Vol.4 No.1
Winter 1993

MOLECULAR ANALYSIS OF THE SULFUR REGULATORY CIRCUIT OF *NEUROSPORA CRASSA*

B.G. Yaghmai* and G.A. Marzluf**

*Department of Biochemistry, Kerman School of Medicine, Kerman, Islamic Republic of Iran
**Department of Biochemistry, Ohio State University, Columbus, Ohio 43210, U.S.A

Abstract

The sulfur regulatory circuit of the filamentous fungus, *Neurospora crassa,* consists of a set of unlinked structural genes which encode sulfur catabolic and two major regulatory genes which govern their expression. The *cys-3* regulatory gene encode a transacting regulatory protein which activates the expression of *cys-14* and *ars,* whereas the other regulatory genes *Scon-1* and *Scon-2* appear to act in a negative fashion.

Introduction

Structural genes within the sulfur control circuits of *Neurospora crassa* are subject to global regulation by the positive-acting CYS3 regulatory protein, a master regulatory protein which governs the expression of an entire set of unlinked stuctural genes which specify catabolic enzymes catalyzing various reactions of sulfur metabolism. The CYS3 protein appears to be a member of the bzip class of DNA-binding proteins. The DNA binding domain of CYS3 is bipartite and is composed of a leucine zipper, which is responsible for dimerization of CYS3 monomers, and an adjacent upstream basic region which appears to make direct contact with DNA. Chemical cross-linking studies have revealed that CYS3 exists as a dimer, and have also revealed the importance of individual leucine residues and their correct spacing within the zipper structure. Although methionine can largely replace leucine in a zipper structure, a mutant CYS3 protein with a pure "methionine zipper" shows reduced function *in vivo* and decreased dimerization and reduced DNA-binding *in vitro*. The zipper is not only essential for dimerization but it also plays a crucial role in aligning the basic region for productive DNA binding.

Keywords: Amino acid synthesis; Neurospora Crassa

Materials and Methods

Neurospora crosses were done according to standard procedures with corn meal agar of Westergaard medium [13]. The *Neurospora Crassa* wild-type strain 740R231A and the *cys-3* mutant (allele p22) were obtained from the fungal Genetic Stock Center, University of Kansas Medical Center, Kansas City. A *cys-3* temperature-sensitive nevertant (allele 65t) was described [14], and two scon mutants were obtained from R.L. Metzenberg. Liquid cultures of *N.Crassa* were grown at 30°C as discribed previously [6,9].

Results and Discussion

The Sulfur Regulatory Circuit

The sulfur regulatory circuit of the filamentous fungus, *Neurospora crassa,* consists of a set of unlinked structural genes which specify sulfur catabolic enzymes. Synthesis of this entire family of enzymes, which includes two sulfate permease species, aryl sulfatase, choline sulfatase, a methionine-specific permease, and an

extracellular alkaline protease, only occurs when the available sulfur becomes limited [1-2]. Expression of these catabolic enzymes is controlled by three regulatory genes, *scon-1*, *scon-2*, and *cys-3* [3-4]. Both *scon-1* and *scon-2* appear to act in a negative fashion, and, as would be expected, mutants of either of them show constitutive synthesis of the sulfur catabolic enzymes [3-5]. The *cys-3* regulatory gene encodes a transacting regulatory protein which activates the expression of *cys-14* and *ars*,, which encode sulfate permease II and aryl sulfatase, respectively, as well as all of the other coregulated structural genes. The cellular content of *cys-14* mRNA and of *ars* mRNA is highly regulated by sulfur repression and by the *cys-3* and *scon-1* genes [6-7], and implies that both *cys-14* and *ars*, and presumably, all of the other structural genes of the sulfur circuit, are controlled at the level of transcription. Of special interest is the unexpected finding that expression of the *cys-3* regulatory gene is itself highly regulated by sulfur availability and by the negative-acting *scon-1* gene [8-9]. Remarkably, *cys-3* also appears to control its own expression, via positive autogenous regulation [8-9].

The CYS-3 Regulatory Gene Encodes a Bzip Protein

The *cys-3* gene has been cloned and its complete nucleotide sequence has been determined [8-10]. Expression of the *cys-3* regulatory gene is itself highly regulated by sulfur availability and by the negative-acting *scon-1* gene. Northern blot experiments showed that *cys-3* mRNA content was greatly elevated in amount in wild-type cells which had been subjected to sulfur limitation [8]. It has been suggested that *cys-3* may autogenously regulate itself in a positive manner. Mutants which lack a functional *cys-3* protein do not turn on *cys-3* expression and contain only a low level of *cys-3* mRNA under all growth conditions examined [8]. The available evidence suggests a complex regulatory mechanism: the negative-acting *scon* genes repress *cys-3* expression when sulfur is readily available to the cells. Upon sulfur limitation, this negative control is lifted and *cys-3* is turned on, giving rise to a *cys*3 protein which itself further increases *cys-3* expression, yielding an increasing pool of *cys*3 protein which activates the expression of the various unlinked structural genes (See Figure 1).

The *cys-3* gene appears to encode a protein composed of 236 amino acids. In one alanine-rich region, the *cys3*

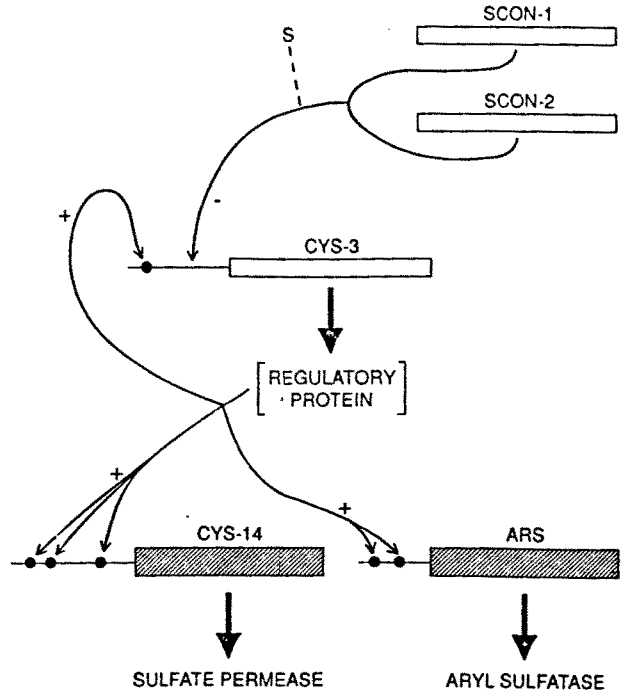

Figure 1. Molecular Model for Operation of the Sulfur Regulatory Circuit of *Neurospora*. The *scon* genes are visualized to encode products which negatively control *cys-3* when sulfur is readily available. The *cys-3* gene encodes a trans-acting protein DNA-binding protein which via positive autogenous control increases *cys-3* expression, and also turns on the expression of *cys-14*, *ars*, and other coregulated sulfur genes. Three CYS3 binding sites upstream of *CYS-14* and a single site serving *cys-3* have been identified; binding sites for *ars* have not been defined.

protein shows homology to histone H1 [8]. A deleted form of the *cys-3* gene, which lacked the sequences which encode the alanine-rich region, was constructed to determine whether this region was required for function *in vivo*. The deleted *cys-3* gene was found to be capable of transforming *cys-3* mutant strains. Thus, the *cys*3 protein lacking the alanine-rich region has partial function, but it is less active than the complete *cys*3 protein (kanaan and Marzluf, unpublished results).

The CYS3 protein appears to be a member of the bzip class of DNA-bindng proteins and possesses a motif similar to that found in the yeast GCN4 protein, the mammalian oncoprctein, FOS, and other members of this group (Figure 2). The CYS3 DNA-binding domain is bipartite and consists of a leucine zipper which is responsible for dimerization of two CYS3 monomers

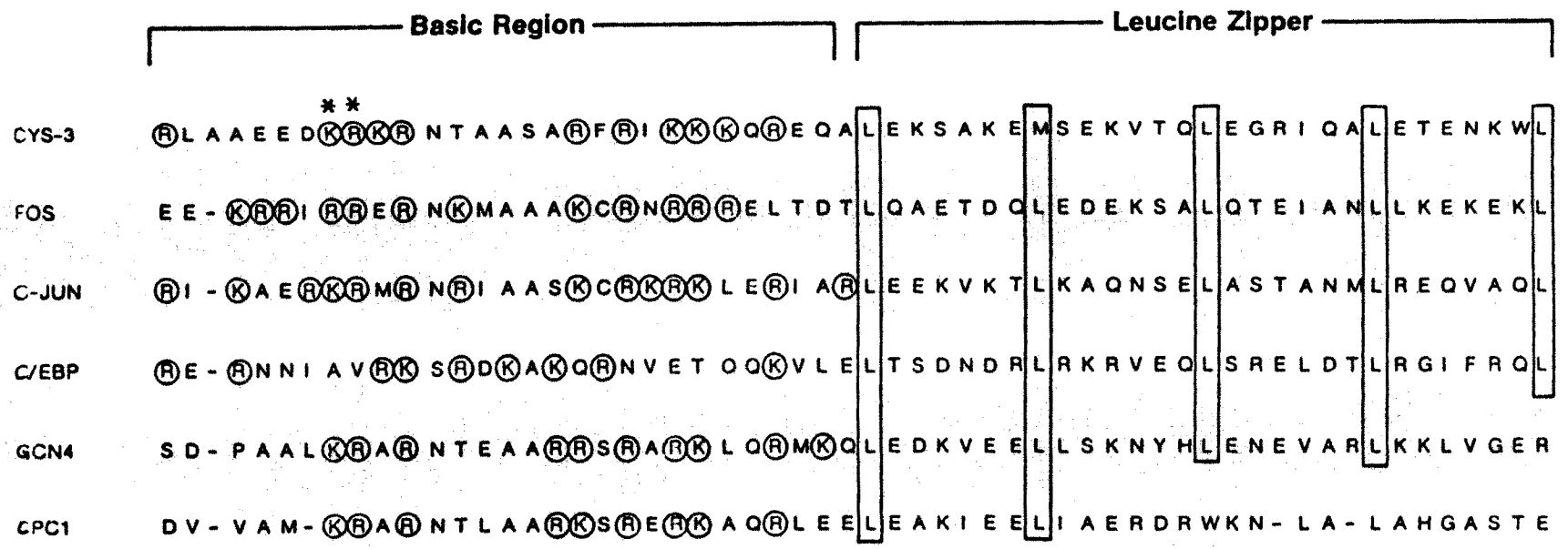

Figure 2. CYS3 is a member of the bzip class of DNA-binding proteins. The leucine zipper, consisting of leucine residues in heptad repeat is shown for various regulatory proteins from fungi and mammalian cells. The leucine zipper provides the dimerization surface for these proteins. Upstream of the leucine residues (boxed) is the basic region which serves as the DNA-contact surface (basic residues are boxed). Two basic residues which lead to loss of CYS3 function when substituted by glutamine are starred.

and an immediately adjacent upstream basic region which appears to make direct contact with DNA. Cross-linking experiments have shown that the CYS3 protein does exist as a dimer, and that a smaller CYS3 protein, encompassing only the bzip region, is also capable of dimer formation.

CYS3 is a DNA-Binding Protein

The **CYS3 protein has been expressed in** *E. coli* and used to examine DNA-binding with gel mobility shift experiments and with DNA footprint analyses [9], as shown in Figure 3. CYS3 binds *in vitro* to three distinct sites upstream of *cys-14*, the sulfate permease structural gene, and to a single site in the 5' promoter region of the *cys-3* gene itself [9]. A shorter version of the CYS3 protein containing only the bzip domain binds to these same sites with the same specificity and affinity as the full length protein, demonstrating that DNA recognition by CYS3 is determined solely by the bzip domain and does not involve other regions of the protein [10].

The three CYS3 binding sites upstream of *cys-14* and the single site upstream of *cys-3* show only limited sequence homology, although all contain at least one and most multiple copies of a core sequence, CAT. These repeated CAT sequences appear to provide a limited dyad symmetry, which may represent the central core of a CYS3 binding site; e.g., the first binding site upstream of *cys-14* has a central ATGCCAT sequence [9]. In agreement with this concept, substitutions which altered either the 5' ATG or the 3' CAT bases of this core sequence resulted in approximately an 80% loss in recognition by the CYS3 protein [11]. Since the CYS3 protein clearly has a global responsibility of turning on an entire family of coregulated genes, presumably at different rates and to different extents, it is not surprising that it is capable of recognizing different sequences.

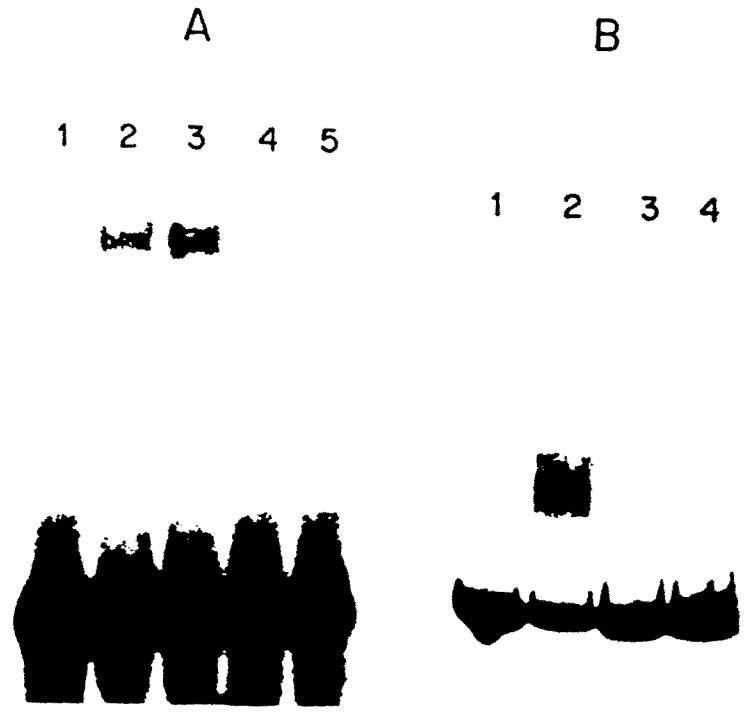

Figure 3. DNA binding by the *E. coli*-expressed CYS3 protein. Gel mobility shift experiments shown here reveal that the wild-type, but not mutant forms, of CYS3 bind to DNA. A 25-nucleotide base pair double-stranded DNA fragment corresponding to the DNA recognition element in the promoter region of *cys*-14 was synthesized. Panel A, Lanes: 1, DNA only; 2, DNA + 1X wild-type CYS3; 3, DNA + 1.5X wild-type CYS3; 4 and 5, DNA + 1.5X mutant CYS3 proteins. Panel B, competition studies, Lanes 1, DNA only; 2, DNA + CYS3 protein; 3, DNA+CYS3 protein+ nonradioactive DNA; 4, DNA + CYS3 protein+nonradioactive *cys*-14 promoter element. These results show that CYS3 binds to DNA in a sequence specific manner.

Major questions which remain unanswered concern the minimum requirement for a CYS3 binding site and the basis for different binding affinities.

Studies of CYS3 Mutants

A conventional *cys-3* mutant gene which had the expected "null" phenotype was cloned and sequenced. Its nucleotide sequence revealed that two basic amino acids within the basic region of the bzip motif were replaced by glutamine in the mutant CYS3 protein of this strain [8]. This mutant protein was expressed in *E. coli* and shown to be incapable of DNA-binding [9]. Site-directed mutagenesis has recently been employed to change various highly conserved amino acids in both the leucine zipper and in the basic region to assess whether they are essential for CYS3 function *in vivo* and for DNA-binding *in vitro*. Substitution of glutamine or glycine for several of the basic amino acids within the positively charged region upstream of the zipper abolished *cys-3* function *in vivo*. Replacing even a single basic amino acid with glutamine resulted in a loss of *cys-3* function [10]. Moreover, expressed CYS3 proteins corresponding to each of these basic region mutants were wild-type with regard to dimerization, but were completely deficient in DNA-binding to both the *cys-3* and the *cys-14* recognition elements [10]. These results support the concept that the basic region constitutes the DNA binding surface. The finding that these mutant CYS3 proteins with changes in the basic region readily dimerize reinforces the concept that dimerization depends only upon the leucine zipper motif and does not involve the basic region.

The CYS3 protein contains a heptad repeat motif designated as a putative "leucine zipper" although its second position is occupied by methionine (See Figure 2). The effect of amino acid substitutions within this putative zipper structure was found to depend upon the position within the zipper and the nature of the substitutions, some replacements being compatible with function, others eliminating *cys-3* function [10]. The change of any single leucine to methionine gave a functional CYS3 protein with activity similar to that of wild-type. Single valine substitutions at positions 1 or 2 destroyed CYS3 function, although valine replacements at posititon 3 or 4 were tolerated; however, a single more drastic change, e.g., a lysine substitution, even at a position 4, led to a nonfunctional CYS3 protein [10]. The substitution of valine at two positions within the zipper led to a nonfunctional CYS3 protein, whereas two or more methionine residues could be substituted in zipper positions with retention of *cys-3* activity. CYS3 proteins which possessed a complete "methionine zipper", i.e., had methionine at all five positions, showed reduced function *in vivo* and decreased dimer formation and reduced DNA-binding *in vitro*. Thus, it appears that leucine plays a special role in a so-called "leucine zipper", although some amino acids, e.g., methionine or isoleucine [10-12], can partially substitute for leucine. Some changes in the zipper e.g. substitution of valine at the first zipper position, permitted CYS3 dimer formation but prevented DNA-binding and, as expected, were nonfunctional *in vivo*. Similarly, changes in the spacing between the zipper and the basic region resulted in a CYS3 protein that formed dimers but was inactive in DNA-binding. These results emphasize that the leucine zipper is not only required for dimer formation but is also crucial for properly positioning the basic region to permit productive DNA-binding.

One interesting question was whether both basic regions of a CYS3 dimer have to be functional in order to allow productive DNA-binding. To answer this question, a hybrid CYS3 dimeric protein was formed between two different monomers, both of which had a wild-type leucine zipper motif. However, one monomer had a wild-type basic region, whereas the other monomer had an amino acid replacement such that a dimer formed with it was inactive for DNA-binding. The hybrid dimer was completely incapable of binding DNA, thus showing that both basic regions of a CYS3 dimer must be functional for DNA-binding activity.

Numerous important questions remain concerning the sulfur regulatory circuit. Still unclear is the mechanism by which the presence of repressing cellular levels of sulfur is detected to control the expression of the *cys-3* gene. This molecular signal probably involves the negative-acting *scon*-1 and *scon*-2 gene products [3-5], but their mode of action is not yet established. Although it seems clear that the *cys-3* gene is subject to positive autoregulation, direct evidence is needed to establish this concept. It is clear that the CYS3 protein is a trans-acting DNA-binding protein; yet, it is unknown how the CYS3 protein, upon binding to upstream promoter elements, activates structural gene expression. It is conceivable that CYS3 interacts directly with RNA polymerase or with accessory factors such as the TATA-box protein, TFIID or TFIIB, another transcriptional

factor.

References

1. Hanson, M.A. and Marzluf, G.A. *Proc. Natl. Acad. Sci.* U.S.A., **72**, 1240-1244, (1975).
2. Pall, M.L. *Biochim. Biophys. Acta*, **233**, 201-214, (1971).
3. Burton, E.G. and Metzenberg, R.L. *J. Bacteriol.*, **109**, 140-151, (1972).
4. Marzluf, G.A. and Metzenberg, R.L. *J. Mol. Biol.*, **33**, 423-437, (1968).
5. Paietta, J.V. *Mol. Cell. Biol.*, **10**, 5207-5214, (1990).
6. Ketter, J.S. and Marzluf, G.A. *Mol. Cell. Biol.*, **8**, 1504-1508, (1988).
7. Paietta, J.V. *ibid.*, **9** 3630-3637, (1989).
8. Fu, Y.H. Paietta, J.V. Mannix, D.G. and Marzluf, G.A. *ibid.*, **9**, 1120-1127, (1989).
9. Fu, Y.H. and Marzluf, G.A. *J. Biol. Chem.*, **265**, 11942-11947. (1990).
10. Kanaan, M. and Marzluf, G.A. *Mol. Cell. Biol.*, **11**, 4356-4362, (1991).
11. Ketter, J.S.,Jarai, G., Fu, Y.H. and Marzluf, G.A. *Biochemistry*, **30**, 1780-1787, (1991)
12. Landschulz, W.H., Johnson P.F. and McKnight, S.L. Science, **243**, 1681-1688, (1989).
13. Davis, R.H. and Deserres, E.F. *Methods Enzymol.*, **17A.**, 79-143, (1970).
14. Marzluf G.A. and Motzenberg, R.L. Motzenberg, *J. Mol. Biol.*, **33**, 423-437, (1968).