Mutational Analysis of the DNA-Binding Domain of the CYS3 Regulatory Protein of *Neurospora crassa*

MOIEN N. KANAAN AND GEORGE A. MARZLUF*

Departments of Molecular Genetics and Biochemistry, The Ohio State University,
484 West 12th Avenue, Columbus, Ohio 43210

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***cys-3*, the major sulfur regulatory gene of *Neurospora crassa*, activates the expression of a set of unlinked structural genes which encode sulfur catabolic-related enzymes during conditions of sulfur limitation. The *cys-3* gene encodes a regulatory protein of 236 amino acid residues with a leucine zipper and an upstream basic region (the b-zip region) which together may constitute a DNA-binding domain. The b-zip region was expressed in *Escherichia coli* to examine its DNA-binding activity. The b-zip domain protein binds to the promoter region of the *cys-3* gene itself and of *cys-14*, the sulfate permease II structural gene. A series of CYS3 mutant proteins obtained by site-directed mutagenesis were expressed and tested for function, dimer formation, and DNA-binding activity. The results demonstrate that the b-zip region of *cys-3* is critical for both its function in vivo and specific DNA-binding in vitro.**

The control of sulfur metabolism in *Neurospora crassa* requires the expression of a set of unlinked structural genes which encode sulfur catabolic-related enzymes (8). The synthesis of these enzymes, which include choline sulfatase, aryl sulfatase, sulfate permease, and a methionine-specific permease, occurs when sulfur is limited and is controlled by at least two regulatory genes. The *scon* gene (for sulfur controller) acts in a negative fashion, turning off sulfur-related enzymes under sulfur repression (3). The *cys-3* regulatory gene acts in a positive fashion, turning on the sulfur-related enzymes during sulfur-limited conditions (3, 17). The two regulatory genes appear to act in a sequential fashion: *scon* negatively controls the expression of *cys-3*; when sulfur becomes limited, *cys-3* is expressed and, in turn, activates the expression of the sulfur-related enzymes (14). In agreement, *scon* mutants show a constitutive expression of *cys-3* message, regardless of the sulfur condition (3). *cys-3* mutants, on the other hand, show a pleiotropic effect and lack all sulfur-related enzymes (3, 14). Detailed studies of *cys-3* mutants, including null and temperature-sensitive mutants, suggest that *cys-3* gene specifies a regulatory protein that is required for expression of the sulfur-related enzymes, presumably by binding a DNA element(s) upstream of each structural gene (7, 17). DNA footprinting and mobility shift analysis showed that an *Escherichia coli*-expressed CYS3 protein binds specifically to 5' upstream DNA sequences of *cys-14* (sulfate permease gene) and the *cys-3* gene itself (7, 8).

The CYS3 protein shows considerable homology with the yeast GCN4, the *Neurospora* CPC1, and the mammalian Fos, Jun, and C/EBP proteins (7). This family of proteins contains a common DNA-binding motif that consists of two regions (11); one region facilitates dimer formation, and the other contacts the DNA. The dimer-forming region, termed the leucine zipper, contains four to five leucine residues in a heptad arrangement within amphipathic alpha helices which adhere tightly to one another in a coiled-coil structure via their hydrophobic surfaces (11). The DNA contact region has a characteristic sequence rich in basic amino acids

residues and thus is termed the basic region (11, 12). This basic region exhibits a consensus of 16 residues that starts exactly 7 residues NH₂ terminal to the first leucine of the zipper. The alpha-helical surfaces of the basic region have been postulated to bifurcate like the arms of a Y (12, 21). The juxtaposition of these arms forms two tightly linked DNA contact surfaces, with the arms of the Y tracking in successive major grooves of DNA (9, 21). Recent studies suggest that the basic regions lack a defined structure and only alpha helices form in the presence of DNA (15, 18).

The CYS3 protein contains a well-defined leucine zipper of four leucines and one methionine and an NH₂-terminal basic region (Fig. 1). Here we report experiments in which the putative b-zip domain of the CYS3 protein was expressed in *E. coli*. Mobility shift experiments show that the b-zip domain can alone mediate DNA binding with an affinity that is equivalent to that of the full-length CYS3 protein. To investigate the character of the *cys-3* DNA-binding domain, site-directed mutagenesis was used to alter amino acid residues within this domain of the protein. Our results demonstrate that both the leucine zipper and the basic region are essential for the function of *cys-3* in vivo and for its sequence specific DNA-binding activity in vitro.

MATERIALS AND METHODS

Strains. The *N. crassa* wild-type and *cys-3* mutant (allele P22) strains were obtained from the Fungal Genetics Stock Center (University of Kansas Medical Center). Mycelia were grown in Vogel's liquid medium with shaking at 30°C as described previously (6).

Site-directed mutagenesis. Site-directed mutagenesis of the cloned *cys-3* gene (7) was carried out by the method of Kunkel (10). Dideoxy sequencing was carried out to confirm the desired changes.

Transformation. Competent *E. coli* cells were prepared by calcium treatment and transformed according to standard techniques (4). *N. crassa* transformation was carried out by the Novozyme 234 spheroplasting technique (22).

Protein expression. Expression of the mutant CYS3 proteins was carried out by replacing a 160-bp *StuI-XhoI* DNA fragment of the *cys-3* gene in the PC3T71 expression vector

* Corresponding author.

FIG. 1. Leucine zipper of the CYS3 protein, comprising amino acids residues from Arg-89 to Gly-164. The leucine residues and the single methionine residue which constitute the heptad repeat are circled. Basic amino acids in the charged region upstream of the leucine zipper are boxed. Asterisks identify the two basic amino acids which are replaced by glutamine in the conventional CYS3 mutant protein. Arrows identify the hydrophobic amino acids residues that may contribute to the amphipathy created by the leucine repeat.

(7) with the corresponding DNA fragment which contained each mutational alteration. To express a *cys-3* DNA fragment containing just the leucine zipper motif (the b-zip region), two *Bam*HI sites flanking the b-zip region were introduced into the *cys-3* gene and a 200-bp *Bam*HI DNA fragment corresponding to the leucine zipper motif was cloned into the pET3b vector. Dideoxy sequencing confirmed the identity of these mutants in the expression vector. The expression plasmids harboring the *cys-3* mutants and the b-zip region were transformed into *E. coli* BL21(DE3) (pLysS) for protein expression. The expressed proteins were recovered and enriched as described previously (7).

Gel band mobility shift experiments. Radioactive DNA fragments containing both the *cys-3* and *cys-14* promoter elements were prepared by fill-in with the Klenow fragment of DNA polymerase (13). A synthetic 27-mer oligonucleotide and its complement were synthesized and hybridized to form a double-stranded oligonucleotide of 25 bp with the sequence ATGT TCGCTGATG CCATTCATTGAT with unpaired bases, GC, at both 5' ends. Mobility shift experiments using the in vivo-expressed proteins were carried out as described previously (7) except that the gels were run in 0.25× Tris-borate-EDTA buffer without circulation.

DNA-binding affinity (off-rate analysis). The procedure of Fried and Crothers (5) was used to determine the dissociation rates of both CYS3-DNA and b-zip-DNA complexes. The complexes were generated by incubating 1.0 pmol of ³²P-labeled synthetic *cys-14* site 1 DNA (7) and 0.3 μmol of the *E. coli*-expressed protein in a 25-μl reaction mixture containing 2.0 μg of poly(dI-dC), 12 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 4 mM Tris-HCl (pH 7.9), 50 mM KCl, 1 mM EDTA, 1 mM dithiothre-

itol, 0.3 mg of bovine serum albumin per ml, and 10% glycerol. Reaction mixtures were incubated for 40 min at 25°C (a time course DNA-binding analysis had shown that the binding had reached equilibrium by this time [data not shown]), when a 600-fold molar excess of nonradioactive *cys-14* site was added. Equal portions were analyzed by band shift assay as a function of time after addition of the unlabeled competitor DNA. The intensities of bands were quantified with a Betascope 603 analyzer (Betagen). The percentage of the logarithmic counts per minute remaining was calculated and plotted as a function of time.

Cross-linking experiments. Glutaraldehyde cross-linking was used to determine whether various CYS3 proteins formed dimers. Expression plasmids (pET vectors) harboring the wild-type and *cys-3* mutant genes and the b-zip domain coding sequence were in vitro transcribed by using T7 RNA polymerase (Stratagene Inc.). The resulting RNAs were translated in vitro, using rabbit reticulocyte extract (Promega) in the presence of L-[³⁵S]methionine according to the manufacturer's instructions. The crude translation reaction was either used directly or purified by heparin-Sepharose chromatography (to remove the heme) and then subjected to glutaraldehyde cross-linking. An aliquot of the translated protein (15,000 cpm) was incubated with 0.01% glutaraldehyde at 4°C in a final volume of 15 nl for 60, 90, and 120 min. The reaction was stopped with ethanolamine at a final concentration of 20 mM. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed the presence of a cross-linked wild-type CYS3 protein dimer, whereas higher-molecular-weight species were not detected even in the presence of excess glutaraldehyde or after prolonged incubation. A more detailed analysis of dimerization will be reported elsewhere.

RESULTS

Expression of the CYS3 protein. The wild-type and mutant *cys-3* genes and the internal region encoding the b-zip domain were each cloned into the expression vector pET3b and transformed into *E. coli* as described in Materials and Methods. Each of these genes was expressed at high levels to give a protein of the expected size, representing the major protein in the bacterial extracts (Fig. 2). *E. coli* cells that contained the expression vector but lacked the *cys-3* gene did not produce any of this protein. The b-zip domain protein was readily visible at the expected size of 12 kDa.

Functional analysis of the *cys-3* b-zip domain. The CYS3 protein appears to possess a DNA-binding domain which consists of a leucine zipper and a highly basic region located

FIG. 2. Expression of CYS3 wild-type and mutant proteins in *E. coli*. Plasmids were constructed and the protein was expressed, extracted, and partially purified as described in Materials and Methods. The protein extracts were run in a 12% polyacrylamide-sodium dodecyl sulfate gel and stained with Coomassie blue. The positions (in kilodaltons [K]) of molecular size markers are shown. Lanes: 1, expression vector only; 2, with the *cys-3* gene; 3 to 16, with *cys-3* mutants M1 to M14; 17 to 22, with *cys-3* mutants B1 to B6; 23, with the b-zip domain. The expressed CYS3 protein is identified with arrows.

TABLE 1. Summary of results obtained mutant CYS3 proteins^a

Construct	Function	DNA-binding activity		Dimerization
		<i>cys-3</i> promoter	<i>cys-14</i> promoter	
Leucine zipper alteration				
Wild type	L---M---L---L---L	+	+	+
Mutant				
M1	-----K-----	-	-	ND
M2	-----V-----	+	+	+
M3	-----V-----	+	-/+	+
M4	---V-----	-	-/+	+
M5	V-----	-	-	+
M6	-----V---V-----	-	-	-
M7	---V---V-----	-	-	-
M8	---V-----V-----	-	-	-
M9	---L-----	+	+	+
M10	-----M-----	+	+	+
M11	-----M-----	+	+	+
M12	-----Q-----	+	+	ND
Spacing alteration				
Wild type	M---S-E-K-V-T-Q---L	+	+	+
Mutant				
M13	M---S-E-K-V*-Q---L	-	-	+
M14	M---S-E-K-V-Q-S---L	+	+	+
Basic region alteration				
W+	K-R-K-R-N-T-A-A-S-A-R-F-R-I-K-K-K-Q-R-E-Q	+	+	+
B1	---G-Q-----	-	-	+
B2	-----Q-Q-Q-----	-	-	+
B3	-----Q-Q-----	-	-	+
B4	-----Q-----	-	-	+
B5	-----Q-----	-	-	+
B6	---basic region---GAATTC---leucine zipper	-	-	+

^a Function in vivo was assayed via transformation, and mobility shift and cross-linking experiments were conducted in vitro to assess DNA-binding and dimerization, respectively. The identities and locations of the amino acid substitutions in each of the *cys-3* mutants are shown under the wild-type sequence. ND, not determined.

five amino acid residues N terminal to the leucine repeat (Fig. 1). Highly conserved amino acid residues in these two regions of CYS3 were changed by site-directed mutagenesis to assess whether these amino acids are required for CYS3 function in vivo and for DNA-binding activity in vitro. Table 1 shows the amino acid substitutions in each of the CYS3 mutants, all of which we made with mutant oligonucleotide primers as described previously (10). These mutants were each introduced into an otherwise wild-type *cys-3* gene as described in Materials and Methods.

Each mutant was tested for function in vivo by transformation into a *cys-3* mutant in parallel with a wild-type *cys-3* gene as a positive control. In addition, each mutant CYS3 protein was expressed in *E. coli* (Fig. 2) and examined for its ability to dimerize and for DNA-binding activity in vitro with a band shift assay. Two different ³²P-labeled DNA fragments were used in the mobility shift assays. One was a 360-bp fragment of the *cys-3* gene promoter region which contains a CYS3-binding site; the second DNA probe was a 27-bp synthetic oligonucleotide which contains the CYS3 recognition site present in the promoter region of the *cys-14* structural gene. These two recognition elements have been previously identified as target sites for the CYS3 protein (7). The results of the transformation, dimerization, and DNA-binding experiments are summarized in Table 1 and described below.

Analysis of the basic region. Substitution of glutamine (or glycine) residues for three (mutant B2) or two (mutants B1 and B3) basic amino acids within the basic region abolished *cys-3* function in vivo (Table 1). We then tested the effect of replacing a single basic amino acid in this region with

glutamine (mutants B4 and B5) and found that a change of even one basic residue resulted in a loss of *cys-3* function in vivo. Moreover, the expressed CYS3 protein corresponding to each of these basic region mutants was found to dimerize but to be completely deficient in DNA binding to both the *cys-3* and the *cys-14* recognition elements (Fig. 3). These results support the concept that the basic region constitutes a DNA contact surface and that substitution of even a single amino acid within it can entirely eliminate its DNA-binding activity. It is also clear that these basic region CYS3 mutant proteins readily dimerize, indicating that dimer formation is determined by the zipper but does not require a functional basic region.

Analysis of the leucine zipper. The CYS3 protein contains a heptad repeat motif that appears to constitute a leucine zipper, although its second position is occupied by methionine (Fig. 1). A mutant in which this methionine was replaced with leucine, yielding a pure leucine zipper, was fully functional (Table 1). The effects of amino acid substitutions within the putative zipper region were found to depend on the position within the zipper and the nature of each substitution, some replacements being compatible with function and others eliminating *cys-3* function (Table 1). For example, the change in the zipper of any single leucine to methionine gave a functional CYS3 protein, comparable with the wild-type CYS3 protein. Interestingly, valine substitution for leucine at residue 1 (mutant M5) or for methionine at residue 2 (mutant M4) is more deleterious than is a valine substitution at position 3 (mutant M3) or 4 (mutant M2) of the zipper, which suggests that individual positions within the zipper heptad repeat are not of equal importance.

FIG. 3. Gel band mobility shift experiments with the 5'-flanking DNA fragments of the *cys-14* gene and the *cys-3* gene. 32 P-labeled DNA fragments were incubated with protein extracts and subjected to polyacrylamide gel electrophoresis as described in Materials and Methods. (A) Analysis with the 27-bp synthetic oligonucleotide containing the *cys-14* recognition element; (B) analysis with the *SalI-XbaI* 5' *cys-3* DNA fragment. Lanes: 1, free probe; 2, protein expressed in cells with vector only; 3, expressed CYS3 null mutant as a negative control; 4, expressed wild-type CYS3 protein; 5, expressed b-zip domain; 6 to 10, CYS3 mutant proteins B1 to B5; 11, CYS3 mutant protein M12; 12 to 14, CYS3 mutant proteins M9 to M11; 15, CYS3 mutant protein B6; 16 and 17, CYS3 mutant proteins M13 and M14. Arrows identify the shifted bands.

For example, the leucine at position 1 in the zipper is more important functionally than is the leucine at position 3 or 4. A valine substitution at the fourth position in the zipper results in an active CYS3 protein; however, a more drastic substitution of arginine for leucine 4 led to loss of function. Mutants M6, M7, and M8, in which two leucines of the zipper were replaced by valine, lack *cys-3* activity.

The results of DNA-binding assays for CYS3 proteins mutated in the zipper region correlated perfectly with their functional activity in vivo. Thus, CYS3 proteins which had a pure leucine zipper, or in which methionine residues replaced individual leucines of the zipper, showed wild-type DNA-binding activity for both the *cys-3* and *cys-14* promoter elements, in agreement with their wild-type function in vivo (Fig. 3). Similarly, mutants in the zipper heptad which had two valine substitutions or a single drastic lysine replacement which lack function in vivo showed a complete loss of DNA binding in vitro (Fig. 3). These mutant proteins failed to form dimers, which clearly precludes any DNA-binding ability. DNA binding by the b-zip class of proteins requires dimerization and also depends on the proper positioning of the two basic regions.

Intriguing results were obtained with CYS3 proteins containing single valine substitutions in the zipper motif. Figure 4 shows the results of DNA-binding assays with these mutant CYS3 proteins with both the *cys-3* and the *cys-14* promoter elements. A CYS3 protein with valine replacing leucine at zipper position 4 (mutant M2) displayed DNA-

FIG. 4. Gel band mobility shift experiments. (A) Shift with the defined oligonucleotide containing the *cys-14* recognition element; (B) shift with the *cys-3* *SalI-XbaI* 5' DNA fragment. Protein extracts from CYS3 mutant proteins M1 to M8 were each incubated with 32 P-labeled DNA fragments and subjected to polyacrylamide electrophoresis as described in Materials and Methods. Lanes: 1, expressed wild-type CYS3 protein; 2 to 9, expressed CYS3 mutant proteins M1 to M8. Arrows identify the shifted bands.

binding activity with both the *cys-3* and the *cys-14* binding elements. However, CYS3 proteins with valine at either zipper position 2 or 3 (mutants M3 and M4) showed DNA binding with the *cys-3* promoter fragment but not the *cys-14* fragment, consistent with previous findings that CYS3 has a higher affinity for its own promoter than it does for the *cys-14* element. A CYS3 protein with valine replacing leucine at zipper position 1 failed to bind to either the *cys-3* or the *cys-14* DNA promoter fragment. Since all of these mutant CYS3 proteins with a single valine substitution dimerize (Table 1), it appears that the loss or reduction in DNA-binding ability reflects a failure to properly position the basic region.

Spacing alteration in the b-zip region. The importance of exact periodicity of the leucine repeat was tested by deletion of a threonine residue which occurs between the methionine (second position) and the leucine in the third position of the zipper (mutant M13; Table 1). The loss of this single amino acid would be expected to change the relative position of the leucine residues in the zipper such that the amphipathic alpha helix would be disturbed since the repeated leucines would not all lie on the same surface. This spacing mutant (M13) results in a protein that lacks any DNA-binding activity (Fig. 3) and fails to transform in vivo. To demonstrate that the threonine residue itself was not functionally important, but rather that the precise spacing within the zipper region was crucial, a different amino acid (serine) was added to reestablish the heptad repeat but at a different position (mutant M14; Table 1). This mutant protein bound both the *cys-3* and *cys-14* promoters in vitro and also was functional in vivo in the transformation assay.

It was also of interest to determine whether the spacing of five amino acids between the zipper motif and the upstream basic region that constitutes the actual DNA-binding surface was critical or whether a different spacing could be tolerated between these two regions of the protein. This question was addressed with mutant B6, which has a wild-type leucine zipper and a wild-type basic region but has an additional two amino acids inserted between these regions (Table 1). The B6 mutant CYS3 protein is nonfunctional in vivo and also lacks any DNA-binding activity (Fig. 3, lanes 15), although it does dimerize to a normal extent.

DNA binding by the b-zip domain. An important question is whether the DNA-binding activity of CYS3 resides entirely in the b-zip region of the protein or depends on tertiary interactions with other parts of the protein. To address this question, a protein of only 88 amino acid residues corresponding just to the CYS3 b-zip DNA-binding domain was expressed in *E. coli* and partially purified as described in Materials and Methods. Mobility shift experiments revealed that this small b-zip domain protein alone binds to both the *cys-3* and the *cys-14* promoter DNA elements (Fig. 3, lanes 5). Competition experiments revealed that this binding by the b-zip domain protein was sequence specific (results not shown).

The DNA-binding affinity of the entire CYS3 protein was then compared with that of the smaller b-zip domain protein by a quantitative mobility shift experiment. Each protein was incubated with a ^{32}P -labeled target DNA sequence, and a nonradioactive competitor DNA was added; at different times, samples of each were run in standard mobility shift gels in order to estimate the rate at which the initial DNA-protein complex dissociated. This analysis revealed that the full-length CYS3 protein and the much shorter b-zip domain protein have nearly identical half-lives for dissociation of their respective DNA-protein complexes (Fig. 5).

DISCUSSION

cys-3, a positive-acting control gene, encodes a regulatory protein that activates the expression of a set of unlinked structural genes which encode sulfur catabolic-related enzymes. The CYS3 protein consists of 236 amino acids and appears to contain a leucine zipper and an upstream basic region (b-zip region) which together constitute a DNA-binding domain (8). Here we report that a protein corresponding to only the CYS3 b-zip domain binds to both the *cys-3* and *cys-14* promoter DNA elements and, moreover, that the full-length CYS3 protein and the much smaller b-zip domain protein have indistinguishable DNA-binding affinities. These results imply that the DNA-binding activity of the CYS3 protein resides entirely in the b-zip region of the protein and that DNA binding requires no tertiary interaction with other parts of the protein. Such a conclusion agrees with results obtained with other b-zip proteins that show nearly identical affinities and specificities for DNA binding with the full-length proteins and fragments encompassing just their b-zip domains (2).

The leucine repeat region has been proposed to exist in an alpha-helix conformation with the leucine side chains projecting out at regular intervals. The amphipathy created by the leucine repeat allows the coiled-coil dimerization of two monomers and also positions the NH_2 -terminal basic region in the precise orientation to interact specifically with DNA (9, 15, 16, 18, 19). To assess the functional importance of highly conserved amino acids residues in both the leucine zipper and the upstream basic region, site-directed mutagenesis was used to obtain specific CYS3 mutant proteins.

Amino acids substitutions in the basic region were selected to affect residues which might be important for function; each such substitution did indeed abolish both the function of *cys-3* in vivo and its DNA-binding ability in vitro. In addition, the recent cloning and sequencing of a conventional *cys-3* mutant have provided insight concerning the importance of the basic region in function of the CYS3 protein. Two nucleotide changes in the mutant resulted in the replacement of lysine 105 and arginine 106 by glutamine residues (Fig. 1). This mutant CYS3 protein was expressed

FIG. 5. Off-rate analysis of both the b-zip-DNA and the CYS3-DNA complexes formed on a defined oligonucleotide containing the *cys-14* recognition element. (A) Off-rate analysis of the b-zip-DNA complex; (B) off-rate analysis of the CYS3-DNA complex. Complexes were formed as described in Materials and Methods. A 600-fold molar excess of nonradioactive *cys-14* site was added, and samples were analyzed by electrophoresis after the addition of the competitor at times indicated above the lanes. Each sample was loaded in the gel as soon as it was taken. As a result, samples were subjected to different times of electrophoresis, leading to small differences in the apparent mobilities of complexes and free DNA. (C) Plot of the off-rate analysis. The intensities of bands were quantified with the Betascope 603 blot analyzer (Betagen), and the percentage of complex remaining was calculated and plotted as a function of time. Data are averages of two independent experiments.

and found to be deficient in DNA binding. The amino acid substitutions of this conventional mutant occur in the basic part of the b-zip region and thus provide a strong correlation between the regulatory function of *cys-3* and its DNA-binding capability. These results support the concept that the basic region constitutes the DNA contact surface, in agreement with the proposed scissors grip model (21), which states that the region responsible for specific DNA binding is immediately NH_2 terminal to the leucine zipper.

The exact spacing of five amino acids between the zipper motif and the upstream basic region in CYS3 appears to be critical for its sequence-specific recognition of DNA. We found that insertion of just two amino acids between the first leucine of the zipper and the basic region yielded a CYS3 mutant protein that is nonfunctional in vivo and lacks any DNA-binding activity. According to the induced helical fork model (15), an alpha-helical conformation is acquired by the basic region only in the presence of the target DNA sequence and is responsible for the sequence-specific interac-

tion of the b-zip domain proteins with DNA. Alterations of the precise spacing between the basic region and the zipper motif of CYS3 apparently causes an incorrect positioning of the two basic regions of the dimer, which completely abolishes DNA-binding activity. Alternatively, it is possible that the altered spacing cause an incorrect folding of the basic region which eliminates its capability for DNA binding. Similarly, insertion or deletion of five amino acids between the zipper motif and the basic region in other b-zip domain proteins has been reported to eliminate DNA binding (1).

The induced fork model suggests that the role of leucine zipper in DNA binding is to position the basic regions of the dimeric protein to interact with the DNA and to initiate helix formation in these basic region (15). The importance of the exact periodicity of the leucine repeat was tested with the M13 and M14 *cys-3* mutant proteins (Table 1). The deletion of a single threonine residue in mutant M13 disturbs the helix periodicity of leucine side chains and produces a protein which dimerizes but which lacks both DNA-binding activity and in vivo function. Both of these functions were restored by insertion of a serine residue (in mutant M14), which changed the amino acid sequence but reestablished the heptad repeat.

Alterations in the amino acid sequence that disturb the amphipathic character of the leucine repeat will interfere with dimer formation and thus also preclude DNA binding. Such detrimental alterations include substitutions of leucines by amino acids less suitable for hydrophobic packing, deletions or insertions that change the relative position of the leucine residues in the zipper, or the presence of helix-breaking residues like proline or glycine (20). The CYS3 protein contains a heptad repeat motif consisting of four leucines and one methionine at the second position. The effects of substitutions within the zipper depend on the position and the nature of each substitution. We found that a valine substitution at either of the first two positions is more deleterious to CYS3 function than is a valine substitution at the third or fourth position of the zipper. These results suggest that the leucines immediately adjacent to the basic region are more important functionally than are the leucine residues of the zipper which are located further downstream. All of these mutant CYS3 proteins with a single valine substitution in zipper positions form dimeric proteins, but only the one with a substitution in the first position of the zipper completely abolished DNA binding. Interestingly, valine substitutions at the second or third position resulted in mutant CYS3 proteins which displayed DNA binding with the *cys-3* but not the *cys-14* promoter fragment. This differential binding presumably reflects the earlier findings that the *cys-3* promoter may bind two or more CYS3 protein molecules, which thus can account both for its stronger binding and for the presence of multiple shifted bands (7). The mutant CYS3 protein with a valine substitution in the fourth zipper position binds both *cys-3* and *cys-14* promoter fragments and functions in vivo. It thus appears that whereas all of the leucine residues in the heptad repeat contribute to dimer formation, the first leucine, and to some extent even the second and third leucines, of the zipper also have a special role in the precise positioning of the basic regions for productive DNA binding. Mutagenesis of both the C/EBP and Jun b-zip domains have similarly emphasized the importance of the first two leucine residues in providing the specific orientation of the DNA-binding domain (9, 12).

Our results show that any individual leucine of the zipper can be replaced by methionine to give a CYS3 protein which is functional in vivo and has DNA-binding activity in vitro.

Moreover, we have found that fully functional CYS3 proteins can contain as many as three methionine residues in zipper positions. These results suggest that methionine, which has an extended, bulky hydrophobic side chain and a free beta-carbon, represents an ideal substitute for leucine in the repeating heptad of the zipper (11). However, a methionine zipper CYS3 protein, containing five methionine residues in heptad repeat, shows reduced (but significant) dimer formation, reduced DNA binding in vitro, and reduced function in vivo (unpublished results). These results emphasize the steric and functional importance of the leucine periodicity and indicate that leucine does have a special role in formation of a functional zipper. Although both methionine, as we have shown, and isoleucine (11, 12) can partially substitute for leucine in a zipper structure, leucine does appear to be the preferred amino acid in this type of dimerization surface, perhaps vindicating the expression "leucine zipper."

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