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The DNA-Binding Domain of the Cys-3 Regulatory Protein of *Neurospora crassa* Is Bipartite†

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ABSTRACT: *Cys-3*, the major sulfur regulatory gene of *Neurospora crassa*, encodes a regulatory protein that is capable of sequence-specific interaction with DNA. The interaction is mediated by a region within the CYS3 protein (the bzip region) which contains a potential dimer-forming surface, the leucine zipper, and an adjacent basic DNA contact region, NH_2-terminal to the leucine zipper. To investigate the bipartite nature of the bzip region, a series of *cys-3* mutants obtained by oligonucleotide-directed mutagenesis were expressed and tested for dimer formation as well as DNA binding and in vivo function. The results demonstrate that CYS3 protein exists as a dimer in the presence and absence of the target DNA and that dimerization of CYS3 is mediated strictly by the leucine zipper, which is required for both *cys-3* function in vivo and DNA-binding activity in vitro. Furthermore, a truncated CYS3 protein corresponding to just the bzip region was found to mediate dimer formation and to possess DNA-binding activity. A CYS3 mutant protein with a pure methionine zipper showed significant, although reduced, function in vivo and in vitro.

Cys-3, the major sulfur regulatory gene of *Neurospora crassa*, controls the expression of a set of unlinked structural genes which encode sulfur catabolic enzymes (Fu et al., 1989; Burton & Metzenberg, 1971; Marzluf & Metzenberg, 1968). Detailed studies of *cys-3* mutants, including null and ts mutants, suggest that the *cys-3* gene specifies a regulatory protein that is required for expression of the sulfur-related enzymes, presumably by binding DNA element(s) upstream of each structural gene (Fu et al., 1990; Paietta et al., 1987). 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 (Fu et al., 1989). *Cys-3* encodes a protein of 236 amino acid residues which shows considerable

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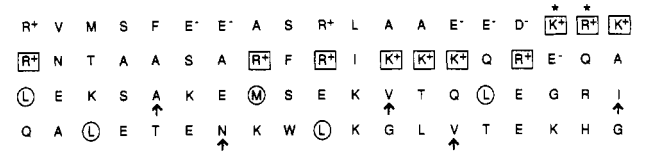

FIGURE 1: Leucine zipper of the CYS3 protein, comprised of amino acids residues from Arg-89 to Gly-164. The leucine residues and the single methionine residue which compose the heptad repeat are circled. Basic amino acids in the charged region upstream of the leucine zipper are boxed. Asterisks identify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

homology to the yeast GCN4, the *Neurospora* CPC-1, and the mammalian Fos, Jun, and C/EBP proteins (Fu et al., 1990). This family of proteins possesses a region required for DNA-binding which includes a leucine zipper as a dimerization domain and a DNA-binding segment that is enriched in basic amino acids. Proteins with this DNA recognition–dimerization motif have been termed the bzip proteins to reflect the bipartite nature of the DNA-binding domain. Mutations that alter the basic nature of the recognition domain prevent DNA-binding activity without altering dimerization; however, mutations in the leucine zipper that prevent dimerization abolish DNA-binding activity as well (Landschulz et al., 1988, 1989).

The CYS3 protein contains a well-defined leucine zipper comprised of four leucines and one methionine plus an NH_2-terminal basic region (Figure 1). Previously, we showed that the bzip domain of CYS3 protein can alone mediate DNA-binding with an affinity that is equivalent to that of the full-length CYS3 protein (Kannan & Marzluf, 1991). Furthermore, mutations that alter single amino acids in the basic domain produce CYS3 proteins that are completely deficient in DNA binding. These results support the concept that the basic region constitutes the DNA contact surface. On the contrary, the effect of substitutions within the zipper depend on the position and the nature of each substitution. Single conservative substitutions of valine for leucine L1 or L2 abolish DNA binding, while valine substitutions for L3 or L5 have no effect on DNA-binding activity. Moreover, the results of DNA-binding assays for CYS3 proteins mutated in the bzip region correlated perfectly with their functional activity in vivo (Kanaan & Marzluf, 1991).

Here we report experiments designed to directly investigate whether CYS3 exists as a dimer. The bzip domain and the full-length CYS3 protein were cotranslated, and mobility shift experiments utilizing the co-translated products show that CYS3 indeed binds DNA as a dimer. Moreover, cross-linking studies show that both the full-length CYS3 protein and the bzip domain protein exist as a dimer even in the absence of DNA. To investigate the bipartite nature of the *cys-3* bzip domain, a series of CYS3 mutant proteins were expressed in vitro and tested for dimerization. The results indicate that CYS3 dimerization is strictly dependent upon the leucine zipper region and does not require an active basic domain and that dimerization is required for both the *cys-3* function in vivo and 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 Vogels liquid medium with shaking at 30 °C as described previously (Fu & Marzluf, 1988).

Site-Directed Mutagenesis. Mutagenesis of the cloned *cys-3* gene was carried out by the method of Kunkel (1985). Dideoxy sequencing was carried out to confirm the desired changes.

Transformation. Competent *E. coli* cells were prepared according to the standard techniques. *N. crassa* transformation was carried out by the spheroplasting technique with Novozyme 234 (Dagert & Ehrlich, 1979). *Neurospora* transformants arise by the stable integration of plasmid DNAs, nearly always at ectopic sites. The transformation procedure has been described in detail previously (Fu et al., 1989; Kanaan & Marzluf, 1991).

Protein Expression. Expression of the mutant CYS3 proteins was carried out by replacing a 160 bp *Stu*1–*Xho*1 DNA fragment of the *cys-3* gene in the PC3T71 expression vector (Fu & Marzluf, 1990) with the corresponding DNA fragment which contained each mutational alteration. To express the *cys-3* bzip domain, a 200 nbp *Bam*H1 DNA fragment corresponding to the bzip domain was cloned into the PET3b vector.

In Vitro Transcription and Translation. The expression plasmids harboring the *cys-3* mutants and the bzip region were in vitro transcribed utilizing the Stratagene kit. The resulting RNAs were translated in vitro, using rabbit reticulocyte extract (Promega) in the presence of L-[^{35}S]methionine according to the manufacture's instructions. ^{35}S-labeled translation products were separated by SDS–PAGE, and the gel was treated with EN3HANCE (Du Pont), dried, and autoradiographed.

DNA-Binding Assay. A DNA fragment containing the CYS3 recognition site present in the promoter region of the *cys-14* structural gene was prepared by annealing two complementary 27-mer synthetic nucleotides. Mobility shift experiments utilizing aliquots (1–5 μL) of in vitro translated protein were carried out as described previously (Kanaan & Marzluf, 1991) except that 8% acrylamide gels were used; the gels were fixed for 30 min in 10% acetic acid and 30% methanol, treated wtih EN3HANCE for 30 min, dried, and autoradiographed.

Glutaraldehyde Cross-Linking. ^{35}S-Labeled wild-type, bzip domain, and mutant CYS3 proteins were translated in vitro. The crude translation reaction was either used directly or purified by heparin–Sepharose chromatography (to remove the heme) and subjected to glutaraldehyde cross-linking, using conditions described previously (Mermond et al., 1989; Turner & Tjian, 1989). 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, 120, and 180 min. The reaction was stopped with ethanolamine at a final concentration of 20 mM. The cross-linked products were analyzed by SDS–PAGE (10% acrylamide for the full-length CYS3 protein and 15% for the bzip domain protein). The gels were fixed, treated with EN3HANCE, dried, and exposed to X-ray film. To purify the in vitro translated CYS3 proteins, the equivalent of three translation reactions (150 nL) was diluted to 50 mM KCL and loaded on 200 nL of a heparin–sepharose column. The column was equilibrated with buffer A (20 mM HEPES, pH 7.9, 50 mM KCL, 1 mM dithiothreitol, and 0.1% Nonidet-P40), and the CYS3 proteins were eluted with the same buffer containing 500 mM KCL. The eluted proteins were diluted to 50 mM KCL utilizing buffer A without KCL and then concentrated to 150–200-nL total volume using Centricom (Amicom) at 6500*g* for 3 h.

Results

Expression of the CYS3 Protein. The wild-type and the mutant *cys-3* genes and the internal region encoding the bzip domain were each cloned into the expression vector pET3b and transcribed and translated in vitro. The bzip domain and

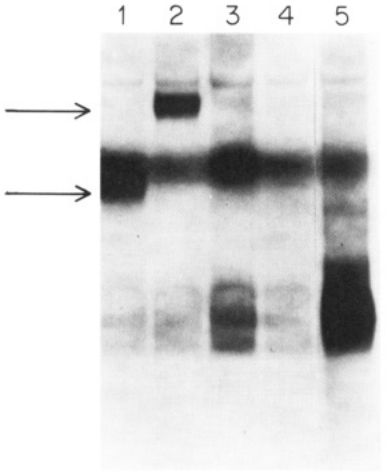

FIGURE 2: Mobility shift experiments with the in vitro expressed ^{35}S-labeled CYS3 and bzip domain proteins, using a DNA fragment with the *cys-14* recognition element. The ^{35}S-labeled CYS3 proteins do not enter the gel in the absence of DNA. Background labeled proteins from the translation system are nonspecific and visible in lanes 3–5. Lanes: 1, mobility shift band with DNA and the bzip domain protein; 2, DNA plus the CYS3 protein; 3, CYS3 protein with no DNA; 4, bzip domain protein with no DNA; 5, mobility shift experiment with DNA and products from the control translation reaction which lacks any CYS3 protein. Arrows identify the bands which show the ^{35}S-labeled CYS3 proteins which enter the gel as a DNA–protein complex.

the full-length CYS3 proteins of 10 and 30 kDa, respectively, were either translated in vitro separately or cotranslated together in the same reaction mixture. An in vitro translation reaction that lacked any *cys-3* message did not produce any of these proteins. Figure 2 shows the DNA-binding activity of the in vitro translated bzip (lane 1) and the full-length CYS3 proteins (lane 2). As expected, a control translation reaction lacking CYS3 protein did not show specific DNA-binding activity (lane 5). Moreover, in the absence of DNA, neither the full length or the shorter bzip domain protein enter the gel (lanes 3 and 4).

CYS3 Binds DNA as a Dimer. The "leucine zipper hypothesis" emphasizes the bipartite nature of this type of DNA-binding domain in which the leucine repeat acts as a dimerization surface. The full-length CYS3 and the bzip proteins both have been shown to recognize DNA elements with limited dyad symmetry. The native CYS3 protein may exist as a dimer in the absence of DNA *or* instead might form dimers only after the binding of two monomers to adjacent palindromic sequences. To examine these possibilities, an *E. coli* expressed, partially purified full-length CYS3 protein was mixed with the truncated *E. coli* expressed bzip domain under conditions that could promote subunit exchange, including freeze–thawing (Markert., 1963) and heating–cooling approaches. If an exchange of subunits occurs, mobility shift experiments should reveal a shifted band of intermediate mobility, representing a heterodimer consisting of one full-length monomer and one truncated monomer.

However, such an intermediate band was not observed (Figure 3A), which indicates that either CYS3 dimers are stable and do not readily exchange subunits (at least under the conditions tested) or that CYS3 is not a multimeric protein. However, when the full-length CYS3 protein and the bzip domain protein were cotranslated together in vitro, mobility shift experiments revealed a complex of intermediate mobility (Figure 3B, lane 6), in exactly the position expected for a heterodimer possessing one full-length and one truncated CYS3 subunit. This result demonstrates that the CYS3 protein binds DNA as a dimer; however, it does not directly

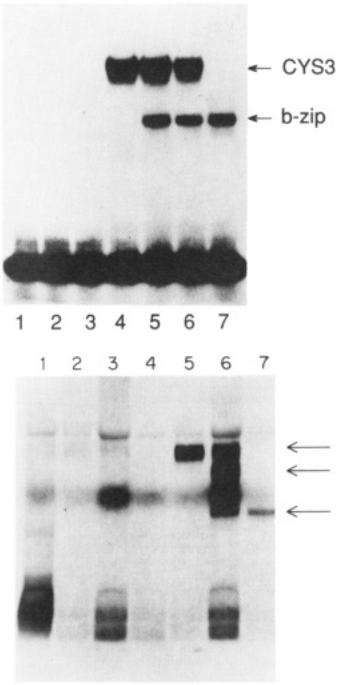

FIGURE 3: (A, top) Gel band mobility shift experiments with the 5′-flanking DNA fragments of the *cys-14* gene. ^{32}P-labeled DNA fragments were incubated with protein extracts and subjected to polyacrylamide gel electrophoresis as described under Material and Methods. All lanes contain the ^{32}P-labeled probe. Lanes: 1, no protein (free probe only); 2, protein expressed in cells with vector only; 3, CYS3 null mutant protein as a negative control; 4, wild-type CYS3 protein; 5, CYS3 and bzip domain proteins mixed at equal proportion in 1 M NaCl, frozen overnight, and thawed; 6, equal proportion of CYS3 and bzip domain proteins in phosphate-buffered saline and heated at 50 °C for 2 min; 7, bzip domain protein. (B, bottom) Heterodimer detection with the mobility shift assay. Gel mobility shift experiments were performed as described under Materials and Methods with the nonradioactive *cys-14* DNA recognition element and in vitro translated, ^{35}S-labeled proteins. Lanes: 1, DNA plus the control translation rection; 2, CYS3 protein with no DNA; 3, the bzip domain protein with no DNA; 4, cotranslated CYS3 and bzip proteins with no DNA; 5, CYS3 protein plus DNA; 6, DNA plus cotranslated CYS3 and bzip proteins; 7, DNA plus the bzip domain protein. Arrows identify the shifted bands.

reveal whether the CYS3 protein exists as a dimer in the absence of the DNA.

CYS3 Is a Dimeric Protein. To investigate whether DNA is required for CYS3 dimerization, we performed glutaraldehyde cross-linking experiments in the absence of DNA. ^{35}S-labeled CYS3 proteins were translated in vitro, and the translation mixtures were examined directly or partially purified (see Materials and Methods). After incubation with a low concentration of glutaraldehyde for varying lengths of time at 4 °C, the reaction products were fractionated by SDS–PAGE. The treatment with glutaraldehyde resulted in the time-dependent appearance of a single new species, corresponding in size to the expected cross-linked dimer (Figure 4A, lanes 4–6); moreover, no larger sized cross-linked forms were detected, even in the presence of excess glutaraldehyde or after prolonged incubation (Figure 4B, lanes 1–2). This result, plus the finding that certain CYS3 mutant proteins fail to show any dimers at all (see below), argues against the possibility that the cross-linked products are due to random collisions of CYS3 monomers. Similarly, control cross-linking experiments with lactoglobulin showed only the expected cross-linked species (not shown). The above results indicate that CYS3 exists as a dimer in solution in the absence of DNA. Interestingly, dimers were also obtained, in a time-dependent manner, when the in vitro translated bzip domain protein was

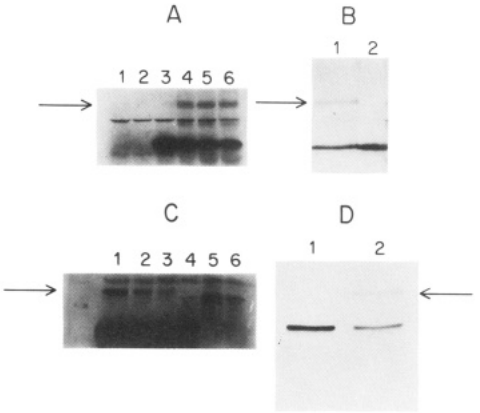

FIGURE 4: Glutaraldehyde cross-linking. The full-length *cys-3* and the bzip domain proteins were translated in vitro. The crude translation reaction was either used directly or purified as described under Materials and Methods. (A) Lanes: 1, the control translation reaction; 2, the control translation reaction incubated with 0.01% glutaraldehyde at 4 °C for 120 min; 3, the crude translation reaction containing the expressed CYS3 protein; 4–6, the crude translation reaction containing CYS3 treated with 0.01% glutaraldehyde at 4 °C for 60, 90, and 120 min, respectively. (B) Lanes: 1, the purified CYS3 protein treated with 0.04% glutaraldehyde at 4 °C for 180 min; 2, the purified CYS3 protein. (C) Glutaraldehyde cross-linking of the bzip domain protein; Lanes: 6, the control translation reaction; 5, the control reaction treated with 0.01% glutaraldehyde at 4 °C for 120 min; 4, the crude translation reaction expressing the bzip domain; 1–3, the crude translation reaction with the bzip protein treated with 0.01% glutaraldehyde at 4 °C for 120, 90, and 60 min, respectively. (D) Lanes: 1, the purified bzip domain protein; 2, the purified bzip protein treated with 0.04% glutaraldehyde at 4 °C for 180 min. Arrows identify the positions occupied by dimers.

crossed-linked (Figure 4C, lanes 3–1, and Figure 4D, lanes 1–2). These combined results strongly argue that the native CYS3 protein exists as a stable dimer even in the absence of DNA and that both DNA-binding and dimerization are inherent in the bzip region of the protein.

Functional Analysis of the bzip Domain. The DNA-binding domain of the CYS3 protein consists of a leucine zipper and a highly basic region located five amino acid residues N-terminal to the leucine repeat (Figure 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 and dimerization in vitro. Table I shows the amino acid substitution in each of the CYS3 mutants, all of which were made with oligonucleotide primers as described under Materials and Methods. Each mutant was tested for function in vivo by transforming into a *cys-3* mutant in parallel with the wild-type *cys-3* gene as a positive control (Kanaan & Marzluf, 1991). In addition, each mutant CYS3 protein was expressed in *E. coli* and examined for DNA-binding activity in vitro with band shift assays utilizing both the *cys-3* and *cys-14* recognition elements (Kanaan & Marzluf, 1991). Here we report cross-linking studies to determine the capacity for dimerization of these various mutant CYS3 proteins; each mutant protein was in vitro translated and tested for dimer formation as described under Materials and Methods. The results of the transformation assays, DNA-binding, and dimerization tests are summarized in Table I and described below.

Analysis of the Basic Region. Substitution of glutamine residues for three (mutant B2), two (mutants B1 and B3), or even one basic amino acid within the basic region abolished *cys-3* function in vivo (Table I). The expressed CYS3 protein corresponding to each of these basic region mutants was found to dimerize (Figure 5A, lanes 5–6) but to be completely deficient in DNA-binding to both the *cys-3* and *cys-14* recognition elements (Table I). These results support the concept that a functional basic region constitutes a DNA contact surface but is not required for dimerization.

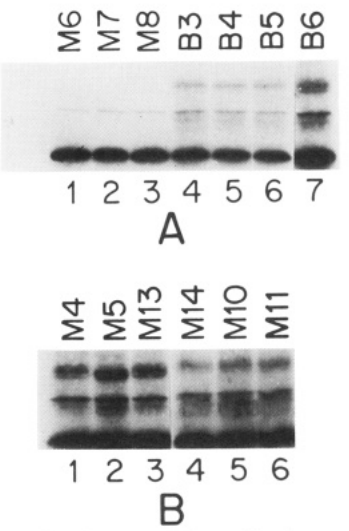

FIGURE 5: Glutaraldehyde cross-linking of in vitro translated CYS3 mutant proteins. (A) Lanes: 1–7, the cross-linked product of mutants M6, M7, M8, B3, B4, B5, and B6, respectively. (B) Lanes: 1–6, the cross-linked products of mutants M4, M5, M13, M14, M10, and M11, respectively. Protein cross-linking was carried out with 0.01% glutaraldehyde at 4 °C for 90 min and dimer formation detected as described under Materials and Methods.

Analysis of the Leucine Zipper. The CYS3 protein contains a well-defined leucine zipper which consists of four leucines and one methionine (Figure 1). The effects of amino acid substitutions within the zipper region were found to depend on the position within the zipper and the nature of each substitution. For example, we previously showed that a valine substitution for leucine at the first zipper residue (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 (Table I), which suggests that leucine at position 1 in the zipper is more important functionally than is leucine at position 3 or 4. Mutants M6, M7, and M8, in which two leucines of the zipper were replaced by valine, lack *cys-3* function in vivo. The results of DNA-binding assays for CYS3 proteins mutated in the zipper were correlated in nearly every instance with their functional activity in vivo (Table I). All of the mutant CYS-3 proteins with single valine substitutions were found to dimerize (Figure 5B, lanes 1–2); it is particularly noteworthy that mutant M5, in which valine replaces leucine 1 of the zipper, dimerizes very well and yet fails to display any DNA-binding activity and fails to function in vivo. Thus, it appears that the complete loss in DNA-binding ability of the M5 mutant CYS3 protein reflects a failure to properly position the basic region. In contrast, CYS3 mutant proteins with two valine substitutions (M6, M7, and M8) fail to form dimers (Figure 5A, lanes 1–3), which clearly precludes any DNA-binding ability. These results indicate that DNA-binding by CYS3, as with other members of the bzip class of proteins, requires dimerization and also depends on the proper positioning of the two basic regions. A series of new mutants in which three or more methionine residues occupy zipper positions were constructed and examined (Table I). Mutants M11a and M11b, which each have three methionines in zipper positions, function in a wild-type manner in vivo and show normal dimer formation and DNA-binding activities. Mutants M11c and M11d, each with four methionine substitutions, and M11f, which has methionine in all five

Table I: Summary of the Results Obtained with Transformation, Mobility Shift, and Cross-Linking Experiments To Analyze the Mutant CYS3 Proteins[a]

		DNA-binding activity		
	dimerization	*cys-3* promoter	*cis-14* promoter	in vivo function
basic region substitutions				
W+ K-R-K-R-N-T-A-A-S-A-R-F-R-I-K-K-K-Q-R-E-Q	+	+	+	+
B1 Q-G----------	+	–	–	–
B2 ----------Q-Q-Q--------	+	–	–	–
B3 ----------Q---Q----------	+	–	–	–
B4 ----------Q----------	+	–	–	–
B5 ----------Q----------	+	–	–	–
B6 ---basic region---GAATTC---leucine zipper	+	–	–	–
zipper Val–Leu substitutions				
wild type L---M---L---L---L	+	+	+	+
M2–mutant ----------V----	+	+	+	+
M3–mutant --------V--------	+	reduced	–	+
M4–mutant ----V----------	+	reduced	–	+
M5–mutant V----------	+	–	–	–
M6–mutant --------V---V----	–	–	–	–
M7–mutant ----V---V--------	–	–	–	–
M8–mutant ----V-------V----	–	–	–	–
zipper Met–Leu substitutions				
M9–mutant ----L----------	+	+	+	+
M10–mutant --------M--------	+	+	+	+
M11–mutant ----------M----	+	+	+	+
M11a–mutant M---M---L---M---L	+	+	+	+
M11b–mutant M---M---M---L---L	+	+	+	+
M11c–mutant M---M---M---M---L	+	reduced	reduced	+
M11d–mutant L---M---M---M---M	+	reduced	reduced	+
M11f–mutant M---M---M---M---M	+	reduced	reduced	+
spacing alterations				
wild type M---S-E-K-V-T-Q---L	+	+	+	+
M13–mutant M---S-E-K-V-*-Q---L	+	–	–	+
M14–mutant M---S-E-K-V-Q-S---L	+	+	+	+
truncated CYS3 protein				
bzip domain	+	+	+	–

[a] The identity and location of the amino acid substitution in each of the CYS3 mutants are shown under the wild-type sequence. 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. Quantitation of DNA binding, described as reduced binding in the table, was determined with a Betascope 603 analyzer (Betagen).

zipper positions, all function in vivo and display DNA binding, although their binding activity is reduced, presumably as a result of a lower efficiency of dimerization.

Analysis of a Spacing Alteration in the bzip Region. The importance of the leucine periodicity 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 I). Such a mutation would be expected to disturb the amphipathic α-helix since the repeated leucines would not all lie on the same surface. The M13 mutant protein lacks any DNA-binding activity and fails to transform in vivo. When the correct spacing within the zipper was reestablished by insertion of a different amino acid (serine) at a different position (mutant M14; Table I), both DNA-binding and in vivo function were regained, thus demonstrating the importance of precise spacing within the zipper. Interestingly, the threonine-deletion mutant (M13) protein dimerizes as well as does the M14 revertant (Figure 5B, lanes 3 and 4). This result suggests that the altered spacing within the zipper prevented proper interaction of the leucine and methionine N-terminal to the deletion, which in turn resulted in improper alignment of the basic region and a loss of DNA-binding activity; the region C-terminal to the deleted threonine apparently provides a sufficient surface for dimerization. This interpretation is consistent with the ability of mutant CYS3 proteins M4 and M5 to dimerize but not to bind DNA. The critical spacing of five amino acids between the zipper motif and the upstream basic region was tested 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 I). The B6 mutant CYS3 protein is nonfunctional in vivo, lacks any DNA-binding activity, but dimerizes normally (Figure 5A, lane 7). These results strongly imply that DNA binding by the CYS3 protein requires both dimerization and the precise alignment of the basic regions.

DNA-binding, Dimerization, and cys-3 Function in Vivo. It was of interest to determine whether a functional basic region must be present in both subunits of a CYS3 dimer for DNA-binding activity and for function in vivo. To address this question, mobility shift experiments were carried out with the in vivo translated truncated bzip domain protein (which has a wild-type zipper and basic region and both dimerizes and binds to DNA) which was cotranslated with either the B3 mutant full-length protein (forms dimers but does not bind to DNA due to a defective basic region), the M8 mutant (does not bind DNA or form dimers), or the M11f mutant (reduced dimerization, DNA binding, and in vivo function, data not shown).

The results showed significant DNA binding by heterodimers formed from subunits of the bzip and M11f mutant protein (Figure 6, lane 2). In marked contrast, no DNA binding at all was detected due to heterodimers that resulted from one bzip subunit associating with a subunit from either the B3 or the M8 mutant proteins (Figure 6, lanes 5 and 8). These experiments suggest that DNA-binding requires that both subunits of a CYS3 dimer must have a basic region which is active for DNA binding; dimers with only one subunit with

a functional basic region appear to be incapable of binding to DNA. Furthermore, these results suggest that both the B3 and M8 might act as dominant mutations, producing nonfunctional heterodimers with wild-type monomers, via negative complementation. In contrast, the M11f mutant would be expected to act as a recessive because it encodes a mutant CYS3 monomer (with a pure methionine zipper) that interacts with a wild-type monomer to form a heterodimer which functions in DNA binding.

These results were further confirmed by cotransformation of each of these mutants with a wild-type *cys-3* gene into a null *cys-3* mutant host. The results of these experiments showed that both the B3 and M8 mutant genes appeared to inhibit the expression of the wild-type gene, detected by a reduction in the frequency of transformation by approximately 30-fold; thus, both mutations (B3 and M8) act dominant to wild-type in vivo, which is consistent with their negative complementation observed in vitro. However, overproduction of the wild-type CYS3 protein, achieved by increasing the ratio of the wild-type:mutant genes in the cotransformation experiments, overcomes the dominance of the *cys-3* mutants. The M11 mutant gene had no effect in the transformation frequency when cotransformed with the wild-type, consistent with its predicted recessive behavior. These results emphasize the bipartite nature of the bzip domain and provide a strong relation between DNA binding and dimerization of CYS3 protein in vitro and *cys-3* function in vivo.

Discussion

Cys-3, the major sulfur regulatory gene of *N. crassa*, encodes a trans-acting regulatory protein which turns on the expression of a set of unlinked structural genes which specify sulfur catabolic enzymes during conditions of sulfur limitation. The CYS3 protein consists of 236 amino acids and contains a leucine zipper and an upstream basic region (bzip region) which together constitute a DNA-binding domain (Kanaan & Marzluf, 1991). The leucine repeat region of the bzip class of proteins appears to assume an α-helix conformation, with the leucine side chains projecting out at regular intervals. The hydrophobic surface 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 (Gents et al., 1989; O'Neil et al., 1990; O'Shea et al., 1989; Shuman et al., 1990; Talania et al., 1990). Previously, we showed that the full-length CYS3 protein and a much smaller protein, encompassing the bzip region of CYS3 protein, have indistinguishable DNA-binding affinities, implying that the DNA-binding activity of the CYS3 protein resides entirely in the bzip region of the protein, as was also found with other bzip proteins (Bohmann & Tjian, 1989).

Here we provide direct evidence that the CYS3 protein exists as a dimer in the absence of DNA and also show that the CYS3 bzip domain alone can mediate dimer formation. The fact that CYS3 binds to DNA as a dimer was demonstrated by the presence of a heterodimer, composed of one full-length *cys-3* monomer and one smaller *cys-3* bzip domain monomer, detected when these proteins were cotranslated in vitro and assayed for DNA binding.

The question remained whether CYS3 exists as a dimer in the absence of DNA or only assumes a dimer structure upon binding to DNA. Glutaraldehyde cross-linking of in vitro translated full-length CYS3 and of the bzip domain protein in the absence of DNA revealed in each case the appearance of a single new species, corresponding in size to the expected cross-linked dimer. These results indicate that the native structure of CYS3 protein is a dimer and that both dimerization and DNA binding are inherent in the bzip region of the protein. We found that the CYS3 dimers were stable and did not exchange subunits, unlike the situation for several other bzip prtoeins, e.g., FOS and JUN, which freely exchange subunits in the absence of DNA (Landschultz et al., 1989).

To examine the bipartite nature of the CYS3 bzip region and to more precisely identify the regions involved in dimer formation and in DNA-binding activity, we employed site-directed mutagenesis to introduce substitutions for highly conserved amino acid residues in both the basic region and the leucine repeat, which might be important for function. Some of these mutants were previously tested for function in vivo and DNA-binding activity in vitro (Kanaan & Marzluf, 1991). Here we report these activities for five additional CYS3 mutant proteins, M11a, b, c, d, and f (Table I), and also give the results of a complete dimerization analysis of all of these CYS3 mutants proteins.

Mutations that alter the basic region by substitution of glutamine residues for even one basic amino acid abolish CYS3 function in vivo and DNA-binding activity in vitro. In agreement, a conventional null *cys-3* mutant was found to have two nucleotide changes, which resulted in the replacement of lysine 105 and arginine 106 by glutamine residues (Figure 1). This mutant CYS3 protein was expressed and found to be completely deficient in DNA binding, thus providing a strong correlation between the regulatory function of *cys-3* in vivo and its DNA-binding capability. Significantly, the expressed CYS3 protein corresponding to each of these basic region mutants was found to dimerize, essentially to the same degree as does the wild-type protein. These results imply that the basic region of the CYS3 protein is essential for productive interaction with DNA but is not required for dimerization.

A particularly informative result was obtained by chainging the critical spacing between an otherwise native zipper motif and wild-type basic region by insertion of two amino acids residues between them (Table I, mutant B6). This CYS3 mutant protein dimerizes normally but lacks both DNA binding in vitro and *cys-3* function in vivo. These results argue that DNA binding by a CYS3 dimer not only requires functional basic regions but also is completely dependent upon a precise orientation of the basic motifs, which is determined by the positioning of the leucine zipper dimerization surfaces.

According to the induced helical model (O'Neil et al., 1990), an α-helical conformation is acquired by the basic region only in the presence of the target DNA and is responsible for the sequence-specific interaction of the bzip domain proteins with DNA; the role of the leucine zipper in DNA binding is proposed to position the basic regions of a dimeric protein to interact with the DNA and to initiate helix formation in these basic regions. Alteration of the precise spacing between the basic region and the zipper may cause incorrect position of the basic regions of a dimer or incorrect folding of the basic region, which eliminate DNA-binding activity, but has no effect on the leucine zipper-mediated dimerization. Similarly, insertion or deletion of three or more amino acids between the zipper motif and the basic region in other bzip domain proteins has been reported to eliminate DNA binding but has no effect on dimerization (Agre et al., 1989).

We examined the importance of exact periodicity within the leucine repeat. The deletion of a single threonine residue in mutant M13 disturbs the helical periodicity of leucine side chains and produces a protein that lacks both DNA-binding activity and in vivo function. Both functions were restored by insertion of a serine residue (in mutant M14) which

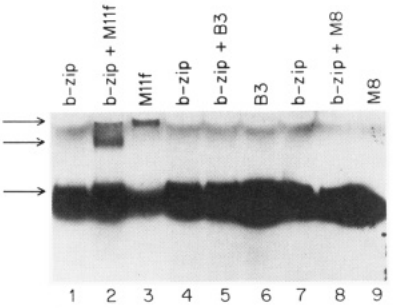

FIGURE 6: Heterodimer formation analysis of selected CYS3 mutant proteins with the cotranslation product of the bzip domain and detected via mobility shift assays. Lanes: 1, 4, and 7, mobility shift with the in vitro translated bzip protein alone; 2, 5, and 8, mobility shift with cotranslated products of the bzip domain protein with mutant M11f, B3, and M8 proteins, respectively; 3, 6, and 9, mobility shift with the in vitro translated M11f, B3, and M8 mutant proteins alone. Arrows identify the shifted bands.

changed the amino acid sequence but reestablished the heptad repeat. However, both the M13 and M14 CYS3 mutant proteins dimerize normally, which suggests that the altered spacing in the M13 mutant has a serious affect upon the packing of the leucine and methionine located N-terminal to the deletion, which results in improper alignment of the basic region and loss of DNA binding; presumably, the leucines C-terminal to the deletion provide a sufficient surface for dimerization.

Amino acid substitutions that distrub the amphipathic character of the leucine repeat might be expected to interfere with dimer formation and thus preclude DNA binding. Such detrimental alterations could include deletions or insertions that change the relative position of the leucine residues in the zipper, substitution of leucines by residues less suitable for hydrophobic packing, or the presence of helix-breaking residues like proline or glycine (Turner & Tjian, 1989). The above hypothesis was examined by substituting specific amino acids in the CYS3 leucine zipper and then testing the effects of such mutations on dimerization, DNA binding, and in vivo function. We found that the substitution of two leucines of the zipper by valine in several mutants (M6, M7, and M8, Table I) gave mutant proteins which failed to dimerize, as demonstrated by direct cross-linking assays. As would be expected, these mutants lack both DNA binding in vitro and transforming ability in vivo.

The effect of other 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. All of these mutant CYS3 proteins with a single valine substitution in zipper positions form dimeric proteins. It is important to note that only those CYS3 mutants with a single valine substitution in one of the first two zipper positions abolished DNA binding. It thus appears that whereas all of the leucine residues in the heptad repeat contribute to dimer formation, the first two 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 the Jun bzip domains have similarly emphasized the importance of the first two leucine residues in providing the specific orientation of the DNA-binding domain (Gentz et al., 1989; Landschulz et al., 1989). It was intruiging that CYS3 proteins with as many as three methionine residues in zipper positions appear to function as well as the wild-type protein in all activities. Moreover, a CYS3 protein containing a "methionine zipper", i.e., one in which all five zipper positions were occupied by methionine, was functional in vivo and showed both dimerization and DNA binding, although reduced, in vitro. It thus appears that leucine is the most suitable amino acid for efficient function of a zipper structure but that methionine, unlike valine and presumably most other amino acids, can replace leucine with retention of significant functional activity. In this regard it was interesting that a heterodimer active in DNA binding was formed between a wild-type monomer, which contains four leucines in zipper positions, and a monomer containing only methionine residues.

Our results also show that DNA binding requires that both subunits of a CYS3 dimer must have a basic region that is active for DNA binding; dimers in which only one subunit possesses a functional basic region are incapable of binding to DNA. Moreover, *cys-3* mutants which encode proteins that are defective in the basic DNA contact region appear to be dominant in vivo via negative complementation to the wild-type *cys-3* gene, which is consistent with the fact that heterodimers do not bind DNA in vitro. In summary, the results presented here strongly support the proposed bipartite nature of the CYS3 bzip domain and clearly demonstrate that this domain of CYS3 is solely responsible for dimerization and DNA binding and is essential for *cys-3* function in vivo.

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