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.

REFERENCES AND NOTES

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 ≤ 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 ± 0.4261 ± 1.1, 7.3 ± 0.5526 ± 0.9; SR1mm, 6.7 ± 0.0272 ± 1.2, 7.6 ± 0.3276 ± 0.9; 1-methyl-4-d 7.1 ± 0.4273 ± 1.1, 7.3 ± 0.6152 ± 0.1; 2-mtl, 5.9 ± 0.3273 ± 1.1, 6.4 ± 0.4249 ± 0.8; 3-mtl, 6.3 ± 0.3219 ± 1.9, 7.1 ± 0.5272 ± 1.5. Plants subsequently receiving NaCl treatment: SR1, 7.1 ± 0.4273 ± 2.0, 7.4 ± 0.2826 ± 1.3; SR1mm, 9.4 ± 0.3430 ± 1.1, 7.1 ± 0.3255 ± 1.0; 1-methyl-4-d 7.4 ± 0.3246 ± 1.4, 7.2 ± 0.3238 ± 1.0; 2-mtl, 6.8 ± 0.3273 ± 1.4, 6.6 ± 0.3274 ± 0.7; 3-mtl, 7.3 ± 0.4263 ± 1.6, 7.1 ± 0.2827 ± 0.9. Values are mean ± SEM; for number of plants evaluated; see Tables 1 and 2.
16. For lines that express 35S miTD, 30 primary transformants were chosen randomly and self-pollinated, and the progeny were selected for single-focus 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 protocol was also used for control SR1mm, except that five primary transformants were chosen. Lines analyzed were SR1, wild type; SR1mm, kanamycin-resistant; and 1-mtl, 2-mtl, and 3-mtl, three different kanamycin-resistant lines that express 35S miTD.
17. The hydroponics system consisted of two genetically identical plants cultured in containers with half-strength Hoagland's nutrient solution (5 litters). (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 experimental bias, evaluations were performed blindly. Growth-room conditions were 18° and 25°C, night and day temperatures, respectively; photoperiod, 12 hours light (500 µE 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.

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-
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 G29 peptides in bis(terpyridyl)iron(II) complexes [G29T2]Fe, [G29T1]Fe, and [G29T2]Fe (17) (Fig. 1). In [G29T2]Fe, the 4'-substituted terpyridyl T2 separated the two G29 carboxamide termini by approximately 11 Å and oriented them at a 180° angle. In [G29T1]Fe, the 4-substituted terpyridyl T1 separated the carboxamide termini by approximately 8 Å and oriented them at a 90° angle. The 4'-substituted terpyridyl T2 enforced the same geometry as T2 but contained a longer and more flexible tether. The ability of these stereochemically defined synthetic scaffolds to organize two G29 peptides into a functional DNA-binding protein was determined by monitoring the DNA binding affinities of the resultant complexes. The disulfide dimer of G29 (G29-SS) (13) and a 54-amino acid peptide containing both the DNA contact and dimerization domains of GCN4 (G29) were studied for comparison.

The binding of [G29T2]Fe complexes to duplex DNA was measured first by gel mobility shift assay (18). Incubation of CRE24a, a 5' end-labeled double-stranded DNA fragment (5'-AGTGGAGATGCCTCAGTCATCTCGTGTCG-3') containing the high affinity GCN4 binding site CRE (ATGACCTCAT; cAMP response element) (19), with 0.13 to 1.2 nM [G29T2]Fe resulted in the gradual appearance of a complex with lower electrophoretic mobility than CRE24 alone or a CRE24-G29-SS complex (Fig. 2A). Monomeric G29T2 failed to bind CRE24, but binding was restored upon addition of Fe(II). Addition of Cu(II) did not result in DNA binding. The affinity of [G29T2]Fe for CRE24 was determined by measuring the fractions of bound and free CRE24 at several [G29T2]Fe concentrations (Fig. 3). The data fit a theoretical equation that describes formation of a 1:1 complex with an equilibrium dissociation constant (Kd) of 0.13 ± 0.01 nM (18), a value comparable to the Kd measured for the G24 homodimer and the Fox-Jam heterodimer at this temperature (11).

DNA binding by [G29T2]Fe was characterized further by deoxyribonuclease I (DNase I) footprinting (20) and circular dichroism (CD) spectroscopy. [G29T2]Fe and G24 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). [G29T35]Fe 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 CRE16 (5'-GAGATGACGT-CATCTC-3') was added (Fig. 4A). The difference spectrum (Fig. 4B) indicated that [G29T35]Fe was almost entirely helical when bound to CRE16 (21). These results demonstrate that a metal ion complex of 612 daltons can replace the function of the leucine zipper, effectively orienting two G29 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 [G29T35]Fe, [G29T35]T1,Fe, and [G29T35]B,Fe. Although these metal complexes are structurally similar, neither [G29T35]T1,Fe nor [G29T35]B,Fe 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 [G29T35]T1,Fe nor [G29T35]B,Fe yielded a detectable DNase I footprint (Fig. 2B) at 4 μM nor reduced the extent of CRE:[G29T35]Fe complex formation in a mobility shift competition assay. The only evidence supporting DNA binding by [G29T35]T1,Fe or [G29T35]B,Fe was obtained in CD experiments performed at a concentration of 10,000 times the Kd of the CRE:[G29T35]Fe 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:[G29T35]Fe complex is 4 kcal mol⁻¹ more stable than either the CRE:[G29T35]T1,Fe or the CRE:[G29T35]B,Fe complex. The spacing of the basic domains in [G29T35]Fe and [G29T35]T1,Fe differs by only four methylene groups, while [G29T35]Fe and [G29T35]B,Fe differ only in the orientation with which the basic domains extend into the major groove. Thus, coordination complex architecture is important in orienting the G29 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 (ATGACGT CAT) (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 API23 (5'-AGTGGAGATGAC-TCATCTCGTG-3') and [G29T35]Fe, even at elevated concentrations (Fig. 5A), whereas G58 bound CRE14 and AP123 with comparable affinity (Fig. 5B).
peptide bound AP2, 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 API sites revealed a footprint only at the CRE site in the presence of [GATA][GAGT][ATC] sequences, whereas footprints were observed at both sites when G35 or G35 were added (Fig. 3B). Finally, [GATA][GAGT][ATC] displayed no increase in helical structure in the presence of API5, (5'-GAGTACGAC- TACCTC-3') (Fig. 4C). A mobility shift competition assay was performed to quantify the differential binding properties of [GATA][GAGT][ATC] to (17, 18, 26). The preference for CREs over API3, exhibited by [GATA][GAGT][ATC] corresponded to a differential binding free energy (DΔG°) of >4 kcal mol−1. Although [GATA][GAGT][ATC] contains the basic and linker domains of GCN4, it had a 1600-fold preference for the CRE site, indicating the sequence specificity of CREB/ATF subfamily. These results demonstrate that the structure 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 differentially activate nearby identical sites with high precision. Here we demonstrate that substitution of the GCN4 coiled coil with a stereocchemically well-defined metal complex generates a molecule capable of differentiating DNA sequences that GCN4 cannot. [GATA][GAGT][ATC] 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.

REFERENCES AND NOTES

18. δG is the sequence on AC-SALKRARK-TEAARRSRKLGRWAKOGCGG-NH2. All peptides and Fe(II) complexes were characterized by amino acid analysis, mass spectrometry, UV/Vis spectrophotometry, and denaturing polyacrylamide gel electrophoresis (邹 Cuenoud and A. Schepartz, Tetrabedlon Lett. 23, 3235 (1991); Proc. Natl. Acad. Sci. U.S.A. 89, 2007 (1992)).
19. M. Fried and D. M. Crothers, Nucleic Acids Res. 9, 6505 (1981); M. M. Garner and A. Revzin, ibid., p. 3047. For a review, see D. D. Goldfarb and J. A. Spring (Harb), New, ed. 2, 1987) and annulated to an unlabelled complementary oligonucleotide. Peptide concentrations were determined by amino acid analysis with normalization to homoserine as internal standards. DNA concentrations were determined using an extinction coefficient at 260 nm calculated as described (G. Farsaan, 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 MgCl2, 10 mM EDTA, 0.1% NP-40, and 5% glycerol (final volume of 5 μl) for 5 min at 4°C. Free and peptide-bound labeled DNA was separated by 10% polyacrylamide gel electrophoresis in 10 mM tris-Cl at 4°C. Radioactivity was quantified using a Molecular Dynamics' 605 Biolayer Analyzer. For lanes containing GATA3 and metal ions, (NH4)2FeSO4 or CuSO4 at a final concentration of 38 μM was added to 10 mM GATA3 before dilution into a buffer containing 100 μM terpyridine. The [Fe2] was obtained by fitting the binding data to the theoretical binding equation Fb = (Fe2)(GATA3)(Fe2)(GATA3) + (Fe2)(GATA3) + (bound CREb + free CREb) with a nonlinear least-squares fitting program (KaleidaGraph 2.1.2). The relative standard error of estimate (SSE) was calculated using the formula: SSE = (100%Fb − 100%)2(Fb)/(Fb).