mental conditions, a contribution in tobacco cells of 100 mM mannitol may be consistent with both mechanisms. However, uniform distribution of mannitol at the subcellular level would likely preclude cytosolic osmoregulation. Determination of both the subcellular location of mannitol and the concentrations of the sugar alcohol in tissues will help to distinguish between these two mechanisms.

Alternatively, mannitol may metabolically predispose tobacco cells to stress tolerance. Thus, the cellular accumulation of mannitol, which is normally foreign in tobacco cells, may increase the response of metabolic pathways normally involved in stress tolerance, which thereby allow cells to withstand stress.

On the basis of pilot experiments, we studied the growth response of plants of a defined age that were exposed to the extreme, or shock, condition of 250 mM NaCl. Additional experiments that vary plant age (development) and NaCl concentration (added all at once or incrementally), as well as experiments that investigate the effects of other environmental stresses such as drought and cold, will help to explain the function of mannitol in higher plants. We have demonstrated that for tobacco, the presence of mannitol in vivo protects against high salinity. Sugar alcohol accumulation may also enhance stress tolerance in other plants.

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14. Data for percent changes in height and fresh weight collected over time for the same individual plants were used to determine the inherent variability in initial measurements and were used in all statistical analyses. Data for percent changes in both height and fresh weight were shown to be normally distributed ($P \leq 0.01$) by calculation of the Shapiro-Wilk statistic that permitted our evaluation with ANOVA.
15. Initial heights/weights for experiment 1; and those from experiment 2, respectively, in percent change, for each line. Plants not receiving NaCl: SR1, 6.1 ± 0.4/21.6 ± 1.1, 7.3 ± 0.5/26.9 ± 0.9; SR1^{km}, 6.7 ± 0.5/27.2 ± 1.2, 7.6 ± 0.3/27.6 ± 0.9; 1-mtl, 6.4 ± 0.6/23.7 ± 1.5, 7.3 ± 0.6/26.0 ± 1.1; 2-mtl, 5.9 ± 0.3/27.3 ± 1.1, 6.4 ± 0.4/24.9 ± 0.8; 3-mtl, 6.3 ± 0.3/21.9 ± 1.9, 7.1 ± 0.5/27.1 ± 1.5. Plants subsequently receiving NaCl treatment: SR1, 7.1 ± 0.4/27.9 ± 2.0, 7.4 ± 0.2/26.1 ± 1.3; SR1^{km}, 9.4 ± 0.4/30.8 ± 1.1, 7.1 ± 0.3/25.5 ± 1.0; 1-mtl, 7.4 ± 0.4/26.4 ± 1.4, 7.2 + 0.3/28.1 ± 0.7; 2-mtl, 6.8 ± 0.3/27.5 ± 1.4, 6.6 ± 0.3/27.4 ± 0.7; 3-mtl, 7.3 ± 0.4/26.3 ± 1.6, 7.1 ± 0.2/27.9 ± 0.8. Values are mean ± SEM; for number of plants evaluated; see Tables 1 and 2.
16. For lines that express 35*S* mtlD, 30 primary transformants were chosen randomly and self-pollinated, and the progeny were selected for single-locus inserts on the basis of expression of kanamycin resistance. Of the lines that contain single locus inserts, three were randomly chosen (1-mtl, 2-mtl, 3-mtl), self-pollinated again, and determined to be homozygous on the basis of expression of kanamycin resistance. This procedure was also used for control SR1^{km}, except that five primary transformants were chosen. Lines analyzed were SR1, wild type; SR1^{km}, kanamycin-resistant; and 1-mtl, 2-mtl, and 3-mtl, three different kanamycin-resistant lines that express 35*S* mtlD.
17. The hydroponics system consisted of two genetically identical plants cultured in containers with half-strength Hoagland's nutrient solution (5 liters). (For Figs. 1 and 2, plants from SR1 and 1-mtl were cultured together in 250 mM NaCl in hydroponic containers.) The nutrient solution was changed every 2 weeks. Plants were inserted into the nutrient solution through two holes in the container lid. The growth room housed eight to ten plots of five randomly placed containers (one for each line). For each experiment, half of the plots were cultured in the absence of added NaCl and the other half were cultured in the presence of 250 mM NaCl. To minimize variability in growth and development of plants before stress, all plants (for the data reported in Tables 1 to 3) were approximately the same age (6 weeks) and were selected from larger groups for inclusion in each experiment for their similar heights and weights. For the two experiments, plants were visually identical before NaCl treatment. To eliminate potential experimenter bias, evaluations were performed blindly. Growth-room conditions were 18° and 25°C, night and day temperatures, respectively; photoperiod, 12 hours light (500 $\mu E\ m^{-2}\ s^{-1}$).
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Altered Specificity of DNA-Binding Proteins with Transition Metal Dimerization Domains

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The bZIP motif is characterized by a leucine zipper domain that mediates dimerization and a basic domain that contacts DNA. A series of transition metal dimerization domains were used to alter systematically the relative orientation of basic domain peptides. Both the affinity and the specificity of the peptide-DNA interaction depend on domain orientation. These results indicate that the precise configuration linking the domains is important; dimerization is not always sufficient for DNA binding. This approach to studying the effect of orientation on protein function complements mutagenesis and could be used in many systems.

Active sites of proteins are typically composed of recognition elements guided into proximity and appropriate orientation by the native protein fold. Individual recognition elements may be remote in primary structure or may be located on different polypeptide chains in multisubunit proteins. With site-directed mutagenesis, amino acids that constitute individual recognition elements can be changed without affecting the overall orientation of the recognition domain. Yet present technology does not allow predictable and routine changes in the orientation of the domains themselves.

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The transcriptional activator protein GCN4 is one of a large family of DNA-binding proteins identified by a bZIP structural motif (*1*); this motif contains a DNA contact domain characterized by conserved basic and hydrophobic residues (b domain), and a dimerization domain identified by a heptad repeat of leucine residues (ZIP domain) (*2*, *3*). The two domains are separated by a six–amino acid linker whose length, but not sequence is conserved across bZIP families (*1*). Previous work has demonstrated that the active DNA-binding entity is generated when the ZIP domains of two protein monomers assemble (*4*) into a parallel coiled coil (*5*, *6*). The scissors grip (*1*) and induced helical fork (*7*) models propose that the coiled coil, the natural dimeriza-

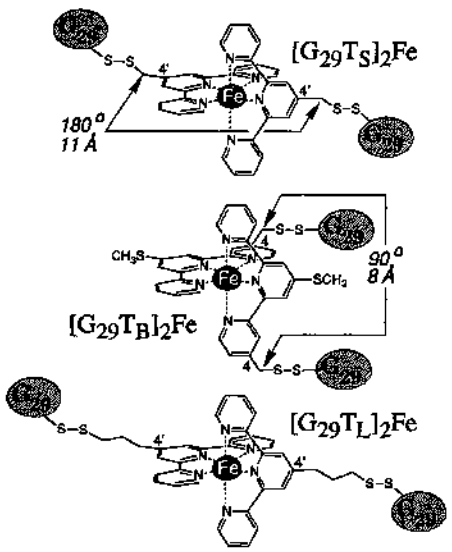


Fig. 1. Schematic representation of $[G_{29}T_S]_2Fe$, $[G_{29}T_L]_2Fe$, and $[G_{29}T_B]_2Fe$ (*17*).

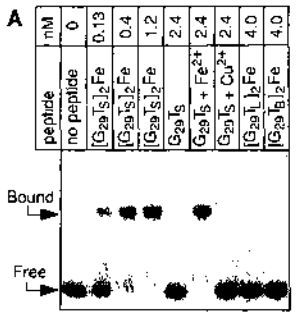
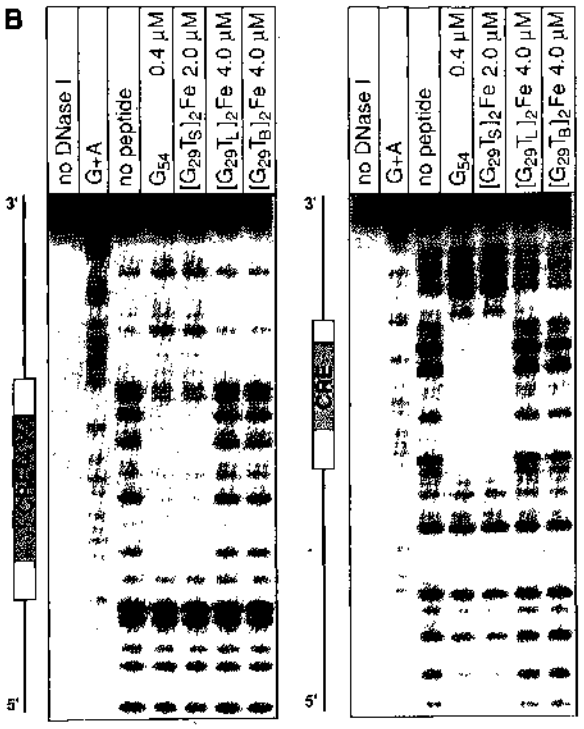


Fig. 2. (**A**) Gel mobility shift assay (*18*) and (**B**) DNase I footprinting analysis (*20*) of peptide-DNA interactions. (B) Left, DNA labeled with ^{32}P at the 5′ end of the + strand; right, label on the − strand. Bars denote regions protected from DNase I.



tion domain, fixes the spatial relationship between the two DNA contact domains to present the correct constellation of functional groups to the DNA (*5*, *8–10*). Even subtle changes in basic domain orientation or organization can have dramatic effects on DNA binding (*5*, *11–13*).

To understand better the role of dimerization domain architecture in modulating the affinity and specificity of bZIP-DNA interactions, we explored an approach to protein design (*14*) that exploited the well-defined geometries of transition metal ion complexes to assemble protein structural domains with defined orientations (*15*). Transition metal ion complexes make ideal synthetic scaffolds: the strong geometric constraint placed on the ligand by the central metal ion allows the relative orientation of two or more domains to be altered systematically by positioning them, through chemical synthesis, at various locations on a ligand of known structure. Molecules were constructed in which the GCN4 coiled coil was replaced by a series of Fe(II) complexes that systematically altered the relative orientation and spacing of the basic DNA contact domains. In our model-building studies we started with the coordinates of the scissors grip model (*1*) and sought a stereochemically well-defined transition metal ion complex whose geometry was complementary to the void produced on removal of the GCN4 coiled coil. These requirements were satisfied by the kinetically inert bis(terpyridyl)iron(II) complex first prepared by Morgan and Burstall in 1932 (*16*).

The terpyridyl substitution pattern dictated the relative orientation of G_{29} peptides in bis(terpyridyl)iron(II) complexes $[G_{29}T_S]_2Fe$, $[G_{29}T_B]_2Fe$, and $[G_{29}T_L]_2Fe$ (*17*) (Fig. 1). In $[G_{29}T_S]_2Fe$, the 4′-substituted terpyridyl T_S separated the two G_{29} carboxamide termini by approximately 11 Å and oriented them at a 180° angle. In $[G_{29}T_B]_2Fe$, the 4-substituted terpyridyl T_B separated the carboxamide termini by approximately 8 Å and oriented them at a 90° angle. The 4′-substituted terpyridyl T_L enforced the same geometry as T_S but contained a longer and more flexible tether. The ability of these stereochemically defined synthetic scaffolds to organize two G_{29} peptides into a functional DNA-binding protein was determined by monitoring the DNA binding affinities of the resultant complexes. The disulfide dimer of G_{29} (G_{29}^{SS}) (*13*) and a 54–amino acid peptide containing both the DNA contact and dimerization domains of GCN4 (G_{54}) were studied for comparison.

The binding of $[G_{29}T]_2Fe$ complexes to duplex DNA was measured first by gel mobility shift assay (*18*). Incubation of CRE_{24}, a 5′ end-labeled double-stranded DNA fragment (5′-AGTGGAGATGAC-GTCATCTCGTGC-3′) containing the high affinity GCN4 binding site CRE (AT-GACGTCAT; cAMP response element) (*19*), with 0.13 to 1.2 nM $[G_{29}T_S]_2Fe$ resulted in the gradual appearance of a complex with lower electrophoretic mobility than CRE_{24} alone or a CRE_{24}:G_{29}^{SS} complex (Fig. 2A). Monomeric $G_{29}T_S$ failed to bind CRE_{24}, but binding was restored upon addition of Fe(II). Addition of Cu(II) did not result in DNA binding. The affinity of $[G_{29}T_S]_2Fe$ for CRE_{24} was determined by measuring the fractions of bound and free CRE_{24} at several $[G_{29}T_S]_2Fe$ concentrations (Fig. 3). The data fit a theoretical equation that describes formation of a 1:1 complex with an equilibrium dissociation constant (K_d) of 0.13 ± 0.01 nM (*18*), a value comparable to the K_d measured for the G_{54} homodimer and the Fos-Jun heterodimer at this temperature (*11*).

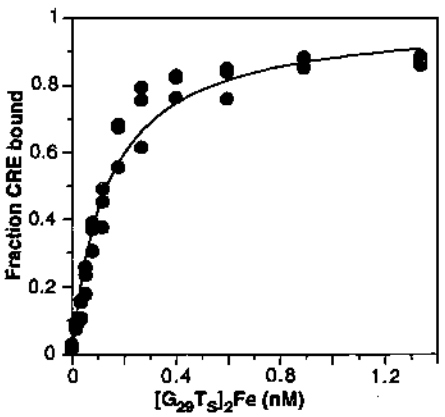


Fig. 3. Determination of the dissociation constant for the complex of $[G_{29}T_S]_2Fe$ and CRE_{24} (*18*).

DNA binding by $[G_{29}T_S]_2Fe$ was characterized further by deoxyribonuclease I (DNase I) footprinting (*20*) and circular dichroism (CD) spectroscopy. $[G_{29}T_S]_2Fe$ and G_{54} exhibited identical DNase I footprints and adjacent hypersensitive sites (Fig. 2B), demonstrating that the two molecules make similar DNA contacts and cause similar structural adjustments of the flanking DNA. Previous work has shown

that the DNA contact domains of bZIP proteins undergo an increase in helical structure when bound to specific DNA targets but not to nonspecific DNA (*7*, *9*, *10*, *13*). $[G_{29}T_S]_2Fe$ had little helical structure in the absence of DNA. The intensity of the CD signal at 222 nm (a helical band) increased significantly when one molar equivalent of CRE_{16} (5′-GAGATGACGTCATCTC-3′) was added (Fig. 4A). The difference spectrum (Fig. 4B) indicated that $[G_{29}T_S]_2Fe$ was almost entirely helical when bound to CRE_{16} (*21*). These results demonstrate that a metal ion complex of 612 daltons can replace the function of the leucine zipper, effectively orienting two G_{29} basic domains to permit high-affinity, sequence-specific recognition of a GCN4 target site.

The importance of orientation in DNA binding is illustrated by comparing the DNA affinities of $[G_{29}T_S]_2Fe$, $[G_{29}T_L]_2Fe$, and $[G_{29}T_B]_2Fe$. Although these metal complexes are structurally similar, neither $[G_{29}T_L]_2Fe$ nor $[G_{29}T_B]_2Fe$ bound CRE in a mobility shift assay (Fig. 2A), even at 0.2 μM. The absence of binding was not due to instability of the relevant DNA complexes under mobility shift conditions; neither $[G_{29}T_L]_2Fe$ nor $[G_{29}T_B]_2Fe$ yielded a detectable DNase I footprint (Fig. 2B) at 4 μM nor reduced the extent of CRE:$[G_{29}T_S]_2Fe$ complex formation in a mobility shift competition assay. The only evidence supporting DNA binding by $[G_{29}T_L]_2Fe$ or $[G_{29}T_B]_2Fe$ was obtained in CD experiments performed at a concentration 10,000 times the K_d of the CRE_{24}:$[G_{29}T_S]_2Fe$ complex, where a moderate increase in peptide helicity was observed (Fig. 4B). From our mobility shift data, we estimated that at 4°C the CRE:$[G_{29}T_S]_2Fe$ complex is 4 kcal mol^{-1} more stable than either the CRE:$[G_{29}T_L]_2Fe$ or the CRE:$[G_{29}T_B]_2Fe$ complex. The spacing of the basic domains in $[G_{29}T_S]_2Fe$ and $[G_{29}T_L]_2Fe$ differs by only four methylene groups, while $[G_{29}T_S]_2Fe$ and $[G_{29}T_B]_2Fe$ differ only in the orientation with which the basic domains extend into the major groove. Thus, coordination complex architecture is important in orienting the G_{29} basic domain for high affinity recognition of the CRE. The transition metal ion complexes studied here provide an independent stereochemical variable in protein design by allowing the assembly of peptide aggregates with defined orientations.

All bZIP proteins share a common domain architecture and conserved basic and hydrophobic residues, yet they differ in their ability to differentiate target sites with alternative half site spacing. The Fos-Jun subfamily prefers the AP1 site (ATGACTCAT) and the CREB/ATF subfamily prefers the CRE site (ATGACGTCAT) (*22*). GCN4 (*9*, *19*) and certain disulfide-linked dimers of the GCN4 basic domain (*23*) bind both sites with comparable affinity. Within a B-DNA context, the additional C:G base pair in the CRE displaces the two ATGA contact surfaces by an axial translation of 3.4 Å and a twist angle of 34° (*24*).

As the results described above demonstrate the effect of basic domain orientation on CRE affinity, we asked whether basic domain orientation would also affect discrimination between CRE and AP1. Mobility shift assays did not detect any interaction between $AP1_{23}$ (5′-AGTGGAGATGACTCATCTCGTGC-3′) and $[G_{29}T_S]_2Fe$, even at elevated concentrations (Fig. 5A), whereas G_{29}^{SS} bound CRE_{24} and $AP1_{23}$ with comparable affinity (Fig. 5B). Neither

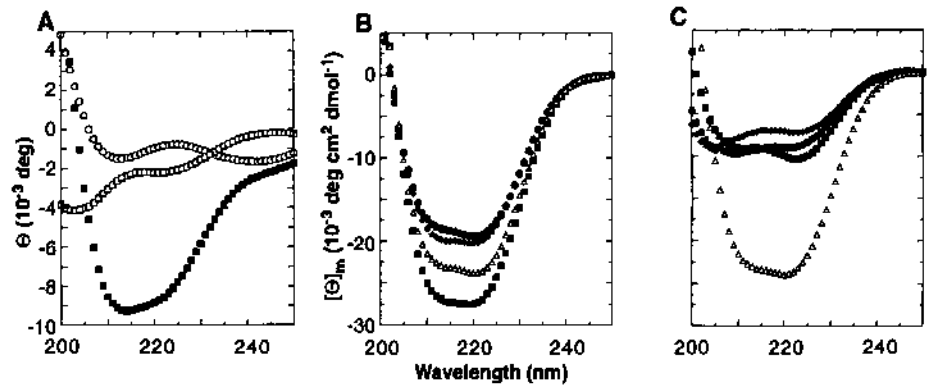


Fig. 4. Circular dichroism spectroscopy. (**A**) CRE_{16} alone (○), $[G_{29}T_S]_2Fe$ alone (□), and CRE_{16} with $[G_{29}T_S]_2Fe$ (■). (**B**) Spectra of $[G_{29}T_S]_2Fe$ (■), $[G_{29}T_L]_2Fe$ (◆), $[G_{29}T_B]_2Fe$ (●), and G_{29}^{SS} (△) peptides with CRE_{16}, calculated as the difference between the observed spectrum of the mixture and CRE_{16} alone. (**C**) Spectra of $[G_{29}T_S]_2Fe$ (■), $[G_{29}T_L]_2Fe$ (◆), $[G_{29}T_B]_2Fe$ (●), and G_{29}^{SS} (△) peptides with $AP1_{15}$, calculated as the difference between the observed spectrum of the mixture and $AP1_{15}$ alone. CD spectra were recorded with an AVIV model 62DS CD spectrometer at 25°C in a 1 mm cell. Samples contained 10 mM phosphate buffer (pH 7.4), 100 mM NaCl, and 4.6 μM peptide and 5.0 μM duplex DNA when present. Θ, the observed ellipticity; $[\Theta]_m$, the mean residue ellipticity. Spectra were the average of 5 scans and were corrected with a spectrum of buffer alone but not smoothed.

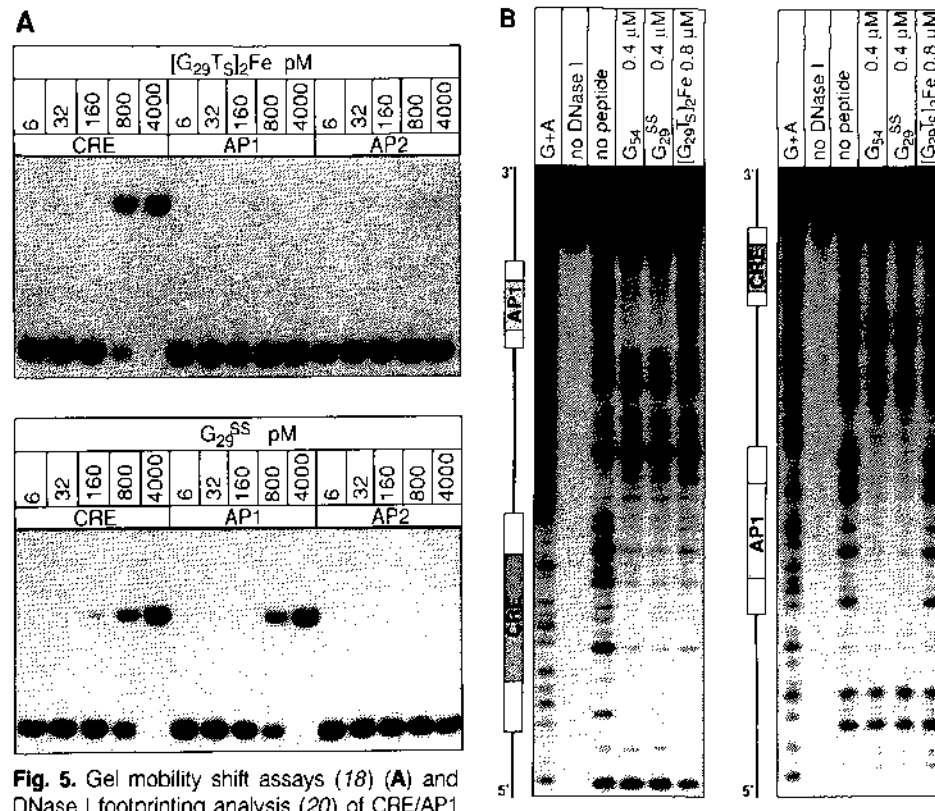


Fig. 5. Gel mobility shift assays (*18*) (**A**) and DNase I footprinting analysis (*20*) of CRE/AP1 discrimination (**B**). The DNase I footprint of the CRE and AP1 sites by $[G_{29}T_S]_2Fe$, G_{54}, and G_{29}^{SS}. The DNA used contains the 10-bp CRE site between positions 21 and 30 and the 9-bp AP1 site between positions 57 and 65. Left, DNA labeled with ^{32}P at the 5′ end of the + strand; right, DNA labeled with ^{32}P at the 5′ end of the − strand.

peptide bound $AP2_{23}$ (5′-AGTGGAGAT-GATTCATCTCGTGC-3′), an AP1-like DNA containing a C to T mutation that is not tolerated by GCN4 in vitro (*25*). DNase I footprint experiments performed with a DNA fragment containing both CRE and AP1 sites revealed a footprint only at the CRE site in the presence of $[G_{29}T_S]_2Fe$, whereas footprints were observed at both sites when G_{54} or $G_{29}{}^{SS}$ were added (Fig. 5B). Finally, $[G_{29}T_S]_2Fe$ displayed no increase in helical structure in the presence of $AP1_{15}$ (5′-GAGATGAC-TCATCTC-3′) (Fig. 4C). A mobility shift competition assay was performed to quantify the differential binding properties of $[G_{29}T_S]_2Fe$ (*17*, *18*, *26*). The preference for CRE_{24} over $AP1_{23}$ exhibited by $[G_{29}T_S]_2Fe$ corresponded to a differential binding free energy ($\Delta\Delta G^{\circ}_{obs}$) of >4 kcal mol^{-1} (*17*). Although $[G_{29}T_S]_2Fe$ contains the basic and linker domains of GCN4, it had a 1600-fold preference for the CRE site, mimicking the sequence-selectivity of the CREB/ATF subfamily. These results demonstrate that the orientation and the relative displacement with which the basic domains emerge from the central dimerization interface are important in controlling CRE affinity and CRE/AP1 selectivity (*27*).

Transcriptional regulation in eukaryotes involves a finely tuned interplay of proteins with a complex set of DNA target sites. A fundamental issue in the study of gene regulation is how regulatory proteins differentiate nearly identical sites with high precision. Here we demonstrate that substitution of the GCN4 coiled coil with a stereochemically well-defined metal complex generates a molecule capable of differentiating DNA sequences that GCN4 cannot. $[G_{29}T_S]_2Fe$ contains the GCN4 basic and linker domains, yet it displays the sequence selectivity of the CREB-ATF subfamily. The bis(terpyridyl)iron(II) dimerization domain modifies the inherent selectivity of the GCN4 basic domain without altering its sequence.

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17. G_{29} contains the sequence Ac-SAALKRARN-TEAARRSRARKLQRMKQGGC-NH_2. All peptides and Fe(II) complexes were characterized by amino acid analysis, mass spectroscopy, UV/VIS spectroscopy, and denaturing polyacrylamide gel electrophoresis [B. Cuenoud and A. Schepartz, *Tetrahedron Lett.* **28**, 3325 (1991); *Proc. Natl. Acad. Sci. U.S.A.*, in press]. Abbreviations for the amino acids are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
18. M. Fried and D. M. Crothers, *Nucleic Acids Res.* **9**, 6505 (1981); M. M. Garner and A. Revzin, *ibid.*, p. 3047. DNA was 5′ end-labeled with polynucleotide kinase [T. Maniatis, E. F. Fritsch, J. Sambrook, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, ed. 2, 1987)] and annealed to an unlabeled complementary oligonucleotide. Peptide concentrations were determined by amino acid analysis with norleucine and homoserine as internal standards. DNA concentrations were determined using an extinction coefficient at 260 nm calculated as described [G. Fasman, Ed., *Handbook of Biochemistry and Molecular Biology, Vol. 1: Nucleic Acids* (CRC Press, Cleveland, OH, ed. 3, 1975)]. Unless stated otherwise, binding reactions were performed by incubating the indicated peptide with <50 pM labeled, double-stranded DNA in a final reaction mixture containing 20 mM tris-Cl (pH 7.4), 4 mM KCl, 2 mM $MgCl_2$, 10 mM EDTA, 0.1% NP-40, and 5% glycerol (final volume of 10 μl) for 5 min at 4°C. Free and peptide-bound labeled DNA were resolved by nondenaturing 5% (80:1) polyacrylamide gel electrophoresis in 10 mM tris-Cl at 4°C. Radioactivity was quantified using a Betascope 605 Blot Analyzer. For lanes containing $G_{29}T_S$ and metal ions, $(NH_4)_2FeSO_4$ or $CuSO_4$ at a final concentration of 38 μM was added to 10 μM $G_{29}T_S$ before dilution into a buffer containing 100 μM terpyridine. The K_d was obtained by fitting the binding data to the theoretical binding equation $F_E = \{[G_{29}T_S]_2Fe)_T/\{K_d + ([G_{29}T_S]_2Fe)_T\}$. F_E is equal to (bound CRE_{24})/(bound CRE_{24} + free CRE_{24})] with a nonlinear least-squares fitting program (KaleidaGraph 2.1.2). The relative standard error of estimate (S_y) = $100[\Sigma(F_T - F_E)^2/n]^{0.5}/(F_E)$ where F_T and F_E are the fractions CRE_{24} bound as determined from theory and experiment, respectively, and n is the number of data points [J. L. Devore, *Probability and Statistics for Engineering and the Sciences* (Brooks/Cole, Monterey, CA, 1987)].
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21. The CD spectra of $[G_{29}T_S]_2Fe$ and $G_{29}{}^{SS}$ were identical between 200 and 600 nm in the absence of DNA, indicating that under these conditions the terpyridyl moiety did not contribute significantly to the CD spectrum. Although we cannot rule out a small contribution to the CD by the DNA-bound terpyridyl moiety, we note that the spectra calculated for CRE_{16}-complexed $[G_{29}T_S]_2Fe$ and CRE_{16}-complexed $G_{29}{}^{SS}$ had similar shapes.
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24. The Jun homodimer and the Jun-Fos heterodimer induce directed bends in DNA containing an AP1 site [T. K. Kerppola and T. Curran, *Cell* **66**, 317 (1991); *Science* **254**, 1210 (1991)]. GCN4 induces little or no bending in DNA containing an AP1 site [M. R. Gartenberg, C. Ampe, T. A. Steitz, D. M. Crothers, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 6034 (1990)]. We have not determined whether $[G_{29}T_S]_2Fe$ bends DNA.
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26. To a mixture of unlabeled $AP1_{23}$ or CRE_{24} (55 pM to 40 μM) and labeled CRE_{24} (<7.5 pM) was added 4 nM $[G_{29}T_S]_2Fe$ in a final reaction mixture containing 20 mM tris-Cl (pH 7.4), 4 mM KCl, 2 mM $MgCl_2$, 10 mM EDTA, 0.1% NP-40, 5% glycerol (final volume 10 μl). After a 1-hour incubation at 4°C, free and peptide-bound labeled CRE_{24} were resolved by gel electrophoresis (*18*).
27. Results similar to those described here were obtained with a series of analogous Fe(II) complexes lacking the Gly-Gly-linker (B. Cuenoud and A. Schepartz, in preparation).

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