites in a similar manner, the fork will be more widely spread in ATF/CREB complexes. Because the Lys-247 and Arg-247 proteins lose affinity for AP-1 sites but not for ATF/CREB sites with normal affinity, we suggest that the Lys-247 and Arg-247 substitutions interfere with the configuration of the fork necessary for AP-1 site binding. It is unlikely, however, that such interference reflects electrostatic repulsion between Lys-247/Lys-247 or Arg-247/Arg-247 pairs, because the corresponding Leu-247 residues in GCN4 are not in close proximity.

DISCUSSION

Functional Analyses of the GCN4–AP-1 Complex. The genetic selection of GCN4 derivatives that function on mutant DNA sequences provides a method for identifying amino acid residues that contribute to DNA-binding specificity. The mutant proteins described here generally retain activity on the optimal AP-1 sequence while gaining the ability to bind specific mutant target sites. Because these GCN4 derivatives were isolated from complex libraries of mutant proteins rather than by directed mutagenesis, it is likely that the residues identified here are important determinants of the strict DNA sequence specificity of GCN4. Indeed, of the five residues that contact the central 7 bp (6), four (Asn-235, Ala-238, Ala-239, and Ser-242) were identified by the GCN4 specificity mutants.

If amino acid substitutions cause only local structural changes, then amino acids and nucleotides identified by the specificity mutants might be predicted to interact in the wild-type complex. Several of the specific contacts inferred in this way (Asn-235 and ±3, Ala-238 and ±3, and Ala-239 and ±1) are indeed observed in the crystal structure. However, GCN4 mutants affecting specificity at ±2 contain substitutions at residues 239, 242, and 246 that are not in contact with base-pair 2 in the wild-type complex. These observations imply that complexes of some of the variant

proteins differ from the wild-type structure in more ways than just local perturbations in the vicinity of the altered residues.

Relationship Between the GCN4 Specificity Mutants and Other bZIP Proteins. Although the specificity mutants were sought primarily to understand the basis of GCN4 DNA-binding specificity, some of them are relevant to other bZIP proteins. First, C/EBP and several other bZIP proteins contain a valine at the position corresponding to Ala-239, where a valine substitution in GCN4 affects specificity at ±1 and ±2, and *Schizosaccharomyces pombe* PAP1 and *Saccharomyces cerevisiae* YAP1 contain a glutamine at this position. Thus, position 239 is likely to play a role in the distinct DNA-binding specificities of GCN4, C/EBP, and YAP1 (3, 10, 18). Second, two of the GCN4 specificity mutants bind with high affinity to ATTACTAAT. Several AP-1 and ATF/CREB proteins also recognize this sequence, and T(G/T)AC has been proposed as the half-site consensus. It is possible that the mutant and natural bZIP proteins recognize ATTACTAAT in the same way. Third, position 247 plays an important role in half-site spacing and is likely to account for some of the differences between AP-1 and ATF/CREB factors; it may also be important for determining half-site relationships in other bZIP proteins.

Adaptability at the Protein–DNA Interface Is a Critical Determinant for DNA-Binding by bZIP Proteins. The basic region of GCN4 and of other bZIP proteins forms an extended α-helix when it binds to DNA, and no other tertiary interactions within the protein stabilize its conformation. By contrast, most of the other well-studied prokaryotic and eukaryotic DNA-binding domains contain compact, globular modules. Constraints within their folded structures restrict adaptability in the DNA recognition surface. Flexibility is instead built into elements such as the arm of λ repressor and the linker segment of GAL4, which fold when the protein binds DNA or the joints in zinc-finger proteins, which allow successive fingers to wrap around DNA in the major groove. Moreover, the globular modules are generally tightly anchored to the DNA backbone through peptide-NH groups or small polar residues. As a result, among proteins with a common structural motif, there is a strong relationship between the amino acid residues on the recognition surface and DNA-binding specificity. Proteins containing similar amino acid residues on the recognition surface generally have similar DNA-binding specificities, whereas proteins with distinct specificities differ at these crucial amino acid positions. Thus, substitutions of amino acid residues that normally contact base pairs usually cause large decreases in affinity, because an altered protein cannot adapt to an unaltered site, and efficient binding of a mutant protein to an altered site can often be explained by new interactions between the substituted amino acids and base pairs.

Our results suggest that adaptability in the local conformation and/or positioning of the basic region is an important aspect of sequence recognition by bZIP proteins. For many of the GCN4 specificity mutants, the substituted residues cannot be accommodated by the structure of the complex. The Val-239 substitution, which affects specificity at ±1 and ±2, requires some adjustment in the protein in order to relieve steric clash with the invariant Arg-243 residue. The Tyr-238 substitution, which broadens specificity at ±3, requires movement of the DNA backbone away from the protein. Other substitutions of larger, hydrophobic residues are permitted at positions 238 and 239 (19), and these presumably cause some perturbation of the protein–DNA interface. The Trp-235 substitution eliminates the only contact to ±2, yet it retains normal DNA-binding specificity at this position. Given the central role of Asn-235 in the wild-type complex (hydrogen bonds to ±2 and ±3 and a possible H_2O-mediated hydrogen bond to ±4), it is striking that some substitutions have relatively modest effects on DNA-binding

affinity (15, 19). Finally, two GCN4 specificity mutants alter specificity at ±2 even though the original (and possibly the substituted) amino acids do not contact ±2. These observations are not simply artifacts of the mutant proteins because, as discussed above, most of them have counterparts in other bZIP proteins.

Adjustments in the α-helical geometry of the GCN4 fork segment and basic region are also likely to accommodate the different half-site spacings of the AP-1 and ATF/CREB sites. Residue 247, which does not contact DNA but lies at the fork between the leucine zipper and basic region, is important for half-site spacing. Assuming that AP-1 and ATF/CREB half-sites are contacted by the same GCN4 residues, then the protein must be sufficiently flexible to allow a rotation of 36° and translation of ≈3.3 Å between half-sites, while maintaining these protein–DNA contacts. This amount of flexibility is unprecedented in other DNA-binding proteins, presumably because tertiary folding constraints limit movement within other DNA-binding domains.

Comparative analysis of bZIP protein sequences and their DNA-binding specificities provides an independent argument for conformational variations in basic regions. The five GCN4 residues that make base-pair contacts (6) are very highly conserved in bZIP proteins; Asn-235 and Arg-243 are invariant, whereas Ala-238/Ala-239 and Ser/Cys-242 are present in >80% of bZIP domains (19). Nevertheless, bZIP proteins can differ considerably in their DNA-binding specificities. This situation is in marked contrast to that observed in helix–turn–helix proteins in which amino acid similarity at the recognition surface is strongly correlated with DNA-binding specificity. Nonconserved residues in the basic region must play a crucial role in the different DNA-binding specificities of bZIP proteins, either by direct base-pair interactions or by indirect effects on the conserved quintet. Both mechanisms require conformational variation in the DNA recognition surface from that of the GCN4–AP-1 complex. These differences may result from variations in the α-helical geometry and/or overall orientation in the major groove of the basic region.

The basic regions of bZIP domains become ordered only upon association with target DNA (7–9) and are not constrained by tertiary interactions within the protein. The absence of a rigid, globular structure makes it plausible that basic regions of bZIP proteins adopt different conformations along DNA. Variable conformations of a given basic region are likely to allow the dual specificities of C/EBP and of the GCN4 derivatives described here. In the case of GCN4, the basic region is held in the major groove primarily by long arginine and lysine side chains, and there is likely some flexibility in the way it is anchored. As these basic residues

are highly conserved, such flexibility is likely to be a general feature of bZIP domains. A precise description of individual protein–DNA complexes will require the high-resolution structures. However, the combined evidence from the GCN4–AP-1 complex structure, the sequence comparison of bZIP proteins, and the structural and functional interpretation of our GCN4 specificity mutants provides a strong case that adaptation at the protein–DNA interface is an important aspect of DNA-binding specificity in bZIP proteins.

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