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Analysis of CYS3 regulator function in *Neurospora crassa* by modification of leucine zipper dimerization specificity

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ABSTRACT

The CYS3 positive regulator is a basic region-leucine zipper (bZIP) DNA-binding protein that is essential for the expression of sulfur-controlled structural genes in *Neurospora crassa*. An approach of modifying the dimerization specificity of the CYS3 leucine zipper was used to determine whether the *in vivo* regulatory function of CYS3 requires the formation of homodimeric or heterodimeric complexes. Two altered versions of CYS3 with coiled coil electrostatic interactions favorable to heterodimerization showed restoration of wild-type CYS3 function only when simultaneously expressed in a Δ*cys-3* strain. In addition, constructs having the CYS3 leucine zipper swapped for that of the oncoprotein Jun or the CYS3 leucine zipper extended by a heptad repeat showed wild-type CYS3 function when transformed into a Δ*cys-3* strain. Gel mobility shift and immunoprecipitation assays were used to confirm the modified CYS3 proteins dimerization and DNA binding properties. The studies, which precluded wild-type CYS3 dimerization, indicate that *in vivo* CYS3 is fully functional as a homodimer since no interaction was required with other leucine zipper proteins to activate sulfur regulatory and structural gene expression. The results demonstrate the utility of leucine zipper modification to study the *in vivo* function of bZIP proteins.

INTRODUCTION

The sulfur regulatory system of *Neurospora crassa* is made up of a genetically defined set of *trans*-acting regulatory genes and a set of structural genes that encode enzymes (e.g., arylsulfatase) used in the uptake and assimilation of a variety of sulfur compounds (1,2). An important part of the sulfur regulatory system involves the positive regulatory gene, *cys-3*$^+$ (3). In *cys-3* mutants there is a loss of expression of the set of sulfur related enzymes and, in contrast to the wild-type, they are sulfur auxotrophs. *cys-3* mutants are also affected in a range of cellular properties such as nucleotide pools, energy charge and ascospore viability (3,4). Such pleiotropic effects have led to the suggestion of a broad regulatory role for CYS3 (4). The *cys-3*$^+$ gene has been cloned (3), and subsequent work has shown the encoded CYS3 product to be a 25.9 kDa bZIP (basic region-leucine zipper) protein (5). CYS3 shows homology to a number of bZIP proteins, including the oncogene *fos* product and the yeast regulator GCN4.

The importance of the leucine zipper in the function of CYS3 has been confirmed by site-directed mutagenesis to produce mutants that are non-functional *in vitro* and *in vivo* (6,7). *In vitro* experiments have also demonstrated that CYS3 can form homodimers (6). Given the frequent occurrence of heterodimerization observed between bZIP proteins (8–10), a more difficult problem is in determining whether other leucine zipper proteins interact with CYS3 *in vivo* and modulate its various regulatory functions. These functions include CYS3's role as a DNA-binding transcriptional activator for the sulfur structural genes, for its own autoregulation, for the feedback control of the negative regulator *sulfur controller-2*, and the potential for cross-regulatory effects involving other cellular processes (11). A genetic approach would be the method of choice for the determination of whether CYS3 function is dependent on heterodimerization since the nature of *in vivo* CYS3 complexes and the range of potential CYS3 binding sites have not been fully determined.

The approach used to detect any potential bZIP interactions for CYS3 was to modify the leucine zipper to eliminate wild-type dimerization interactions and to assay the function of the mutant CYS3 protein *in vivo*. The effect of three types of leucine zipper alterations have been explored. The first alteration examined was to mutate the CYS3 protein to generate two versions of the protein which have altered electrostatic interactions in the leucine zipper that favors heterodimerization. The second alteration tested was to swap leucine zippers between bZIP proteins with different dimerization specificities (i.e., CYS3 and Jun). The final alteration tested was to extend the CYS3 leucine zipper by a heptad repeat in order to prevent formation of functional heterodimeric complexes due to misalignment of the basic regions. A strain deleted for the *cys-3*$^+$ gene (Δ*cys-3*) (6) was used to test the *in vivo* function of the CYS3 constructs. The modified CYS3 proteins demonstrated wild-type CYS3 function in Δ*cys-3* and restored the strain to prototrophy. Further, the modified CYS3 proteins show wild-type regulatory control in terms of the regulation of sulfur structural genes and the negative regulator *scon-2*$^+$, as well as the autoregulated expression of *cys-3*$^+$ itself.

MATERIALS AND METHODS

Strains and culture conditions

N.crassa Δ*cys-3* (18-4) was constructed by gene replacement as previously described (6). Vogel minimal medium (12), with

supplements as required, was used. Sulfur derepression and repression experiments were done by filtration harvesting of mycelia and transfer to Vogel-minus-sulfur medium plus high-sulfur (5.0 mM methionine) or low-sulfur (0.25 mM methionine) medium. Chromate resistance was tested on low-sulfur medium with 10 mM potassium chromate (11).

Plasmid constructs and *in vitro* mutagenesis

Site-directed mutagenesis was carried out by the phosphorothioate method (13). All constructs that involved site-directed mutagenesis were completely sequenced by the dideoxy method (14). *cys-3A* and *cys-3B* were constructed by site-directed mutagenesis with oligonucleotides having several internal mismatches (5′-GTCAC-CCAACTTAAGGGAGAGATCCAGGCTCTCGAGACGGA-GAACAAGTGGCTCGAGGGCCTCGTCA-3′ for *cys-3A* and 5′-GAGATGAGCGAGGAGGTCACCCAACTTGAGGGAC-GCATCCAGGCTCTCAAGACGAAGAACAAGTGGCTC-3′ for *cys-3B*; mutated bases underlined). *cys-3*^{Fos} and *cys-3*^{Jun} were constructed by introducing *Xho*I sites to each side of the *cys-3*$^{+}$ leucine zipper and then inserting a synthesized leucine zipper. Orientation of the insert was confirmed by sequencing. The inserted segment followed codon usage in the *cys-3*$^{+}$ gene. The resulting sequences encoding the leucine zippers were as follows: *cys-3*^{Jun}, 5′-CTC GAG GAG AAG GTC AAG ACC CTC AAG GCC CAG AAC AGC GAG CTT GCC AGC ACG GCC AAC ATG CTC CGC GAG CAG GTC GCC CAG CTC-3′ and *cys-3*^{Fos}, 5′-CTC GAG GCC GAG ACC GAC CAG CTC GAG GAC GAG AAG AGC GCC CTC CAG ACG GAG ATC GCC AAC CTC CTT AAG GAG AAG GAG AAG CTC-3′. In *cys-3*^{Fos} the underlined codon resulted in a glutamate rather than a glutamine in the Fos sequence due to the introduction of the *Xho*I site. CYS3^{In} was produced by introduction of *Apa*LI and *Xho*I sites by site-directed mutagenesis preceding the start of the first heptad repeat in the CYS3 leucine zipper (mutagenized bases underlined, 5′-GAAGAAGCAGCGCGTGCACGCGCTCGA-GAAGTCGGCCAAGG-3′). After digestion of the mutagenized construct with *Apa*LI and *Xho*I, the following segment (as double-stranded) was inserted: 5′-TGCAGGCGCTCGAGGA-CAAGGTGGAGGAGC-3′. A valine for glutamate substitution in the spacer region between the basic region and leucine zipper was present due to the introduced *ApaLI* site.

To construct clones for *in vitro* transcription and translation, mutagenized segments were introduced into pGEM4Z and digested with *Bam*HI or *Ppu*MI to produce templates for transcription (6). The *Ppu*MI site, as described in previous work (6), allowed for the production of a truncated *cys-3* transcript, while the *Bam*HI site allowed for a full-length transcript. *In vitro* transcription and translation were carried out as described previously using SP6 RNA polymerase and a rabbit reticulocyte lysate (6). Translation mixtures to be immunoprecipitated were labeled with ^{35}S-methionine.

Immunological procedures

The anti-CYS3 antibody has been characterized previously (6). The polyclonal antibody was produced against a peptide (pep a) (Fig. 2A) represented in the sequence of the full length CYS3 protein but absent in the truncated version. Immunoprecipitation was performed as described previously with the anti-CYS3 antibody and protein A–agarose (6), followed by SDS–PAGE.

Transformation and homokaryon isolation

N.crassa was transformed by the Novozyme 234 spheroplasting technique (19). Homokaryons were isolated by filtration of iodoacetate-grown conidial cultures through 5 μm Millex filters (20).

Arylsulfatase assays

Arylsulfatase was assayed by incubation of mycelial extracts with *p*-nitrophenol sulfate according to standard methods (15,16).

RNA isolation and analysis

RNA was isolated by phenol extraction, washed with sodium acetate and poly(A)$^{+}$ mRNA isolated by oligo(dT)–cellulose chromatography (16,17). Northern blot and hybridization conditions were as described previously (6). Quantitative comparison of mRNA levels was assured by probing blots with the constitutively expressed *am*$^{+}$ gene of *N.crassa*. ^{32}P-labeled probes were prepared by oligolabeling (18).

Gel mobility shifts

An oligonucleotide of the sequence 5′-GACAACGCTC-CCCGAGAATGGTGTCATTCTCGTGACTTT-3′ and its complement, representing a single CYS3 binding site on the *cys-3*$^{+}$ promoter was end-labeled by T4 polynucleotide kinase with [γ-^{32}P]ATP, annealed and gel purified. The mutant site (Fig. 3A) had a single base change substituting the underlined G in the above sequence for a T. DNA binding and electrophoresis conditions have been previously described (6).

RESULTS AND DISCUSSION

Modification of CYS3 to a heterodimeric form

An important question with regard to the sulfur regulatory system is whether CYS3 homodimers are responsible for the entire range of CYS3 regulatory functions or whether there are essential heterodimeric interactions between CYS3 and other induced or constitutively produced bZIP proteins. The approach used here was to modify CYS3 to preclude the possibility of interaction with other bZIP proteins.

In a number of cases, bZIP proteins have been mutated into alternative versions which preferentially heterodimerize (21–25). A principal method is through amino acid substitutions at positions *e* and *g* of the coiled coil heptad repeat (designated as *abcdefg*) that modify electrostatic interactions (Fig. 1). The modified proteins will remain as non-functional monomers when expressed separately *in vivo*, but can form a functional heterodimer when simultaneously expressed. In the case of CYS3, three *e* and *g* residues in the leucine zipper were reciprocally altered to either lysine or glutamate in each of two versions, CYS3A and CYS3B, as shown in Figure 1D.

In vitro tests were used for initial characterization of the CYS3A and CYS3B mutant proteins. CYS3A and CYS3B proteins were generated as either full-length (F) or truncated (T) versions by *in vitro* transcription and translation (Fig. 2A). In gel mobility shift assays, using the F and T forms there were no detectable dimeric complexes formed when CYS3A and CYS3B were tested separately (Fig. 2B). However, DNA-binding heterodimers could be detected when a cotranslated mixture of the proteins was tested [CYS3A(F) + CYS3B(T)] (Fig. 2B). The

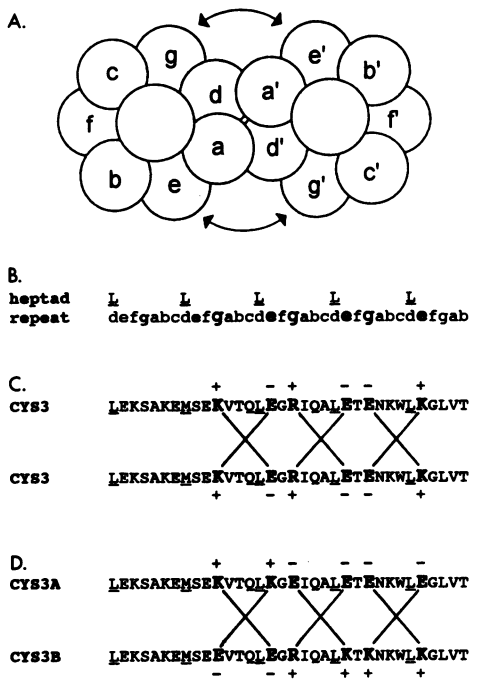

Figure 1. Leucine zipper dimerization mutants CYS3A and CYS3B. (**A**) End view of coiled-coil structure. Letters *a–g* designate amino acid positions in the coiled-coil. Arrows indicate putative electrostatic interactions between amino acids at the *e* and *g* positions. (**B**) Heptad repeat nomenclature of coiled-coils (positions *a–g*). Leucines occur at the *d* positions of the heptad repeat. Residues at positions *e* and *g* represent the positions to be modified and are shown in bold. (**C**) Alignment of wild type CYS3 as a homodimer. Solid lines indicate putative electrostatic interactions between residues of opposite charges at *g* and *e* positions in a CYS3/CYS3 dimer. (**D**) Alignment of mutant versions of CYS3 as a heterodimer. The two versions of CYS3 (A and B) were produced by site directed mutagenesis as described.

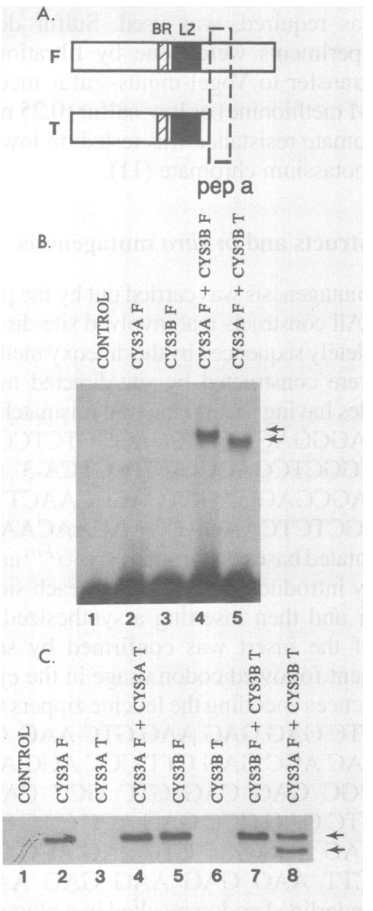

Figure 2. *In vitro* dimerization analysis of CYS3A and CYS3B. (**A**) Schematic diagram of CYS3 constructs used for *in vitro* analysis by gel mobility shifts and immunoprecipitation. The CYS3 protein is shown with BR (basic region) and LZ (leucine zipper) regions designated. Mutant or wild type CYS3A or CYS3B proteins were produced by *in vitro* transcription and translation as full length (F) or truncated (T) as described in Materials and Methods. Pep a represents a synthetic peptide used to generate an anti-CYS3 polyclonal antibody immunoreactive to the full-length (F) form but not the truncated (T) form. (**B**) Gel mobility shift analysis. ^{32}P-labeled DNA with the CYS3 binding site was incubated with *in vitro* translated unlabeled protein as indicated and electrophoresed. Lanes: 1, control, no RNA in translation mix; 2, full-length CYS3A(F); 3, full-length CYS3B(F); 4, full-length CYS3A(F) + full-length CYS3B(F); 5, full-length CYS3A(F) + truncated CYS3B(T). Note lack of DNA binding activity in lanes 2 and 3. Lane 4 has a single band (upper arrow) representing a CYS3A(F)/CYS3B(F) dimer. Lane 5 has a band of greater mobility (lower arrow) as compared to lane 4 and represents a CYS3A(F)/CYS3B(T) dimer which confirms the nature of the binding complex. (**C**) Immunoprecipitation. Anti-CYS3 (pep a) antibody was used to immunoprecipitate *in vitro* translated ^{35}S-labeled protein and was immunoreactive only to full-length versions of CYS3A and CYS3B (see Fig. 2A). Lanes: 1, control, no RNA; 2, full-length CYS3A(F); 3, truncated CYS3A(T); 4, full-length CYS3A(F) + truncated CYS3A(T); 5, full-length CYS3B(F); 6, truncated CYS3B(T); 7, full-length CYS3B(F) + truncated CYS3B(T); 8, full-length CYS3A(F) + truncated CYS3B(T). Note lack of immunoprecipitation in lanes 3 and 6 with truncated versions showing antibody specificity. Lanes 4 and 7 showed immunoprecipitation of full-length only (upper arrow), indicating no dimerization was occurring. Lane 8 showed co-immunoprecipitation of the truncated (lower arrow) with full-length version; i.e., indicating heterodimerization between CYS3A(F)/CYS3B(T).

CYS3A/CYS3B heterodimers retained the expected sequence specific DNA-binding properties as demonstrated by gel mobility shift assay with oligonucleotides containing normal (Fig. 2B) and mutated (data not shown) CYS3 binding sites. Immunoprecipitation studies confirmed the presence of CYS3A/CYS3B heterodimers and lack of homodimers. Heterodimers were detected by the co-immunoprecipitation of the truncated form of CYS3B by the full-length form of the other (CYS3A) (Fig. 2C). The anti-CYS3 antibody used was reactive only to the full-length version and not the truncated version (Fig. 2A). No co-immunoprecipitation took place when the individual forms of the monomers were tested [e.g., CYS3A(F) + CYS3A(T)] (Fig. 2C). Further, no co-immunoprecipitation took place between CYS3 and either of the CYS3A or CYS3B monomers (data not shown).

In order to test the function of CYS3A and CYS3B *in vivo*, transformation of the constructs into the Δ*cys-3* strain was carried out. Δ*cys-3* is a sulfur auxotroph and the recovery of viable transformants depends on the restoration of prototrophic growth. The Δ*cys-3* strain carries a deletion of both the basic region and leucine zipper in the *cys-3*$^+$ gene. Use of Δ*cys-3* eliminates any possibility of background interference with the introduced constructs. When the *cys-3A* and *cys-3B* constructs were used to transform Δ*cys-3* separately, no viable colonies were recovered. Only when both versions of *cys-3* (*A* and *B*) were simultaneously introduced into Δ*cys-3* by transformation were colonies recovered. The chromosomal integration of both versions of *cys-3* in the transformants was confirmed by Southern blot analysis (data not shown). In all cases, homokaryotic isolates of the primary transformants were used for phenotypic and Southern analysis. The CYS3A and CYS3B double transformants showed normal

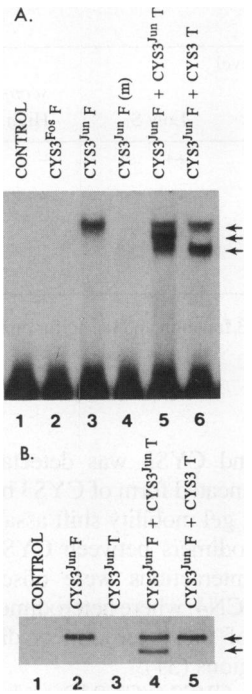

Figure 3. *In vitro* dimerization analysis of CYS3^{Jun}. (**A**) Gel mobility shift analysis. *In vitro* translated protein was incubated with ^{32}P-labeled DNA containing a CYS3 binding site. Lanes: 1, control, no RNA in translation mix; 2, full-length CYS3^{Fos}(F), control construct shows no DNA binding activity; 3, full-length CYS3^{Jun}(F), note band representing homodimer (upper arrow); 4, full-length CYS3^{Jun}(F), (m) represents mutated CYS3 site and the lack of detectable DNA binding confirms sequence specific binding by the construct; 5, full-length CYS3^{Jun}(F) + truncated CYS3^{Jun}(T), note presence of homodimeric [CYS3^{Jun}(F)/CYS3^{Jun}(F), upper arrow; CYS3^{Jun}(T)/CYS3^{Jun}(T); lower arrow] and heterodimeric [CYS3^{Jun}(F)/CYS3^{Jun}(T), middle arrow] complexes; 6, full-length CYS3^{Jun}(F) + truncated CYS3(T), note presence of only homodimeric complexes [CYS3^{Jun}(F)/CYS3^{Jun}(F), upper arrow and CYS3(T)/CYS3(T), lower arrow] and lack of heterodimer formation. (**B**) Immunoprecipitation. Anti-CYS3 antibody was used to immunoprecipitate *in vitro* translated ^{35}S-labeled protein. Full-length and truncated versions were generated as in Figure 2A. Lanes: 1, control; 2, full-length CYS3^{Jun}(F); 3, truncated CYS3^{Jun}(T); 4, full-length CYS3^{Jun}(F) + truncated CYS3^{Jun}(T), note co-immunoprecipitation of truncated (lower arrow) with full-length form (upper arrow) which demonstrates the presence of CYS3^{Jun}(F)/CYS3^{Jun}(T) dimers; 5, full-length CYS3^{Jun}(F) + truncated CYS3(T), note absence of immunoprecipitation of CYS3(T) showing a lack of dimerization with CYS3^{Jun}(F). Lanes 2 and 3 demonstrate the specificity of the antibody for CYS3^{Jun}(F) protein.

CYS3 regulatory functions (Table 1). Arylsulfatase and sulfate permease (reflected by chromate resistance) levels were regulated as in wild-type. The arylsulfatase and sulfate permease levels were used as measures of restoration of sulfur controlled structural gene expression. The level of *sulfur controller-2* mRNA, which is subject to feedback control by CYS3 was also normally regulated. In addition, the transformants showed wild-type autoregulation of *cys-3A* and *cys-3B* mRNA level. Further, the *cys-3A cys-3B Δcys-3* strain had a wild-type growth rate and no nutritional requirements or phenotypic changes were detectable.

The non-function of individual monomers (CYS3A or CYS3B) *in vivo* demonstrates clearly that no other cellular bZIP proteins were capable of interacting and restoring CYS3 function. Only when the complementary CYS3A and CYS3B versions were simultaneously introduced was prototrophic growth and sulfur gene regulation restored. These results eliminate the possibility that interactions with other bZIP proteins are essential for CYS3 function and establish the role CYS3 homodimers play. The *e* and *g* position substitutions have now been successful in a number of bZIP proteins (21–23). Although other interactions in the leucine zipper affect dimerization (26), changes in the *e* and *g* heptad repeat would appear to be a generally useful means of altering dimerization specificity for *in vivo* analyses.

Leucine zipper exchange to form a CYS3^{Jun} chimeric protein

Another type of leucine zipper modification was evaluated to provide confirming data regarding *in vivo* CYS3 function. Leucine zippers have been swapped between a number of bZIP proteins with the dimerization specificity following the particular leucine zipper (27,28). Two forms of CYS3 having Jun or Fos leucine zippers were constructed (i.e., CYS3^{Jun} and CYS3^{Fos}). With the Jun and Fos oncoproteins, it is known that Jun can homodimerize while Fos cannot and that Jun/Fos heterodimers can be formed (29,30). When the chimeric proteins were tested *in vitro*, CYS3^{Jun} bound DNA in gel mobility shift assays with normal sequence specificity as demonstrated by binding wild-type and non-binding to mutated CYS3 binding sites (Fig. 3A). That CYS3^{Jun} could homodimerize was shown by the formation of dimers between CYS3^{Jun} full-length (F) and CYS3^{Jun} truncated (T) in gel shift or immunoprecipitation assays (Fig. 3). Further, both gel mobility shift and immunoprecipitation experiments indicated that CYS3 and CYS3^{Jun} did not heterodimerize indicating that it would be unlikely for CYS3^{Jun} to interact with any putative bZIP protein normally dimerizing with CYS3 (Fig. 3B). The CYS3^{Fos} construct served as a control and did not bind DNA nor form homodimers, as would be expected based on the established properties of the Fos leucine zipper (9).

When *cys-3^{Jun}* was used to transform the *Δcys-3* strain, prototrophic transformants were recovered. As expected, the CYS3^{Fos} control construct did not transform the *Δcys-3* strain to prototrophy. As with the CYS3A/CYS3B heterodimeric constructs above, CYS3^{Jun} transformants showed recovery of the entire range of wild-type CYS3 functions (Table 1). The results from this swap provides additional evidence for a lack of essential *in vivo* heterodimeric interactions involving CYS3, but must be interpreted with caution since it is difficult to rule out the possibility that new bZIP interactions may have been generated with the CYS3^{Jun} construct.

Heptad repeat extension of the CYS3 leucine zipper

The final dimerization modification examined was to extend the CYS3 leucine zipper by exactly a single heptad repeat as has been done with GCN4 (31). To produce CYS3^{In}, an additional heptad repeat was inserted between the basic region and the first heptad repeat of the leucine zipper of CYS3. Figure 4A shows the sequence of the CYS3^{In} construct in relation to CYS3. Extending the leucine zipper produces a mutant protein which can form functional homodimers, but would not be predicted to form functional DNA-binding heterodimers with wild-type CYS3 subunits. The lack of DNA-binding activity expected from CYS3/CYS3^{In} heterodimers is a consequence of the misalignment of the DNA-binding basic regions adjacent to the leucine zipper in each subunit. Insertions of less than exactly a heptad repeat (e.g., 4, 5 or 6 amino acids) resulted in non-functional CYS3 constructs, thus demonstrating the importance of the relative spacing of the basic region–spacer–leucine zipper sequence (unpublished data).

Table 1. *In vivo* function of CYS3 leucine zipper constructs

	Arylsulfatase sp act[b]		Chromate resistance		mRNA level *cys-3*		*scon-2*	
Strain[a]	High S[c]	Low S[d]	High S	Low S	High S	Low S	High S	Low S
cys-3^{+} Δ*cys-3*	<0.05	7.1	+	–	–	++	–	+
cys-3^{Jun} Δ*cys-3*	<0.05	6.4	+	–	–	+	–	+
cys-3^{In} Δ*cys-3*	<0.05	7.2	+	–	–	++	–	+
cys-3A cys-3B Δ*cys-3*	<0.05	6.9	+	–	–	++	–	+

[a]Note: not shown are the *cys-3*^{Fos} Δ*cys-3*, *cys-3A* Δ*cys-3*, *cys-3B* Δ*cys-3* strains which lack detectable CYS3 function and are sulfur auxotrophs.
[b]Expressed as nmol/min/mg total protein.
[c]High-sulfur medium with 5.0 mM methionine.
[d]Low-sulfur medium with 0.25 mM methionine.

In vitro testing revealed that CYS3^{In} bound DNA in gel mobility shift assays with normal sequence specificity as shown by binding a wild-type CYS3 site and non-binding to a mutated CYS3 binding site (Fig. 4). The dimerization of CYS3^{In} was demonstrated by the formation of dimers between CYS3^{In} full-length (F) and CYS3^{In} truncated (T) forms in gel shift or immunoprecipitation assays (Fig. 4B and C). Heterodimerization between CYS3^{In} and CYS3 was detectable by co-immunoprecipitation of a truncated form of CYS3 by full-length CYS3^{In} (Fig. 4C). However, gel mobility shift assays did not detect any DNA-binding heterodimers between CYS3^{In} and CYS3 (Fig. 4B). Comparable interactions were observed with similarly modified forms of GCN4, where heterodimer formation occurred but DNA-binding of the heterodimers did not occur due to misaligned basic regions (31).

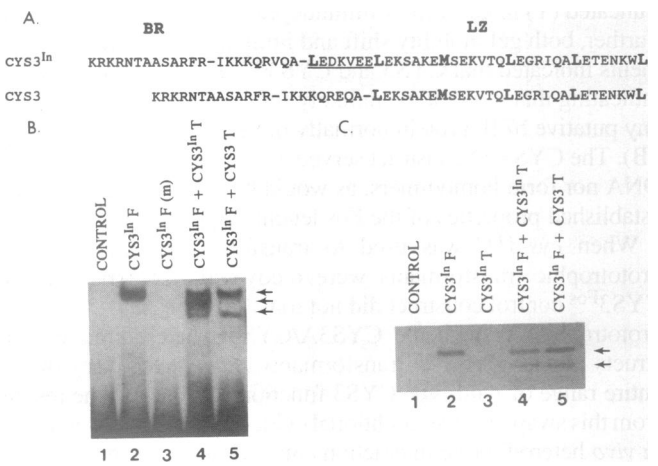

Figure 4. *In vitro* dimerization analysis of CYS3^{In}. (**A**) Sequence of CYS3^{In} as compared to CYS3 showing predicted alignment of basic region–spacer–leucine zipper segment. Inserted heptad repeat is underlined. The designations BR and LZ refer to the basic region and leucine zipper, respectively. (**B**) Gel mobility shift analysis. ^{32}P-labeled DNA with the CYS3 binding site was incubated with *in vitro* translated unlabeled protein as indicated and electrophoresed. Lanes: 1, control, no RNA in translation mix; 2, full-length CYS3^{In}(F); 3, full-length CYS3^{In}(F), (m) represents mutated CYS3 site and the absence of DNA binding confirms sequence specific binding by the construct; 4, full-length CYS3^{In}(F) + truncated CYS3^{In}(T), note presence of homodimeric [CYS3^{In}(F)/CYS3^{In}(F), upper arrow; CYS3^{In}(T)/CYS3^{In}(T); lower arrow] and heterodimeric [CYS3^{In}(F)/CYS3^{In}(T), middle arrow] complexes; 5, full-length CYS3^{In}(F) + truncated CYS3(T), note presence of homodimeric complexes [CYS3^{In}(F)/CYS3^{In}(F), upper arrow and CYS3(T)/CYS3(T), lower arrow] and lack of any DNA-binding heterodimers. (**C**) Immunoprecipitation. Anti-CYS3 antibody was used for the immunoprecipitation of *in vitro* translated ^{35}S-labeled protein. Full-length and truncated versions were produced as shown in Figure 2A. Lanes: 1, control; 2, full-length CYS3^{In}(F); 3, truncated CYS3^{In}(T); 4, full-length CYS3^{In}(F) + truncated cys3^{In}(T), note co-immunoprecipitation of truncated (lower arrow) with full-length form (upper arrow) which demonstrates the presence of CYS3^{In}(F)/CYS3^{In}(T) dimers; 5, full-length CYS3^{In}(F) + truncated CYS3(T), note presence of immunoprecipitation of CYS3(T) demonstrating that dimerization occurs. Based on the results in (B, lane 5), above, the CYS3^{In}(F)/CYS3(T) heterodimers are non-functional for DNA-binding.

As with both the CYS3A/CYS3B and CYS3^{Jun} constructs, CYS3^{In} was used to transform Δcys-3 and was capable of restoring the Δcys-3 strain to prototrophy. In addition, CYS3^{In} transformants showed recovery of the full range of wild-type CYS3 functions (Table 1). As found with the other constructs, there were no detectable changes in overall nutritional requirements (i.e., a normal growth rate occurred on minimal medium) or in development (e.g., conidial formation, necessary for homokaryon purification, was normal) of the transformants. Heterodimeric interactions of CYS3^{In} with other bZIP proteins would appear to be unlikely since such interactions would require the alignment of basic regions between subunits having leucine zippers with differing numbers of repeat units and altered dimerization specificity. Extension of the leucine zipper provides, therefore, another potentially useful class of modification for *in vivo* analysis. Taken together, the three types of dimerization modification provide convincing evidence for the absence of essential *in vivo* CYS3 heterodimeric interactions.

In conclusion, the dimerization experiments presented here demonstrate that CYS3 functions *in vivo* as a homodimer and not by dimerizing with a constitutively produced bZIP protein or one induced by CYS3 or growth conditions. Non-essential interactions cannot be ruled out at this point, but seem unlikely due to the normal growth and development observed on minimal medium in transformants expressing the modified forms of CYS3. A major question with regard to bZIP proteins has involved the definition of *in vivo* interactions involving leucine zipper-mediated dimerization. The general adaptability of bZIP proteins to the modification of dimerization specificity, as shown by CYS3, provides a valuable approach for the dissection of regulatory interactions.

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