Curr Genet (1996) 30: 298–304

ORIGINAL PAPER

Qunhui Li · George A. Marzluf

Determination of the *Neurospora crassa* CYS 3 sulfur regulatory protein consensus DNA-binding site: amino-acid substitutions in the CYS3 bZIP domain that alter DNA-binding specificity

Received: 21 April / 9 June 1996

Abstract CYS3 is the positive-acting global regulatory protein involved in the sulfur control circuit in *Neurospora crassa* and belongs to the family of bZIP DNA-binding proteins. Here we report a characterization of native DNA-binding sites recognized by CYS3. DNA footprinting experiments and systematic mutational analysis were used to define the consensus CYS3-binding sequence, 5′-ATGPuPyPuPyCAT, a 10-bp palindrome. The sequence 5′-ATGACGTCAT acts as a strong binding site, and all single nucleotide changes within this sequence resulted in a reduction, or even complete loss, of CYS3 DNA-binding. Site-directed mutagenesis was employed to study two uncharged residues, serine 113 and phenylalanine 116, in the basic region of the CYS3 protein bZip DNA-binding domain. Ser^{113} appears to be directly involved in a specific interaction with nucleotide 2 of the binding site, possibly by making a direct contact with this base, and Phe^{116} contributes significantly to DNA-binding affinity.

Key words *Neurospora crassa* · Sulfur regulation · bZip proteins · CYS3 · DNA-binding

Introduction

In *Neurospora crassa*, sulfur utilization is subject to sophisticated genetic and metabolic controls. When favored sulfur sources are not available, secondary sulfur sources such as inorganic sulfate, choline-0-SO_4, or tyrosine-0-SO_4 can be used for growth. Under conditions of sulfur limitation, sulfur-related permeases and catabolic enzymes, including two sulfate permeases, a methionine-specific permease, aryl sulfatase, choline sulfatase and an extracellular protease, are expressed (Metzenberg and Parson 1966; Marzluf 1970; Pall 1971; Hanson and Marzluf 1975; Marzluf 1994). CYS3 is a global positive-acting regulatory factor that is required to activate the expression of numerous unlinked structural genes which encode sulfur catabolic enzymes. The expression of the *cys-3* gene is itself sulfur-regulated and is also subject to autoregulation (Kanaan and Marzluf 1993).

Four CYS3-binding sites have been identified by DNase I footprinting experiments (Fu and Marzluf 1990). It was important to determine the specific nucleotides in these binding sites which are required for recognition by the CYS3 protein, since regions protected in DNase I footprints can extend beyond the essential element. Definition of a consensus CYS3-binding sequence will also facilitate the identification of regulatory elements associated with other sulfur-related genes. We present results below that demonstrate that a 10-bp palindromic sequence, 5′-ATGPuPyPuPyCAT, represents the CYS3 recognition element.

CYS3, which consists of 236 amino-acids residues, is a member of the bZIP protein family and binds DNA as a dimer (Kanaan and Marzluf 1991). The bZIP region (residues 87–160) of CYS3 is necessary and sufficient for specific DNA-binding (Kanaan et al. 1992). An X-ray crystallographic structure of GCN4, one of the well-studied members of the bZIP family, has been solved to high resolution (Ellenberger et al. 1992). It was proposed that many, if not all, bZIP proteins anchor within the major groove of their DNA-binding sites with a similar registration. Five highly conserved amino-acid residues in the basic region of bZIP proteins appear to directly contact bases of DNA, and these residues largely determine the DNA-binding specificity of GCN4 (Ellenberger et al. 1992). In the basic region of the CYS3 protein, Ser^{113} and Phe^{116} occur at the corresponding position of two of the five conserved residues, but differ from the amino acids usually found at these positions, with alanine rather than ser^{113} and either alanine or cysteine instead of Phe^{116}. Thus it was of considerable interest to determine what role, if any, Ser^{113} or Phe^{116} might play in the specificity or strength of CYS3 DNA-binding. Phe^{116} is also of particular interest for an-

Q. Li · G. A. Marzluf (✉)
Department of Biochemistry, The Ohio State University,
484 West 12th Avenue, Columbus, OH 43210, USA

Communicated by K. Esser

other reason. A CYS3 mutant protein (encoded by *cys-3* allele P22) has two basic amino-acid residues substituted with glutamine (K105Q; K106Q), and it fails to bind DNA in vitro and is nonfunctional in vivo (Fu et al. 1989). A revertant of this mutant, *cys-3* Rev 21, regained the ability to utilize inorganic sulfate for growth; in this revertant a third amino-acid substitution in the CYS3 protein has occurred, a replacement of Phe^{116} by tyrosine (unpublished results). We speculated that the presence of tyrosine at this position (residue 116) may in some fashion compensate for the binding strength lost in the original double mutant, thus restoring productive CYS3 DNA-binding. Results are presented which indicate that the amino acid at residue 116 effects binding-site strength and may contact a specific nucleotide within the CYS3-binding-site.

Materials and methods

CYS3 DNA-binding studies. Gel-mobility shift experiments wer conducted as described before (Fu and Marzluf 1990) except that DNA probes were added last to the binding mixture. The reactions were loaded to 4% native polyacrylamide gels and run at 200 V for 1–2 h. The gels were then dried and autoradiographed. For quantitative analysis, gels were scanned with a Betascope 603 (Blot Analyzer).

Chemical probing of DNA-protein interaction. DNA fragments were end-labeled with α-^{32}P, by Klenow DNA polymerase, and chemically modified with (1) dimethyl sulfate for restricted methylation of guanines or, (2) 4% vol/vol formic acid for partial depurination or (3) anhydrous hydrazine for partial depyrimidation. The detailed experimental procedures followed were as described by Brunelle and Schleif (1987). The modified DNA probes were then subject to CYS3-binding. The protein-bound and free DNA probes were separated by the gel mobility shift technique. Approximately 50–70% shifting of the total probe was achieved. The shifted DNA bands and free DNA probes were electroeluted from gel slices. The isolated DNA was treated with piperidine and resolved on a sequencing gel, along with Maxam-Gilbert G+A sequencing reactions (Maxam and Gilbert 1980). Band intensities were measured by densitometry.

Site-directed mutagenesis. Mutagenic oligonucleotides were obtained from DNA International, Inc. Site-directed mutagenesis was carried out by the method of Kunkel (1985), and desired changes were confirmed by dideoxy sequencing (Sanger et al. 1977). To generate the series of mutated CYS3 DNA-binding sites, a 120-bp DNA fragment which contains the *cys14* site A was employed as the template.

Expression of mutant CYS3 proteins. A 160-bp *StuI-XhoI* DNA fragment of the *cys-3*$^+$ gene in expression vector pc3T7-1 was replaced with the corresponding DNA fragment of each of the mutated *cys-3* genes; the mutations give rise to individual amino-acid substitutions. Overexpression of CYS3 proteins was achieved in *E. coli* host strain BL21(DE3)LysS. Partial purification of CYS3 was carried out as described by Fu and Marzluf (1990).

Transformation of Neurospora crassa and sulfate transport assays. Plasmids which contain either the wild-type or mutant *cys3* genes were transformed into protoplasts of the *cys3* null mutant P22 and plated on Vogel's medium (Akins and Lambowitz 1985; Cherniack et al. 1990). Only transformants that were able to utilize sulfate as the sole sulfur source can grow. Conidia were grown in Vogel's-minus-sulfate-medium supplemented with 0.25 mM methionine at 30°C for 10–12 h (Marzluf 1970). Samples of approximately 500 mg of mycelia were incubated with radioactive sulfate at 25°C for 5 min, collected on glass fibers and thoroughly washed with a nonradioactive sulfate solution. Radioactivity retained by the cells was determined by Scintillation counting. The dry weight of mycelial samples was obtained, and specific activity of sulfate permease is represented as counts per min per mg of mycelia.

Results

Characterization of natural CYS3-binding sites

Four CYS3-DNA-binding sites, designated A, B and C in the *cys-14* and D in the *cys-3* gene upstream promoter regions, were amplified by the polymerase chain reaction and subcloned to pBluescript. Only limited sequence similarity was evident among these four sites, previously recognized as *cys-14* sites 1, 2, and 3 and one *cys-3* site (Fu and Marzluf 1990). DNase I protection analysis previously identified the regions in which these binding sites were located. To more-precisely identify the sequence elements at the binding sites for mutation analysis, and to detect some CYS3-DNA contacts that are essential for binding, several chemical interference footprinting assays were undertaken. Methylation interference analysis was conducted with sites A and B. Representative results showing the interference footprint for site B are presented (Fig. 1 a) and the results obtained with both sites are summarized (Fig. 1 b). In site A, DNA-binding was strongly reduced by methylation of six guanine residues, located on both DNA strands, which define a 10-bp segment within the longer 17-bp sequence originally recognized by DNase I footprinting experiments (Fig. 1 b). A single G residue immediately adjacent to the DNase I footprint sequence was also identified by this interference experiment. Interference of CYS3 DNA-binding at site B was similarly observed by methylation of five closely spaced guanines within the even-longer (22 bp) DNase I footprint (Fig. 1 b).

We then undertook more sensitive missing-contact DNA footprinting experiments with all four CYS3-binding sites A, B, C and D. The results obtained with sites A and B were consistent with those described above and further helped to precisely identify the CYS3-binding sites and critical base contacts within the longer sequences earlier identified via DNase I footprints (Fig. 2). Significantly, two separate CYS3-binding sites (C1 and C2), 12 bp apart, were revealed in site C. The possibility that C represented duplex binding sites was suggested previously because the sequence revealed by DNase I footprinting was twice as long as that of site A and B (Fu and Marzluf 1990). Despite several attempts, we did not observe a clear interference pattern using depurination or depyrimidination assays with site D. However, inspection of site D suggests that it also contains two distinct binding sites, resembling C1 and C2, separated by 19 bp. In previous experiments, we demonstrated that the CYS3-binding affinity differs significantly among these four natural binding sites, D > C > B > A (Marzluf et al. 1995). These native binding sites all seem to be imperfect 10-bp palindromes, and strong similarity is observed between them when their half-sites are com-

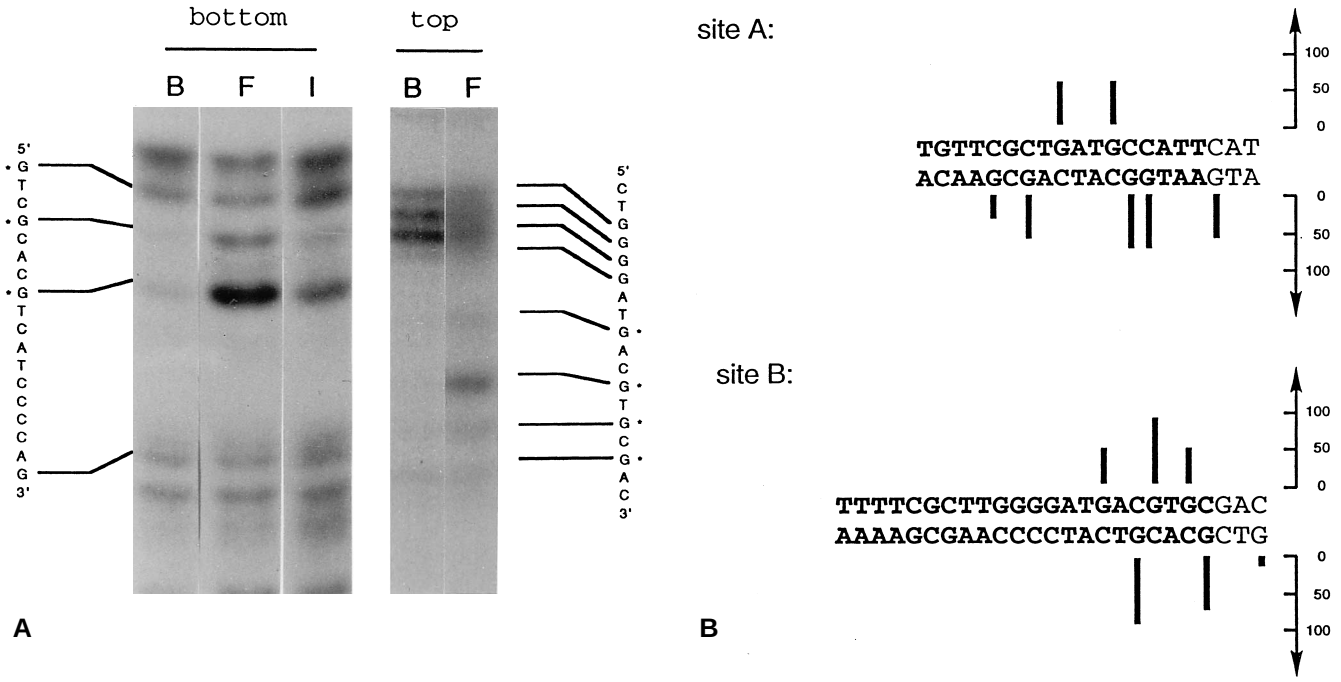


Fig. 1A, B Methylation interference of contacts between CYS3 protein and DNA-binding site. Panel **A** interference pattern for the top strand and bottom strand of CYS3-binding site B. *Lanes B* bound DNA isolated from DNA-protein complexes; *F* free DNA, not retarded by protein in a mobility shift assay; *I* input DNA (not subjected to protein binding). The sequences are aligned to identify bands that correspond to specific nucleotides. Nucleotides whose methylation have an effect upon CYS3 DNA-binding are indicated with *asterisks*. Panel **B** summary of methylation interference assays of CYS3-binding sites A and B. The band intensity of bound DNAs was compared with that of input probes, and the percentage of interference is represented by the height of the bar above or below individual nucleotides. The nucleotides in *bold face* were protected in previous DNase I protection footprints

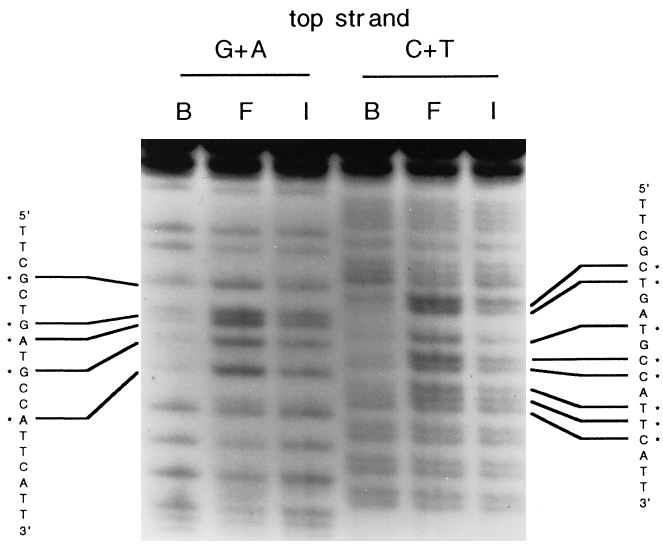
pared (Fig. 3). Based on these observations, we predicted that CYS3-binding sites are composed of two abutting five-nucleotide half-sites, 5′-ATGPuPy, and that the sequence 5′-ATGACGTCAT would constitute a strong CYS3-binding site (Fig. 3).

Mutational analysis of CYS3-binding sites

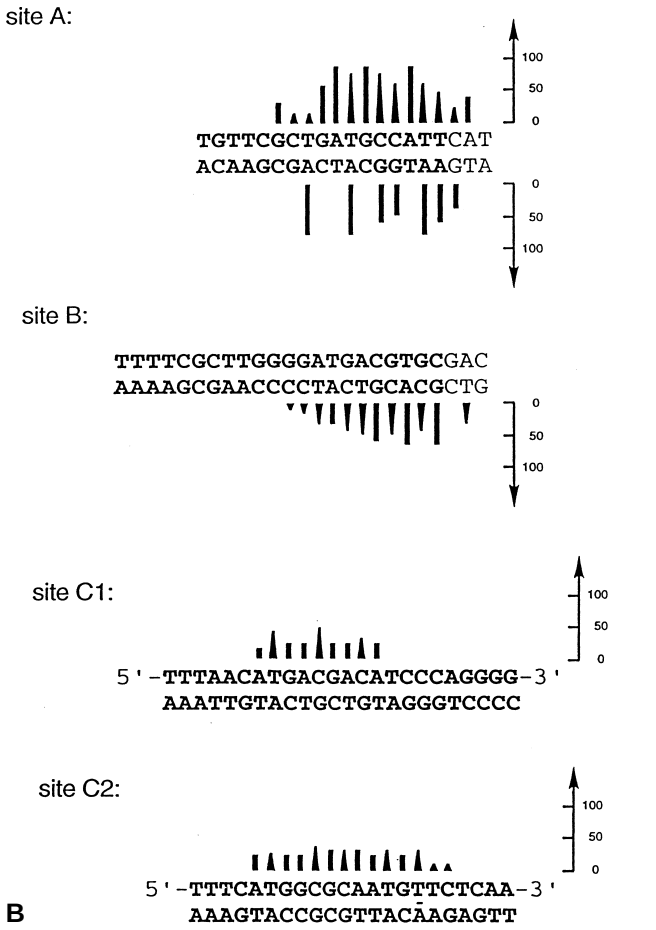
To test the prediction that the deduced consensus sequence is important for CYS3-binding, a series of mutations were introduced at individual nucleotide positions of a half-site of the presumed strong binding sequence 5′-ATGACGTCAT. To determine the relative affinities of the various CYS3- binding sites, quantitative mobility shift experiments were conducted. Identical concentrations of radioactively labeled DNAs containing the wild-type and mutant binding sites were incubated with the same amount of CYS3 protein under conditions in which the fraction of DNA bound was linear with respect to protein concentration. Under these conditions, the ratio of DNA in the shifted band to total DNA in EMSA reflects the relative affinity of the protein for the different DNA elements.

Table 1 describes these binding-site mutations and reports their relative binding affinity, compared with that of the starting sequence. Nucleotide substitutions for each of the five bases of the half-site within the starting sequence resulted in a decreased binding affinity for CYS3, some having drastic effects, others only moderately reducing the binding strength. Three different substitutions (G,T, or C) for the adenine at position 5 all resulted in a modest decrease in the affinity of the site for CYS3 (Table 1). Although no single substitutions were obtained at position 4, thymine at this position appears to confer strong binding and its substitution by guanine led to reduced CYS3-binding (Table 1, compare 4C and 23–2). To constitute a strong binding site, guanine is the preferred nucleotide at position 3, but substitutions for it are possible with the retention of some binding. Either adenine or guanine at position 2 allow strong CYS3-binding, whereas cytosine significantly reduced the binding-site affinity, as shown with sequence 1–29 (Table 1).

The central two bases found in the decanucleotide CYS3-binding site are 5′-PyPu-3′(a pyrimidine at 1, a purine at 1′); the sequences ATGA**CG**TCAT, ATGA**TG**TCAT, and ATGA**TA**TCAT are all functional but, listed in order of decreasing binding affinity, are 100%, 45% and 20% effective, respectively (Table 1). If either two purines or two pyrimidines occur at these central positions (1 and 1′), binding by the CYS3 protein is completely eliminated, which demonstrates the critical role played by these central bases. Perhaps surprisingly, a site (0–5) with the central bases reversed, ATGA**GC**TCAT, retained 12% of binding affinity. Significantly, when one central base pair (1′) was deleted, the resulting 9-bp overlapping palindromes (0–6 and 0–7), which resemble GCN4 recognition sites,



A



B

Fig. 2A, B Missing-contact DNA footprints. Panel **A** depurination and depyrimidation missing-contact footprints for the CYS3 protein and site A upper strand were conducted as described in Materials and methods. Lane designations are identical to those given in the legend of Fig. 1. Panel **B** summary of missing contact analyses of CYS3-binding sites A, B, C1 and C2. The data are presented in the same way as in Fig. 1; *bars* interference due to loss of purines; *triangles* interference due to loss of pyrimidines

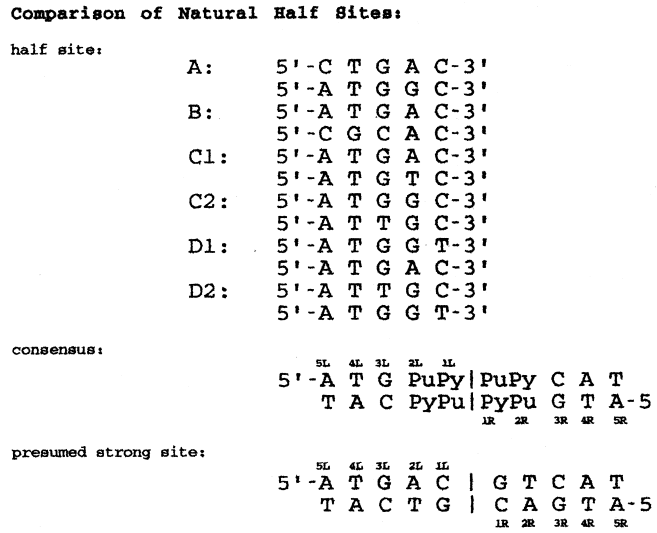


Fig. 3 Sequence of half-sites of the core sequence of CYS3-binding sites A, B, C1, C2 and putative D1 and D2 sites. The deduced CYS3 consensus binding site is shown and a strong CYS3 recognition sequence is given. *Pu* purine; *Py* pyrimidine

Table 1 Nucloetides in each sequence that differ from the starting sequence *(opt)* are underlined; *, deleted nucleotides. CYS3 protein (0.1 μg) and approximately the same amount of each DNA probe (2 – 5 ng) were used in individual binding reactions. The percentage of shifted DNA probe for each mutated sequence was determined as described in Materials and methods, and binding affinities are given relative to that obtained with the starting sequence, *opt*

DNA no.	Sequence	Rel. affinity (%)*
opt	ATGACGTCAT	100
4-C	CTGACGTCAT	57
4-G	GTGACGTCAT	68
4-T	TTGACGTCAT	46
23-1	CTTACGTCAT	33
23-2	CGGACGTCAT	5
23-3	CGTACGTCAT	13
23-4	CGCACGTCAT	33
0-1	ATGATGTCAT	45
0-2	ATGATATCAT	20
0-3	ATGAAGTCAT	0
0-4	ATGATATCAT	0
0-5	ATGAGCTCAT	12
0-6	ATGAC*TCAT	0
0-7	ATGAT*TCAT	0
1-31	ATGCCGTCAT	15
1-29	ATGGCGTCAT	82

were completely deficient for CYS3-binding (Table 1). This result confirms that the CYS3-binding site is a 10-bp palindromic sequence.

Native CYS3 half-binding sites include examples with sequences **C**TGAC, AT**T**AC and **CGC**AC, which differ from the starting sequence (opt) by 1, 1 and 3 nucleotides, respectively; each of these sites displays significant CYS3-binding.

A binding element (23–2) with the half site **CG**GAC, in which two positions (4 and 5) differ from the consen-

Table 2 Binding affinities are given relative to that obtained with the wild-type (wt) CYS3 protein with the opt binding site, which is assigned as 100%. Sulfate permease assays were conducted as described in Materials and methods and specific activities are reported compared to that obtained in transformants with the wild-type *cys-3*$^+$ gene. The triple mutant is K105Q K106Q F116Y. ND, not determined

CYS3 protein	Rel. binding affinity (%)		Phenotype
	ATGACGTCAT (opt)	ATGGCGTCAT (2-29)	Sulfate permease spec. activ. (%)
Wt	100	82	100
S113A	119	47	67
S113C	<5	<5	ND
S113G	12	<5	ND
S113F	0	0	ND
S113T	11	<5	ND
F116C	40	ND	38
F116G	<5	ND	ND
F116I	<5	ND	ND
F116N	<5	ND	ND
F116S	68	ND	39
F116V	10	ND	ND
F116Y	ND	ND	41
Triple	ND	ND	23

sus, binds the CYS3 protein very poorly (Table 1). In contrast, two binding sites (23–3 and 23–4) with the half-site sequences **CGT**AC and **CGC**AC, which have three nucleotide substitutions, have significant binding strength (Table 1). This shows that the effect of a particular nucleotide substitution differs depending upon the sequence context of other bases within the half-site, and suggests that the DNA bases and protein residues are capable of local structural adjustments to maintain maximum contacts and binding strength.

Specific interaction of CYS3 residue Ser113 with DNA

It was predicted that Ser^{113} within the basic region of the CYS3 protein bZIP domain might directly interact with a single nucleotide in the DNA-binding site. Site-directed mutagenesis was employed to obtain *cys-3* mutants which encoded proteins with serine 113 substituted for by alanine, cysteine, glycine, phenylalanine or threonine. The CYS3 proteins were expressed, partially purified, and subjected to DNA-binding studies as described in Materials and methods. The DNA probes used were "opt" (ATGACGTCAT) and "2–29" (ATGGCGTCAT), which differ only at position 2 (underlined). The relative binding affinity of each CYS3 protein with these two different binding sites is given in Table 2. The wild-type and three of the mutant CYS3 proteins bind to the opt site better than to the 2–29 sequence, which shows their preference for adenine over guanine at position 2 (Table 2). The S113F and S113C mutant proteins failed to bind at a detectable level to either DNA segment. Interestingly, the S113A mutant CYS3 protein displayed a 20% greater affinity for the opt site than did the wild-type CYS3 protein, and also showed significantly reduced binding to the 2–29 site. These results suggest that amino-acid residue 113 of CYS3 is involved in the recognition of nucleotide 2 of the binding site, possibly by making a direct contact with this base. This concept is consistent with the finding that both the wild-type protein and the S113A mutant protein show substantially reduced binding to the "1–31" sequence, which also differs only by having a C at nucleotide 2 (Table 1).

This group of mutant *cys-3* genes were also tested for their ability to transform the *cys-3* null mutant (allele P22) strain. The S113A mutant was capable of complementing the *cys-3* mutant to allow use of sulfate at an efficiency slightly lower than that achieved with the *cys-3*$^+$ gene. Transformants with the *cys-3* S113A gene possessed approximately 70% of sulfate permease activity, as compared to *cys-3*$^+$ transformants (Table 2). All of the other *cys-3* mutants with substitutions for Ser^{113} showed little or no DNA-binding in vitro and failed to complement the *cys-3* mutant.

Importance of CYS3 residue Phe116

Most bZIP proteins have either cysteine or serine at the position corresponding to CYS3 residue Phe^{116}, and only a few members of this family have an aromatic amino acid, phenylalanine or tyrosine, at this position (Ellenberger et al. 1992). This feature suggested that this residue might be important in the specificity of DNA-element recognition by CYS3 and other members of the bZIP protein family. Site-directed mutagenesis was used to obtain *cys-3* mutants which had substitutions of various amino acids for F116. Mutant CYS3 proteins with Phe^{116} replaced with cysteine or serine were capable of significant DNA-binding in vitro, 40% and 68%, respectively, when compared with wild-type CYS3. Moreover, these mutant *cys-3* genes (F116C and F116S) transformed the *cys-3* null mutant (allele P22) with an efficiency nearly equal to that achieved with the wild-type cys-3$^+$ gene; these transformants possessed approximately 40% of the wild-type level of sulfate permease activity (Table 2). The importance of CYS3 residue 116 was underscored by several other amino-acid substitutions. The cys-*3* mutant genes which result in replacement of F116 with valine, isoleucine, asparagine, or glycine are nonfunctional in vivo and their respective proteins bind DNA very weakly or else totally lack DNA-binding activity (Table 2).

A *cys-3* mutant F116Y was also created, but we were unable to express the F116Y CYS3 protein in *E. coli*, despite numerous attempts, because it inhibited the growth of the host cells. However, this mutant *cys-3* gene transformed the *cys-3* null mutant with an efficiency equivalent to the wild-type *cys-3*$^+$ gene (Table 2). In this context, it is significant that the *cys-3* double mutant (K105Q K106Q) gene is nonfunctional in vivo and the mutant protein is completely deficient in DNA-binding, whereas the triple mutant K105Q K106Q F116Y is functional in vivo and transforms the *cys-3* null mutant. Transformants of F116Y

and the triple-mutant *cys-3* gene showed 41% and 24% of the wild-type level of sulfate permease activity, respectively (Table 2). This result argues that the presence of tyrosine at residue 116 allows an otherwise-inactive CYS3 protein to be functional by restoring DNA-binding activity, and emphasizes the functional importance of uncharged amino-acid residues within the basic region of the bZIP DNA-binding domain.

Discussion

Previous DNase I footprinting experiments identified four CYS3-binding sites, three in the *cys-14* and one in the *cys-3* 5′ promoter region. Because of stearic hindrance, a DNA-binding protein such as CYS3 often protects a longer sequence than the nucleotides at the element which it actually contacts from DNase I digestion. Chemical probing has the advantage of largely avoiding stearic hindrance, and thus makes it possible to narrow down a binding site to a smaller and more precise region (Brunelle and Schleif 1987). Sequence-specific protein-binding to DNA can be inhibited by the methylation of guanines within, and immediately adjacent to, the actual binding site. Similarly, missing contact analysis can detect individual bases which are required for productive DNA-binding by a trans-acting protein. In the present studies, these chemical-modification types of DNA footprints allowed a more exact definition of CYS3- binding sites than was earlier observed via DNase I footprints. Of particular significance was the finding that the most distal CYS3-binding site (C) in the *cys-14* promoter identified by DNase I protection actually contains two separate binding sites separated by 12 bp. Similarly, site D in the *cys-3* promoter appears to contain two distinct CYS3-binding sites 19 bp apart. The fact that sites C and D are each actually duplex binding sites helps explain their considerably stronger affinity for the CYS3 protein than the single sites A or B. Since site D has the strongest affinity for CYS3, and appears to actually be two tandem sites, limited chemical modification of one site would be hidden by CYS3-binding to the adjacent intact one. Moreover, we have recently determined that the *cys-3* promoter has another more-proximal CYS3-binding site, 5′-ATTGTGTCAT (located centered at -60 with respect to the ATG start codon) which agrees very well with the consensus binding sequence. The presence of three binding sites may explain the failure to detect an interference footprint with site D since the binding of CYS3 protein to even one of these three elements would result in that DNA fragment being included in the shifted band during EMSA.

The binding sites recognized by bZIP proteins can be classified into two categories: (1) AP-1-like, with one central cytidine-guanine base pair as the dyad axis of an overlapping palindrome; (2) ATF/CREB-like, with two central C-G base pairs and adjacent half-sites (Hai and Curran 1991). In the present study we demonstrated that the cognate binding site for CYS3 is a 10-bp abutting palindrome; moreover, a strong CYS3-binding sequence is identical to an ATF/CREB-binding site. Isolation of GCN4 mutants with altered affinity for AP-1 or ATF/CREB sites demonstrated that the a-helical fork between the GCN4 DNA-binding surface and its leucine zipper is important for half-site spacing preferences. The position corresponding to leucine 247 of GCN4 is particularly relevant in this regard. The ATF/CREB class of bZIP proteins usually have a lysine at this position, while AP-1 proteins do not. GCN4 binds to an AP-1 site with 2–5-times greater affinity than to an ATF/CREB site (Gartenberg et al. 1990; Sellers et al. 1990; Kim et al. 1993). In contrast, CYS3, which has a lysine at this pivotal position, has an absolute requirement for ATF/CREB-like spacing, which suggested that the a-helical fork between the DNA-binding surface and its leucine zipper might be less flexible than that of GCN4. In the crystal structure of the GCN4/AP1 complex, Arg243 from one of the monomers interacts with the single central G of the AP-1 site by the formation of two hydrogen bonds (Ellenberger et al. 1992). Since arginine at this position is absolutely conserved among bZip proteins, we assume that Arg117 in each CYS3 monomer (which correspond to Arg243 of GCN4) contacts one of the two central guanine residues. If one central G is substituted by A, only a single hydrogen bond will be formed between Arg17 and the adenine base, resulting in a weaker protein-DNA interaction. If both central G bases are replaced with A, the binding affinity will be reduced even more. This concept is supported by the fact that the DNA-binding sites ATGAC^{1}G^{1}TCAT, ATGAT^{1}G^{1}TCAT, and ATGAT^{1}A^{1}TCAT showed 100%, 45%, and 20% of relative CYS3-binding affinity, respectively.

According to the model for the GCN4 protein DNA binding, van der Waals interaction between GCN4 alanine 239 and a thymine methyl group specifies position 1 of its half-site as adenine (Ellenberger et al. 1992). Our results suggest that amino-acid residue 113 of CYS3 might be involved in the recognition of the nucleotide at position 2 (and presumably also 2′) of the binding site. The wild-type CYS3 protein has a dual preference for either A or G at position 2, while the CYS3 mutant protein S113A binds A^{2} even better and discriminates against G^{2}. Thus, our molecular studies suggest the possibility that a direct contact may occur between this particular amino acid (residue S113 of CYS3) and positions 2 and 2′ of the DNA recognition site, and underscore the importance of uncharged amino acids within the bZip basic region in determining the specificity and strength of DNA-binding. However, direct structural studies of the DNA-protein complex are necessary to determine actual contacts between individual amino acids and nucleotide bases.

The specificity of DNA-binding of bZip proteins is largely determined by uncharged residues within the basic region adjacent to the leucine zipper; however, the basic residues contribute significantly to the binding strength by non-specific ionic interaction with the phosphate backbone. These binding interactions seem to be additive. Thus, a mutant CYS3 protein with a glutamine substitution for one basic residue displays a temperature-sensitive DNA binding, whereas a CYS3 protein with two basic residues

replaced by glutamine is completely non-functional in DNA binding (Fu and Marzluf 1990; Kanaan et al. 1992). The CYS3 F116Y mutant protein appears to function as well as, or even better than, wild-type CYS3 and, as a third-position gain-of-function mutation, F116Y is able to compensate for some of the binding strength lost in the substitution of glutamine for two basic residues. The residue Phe^{116} is important in CYS3 DNA-binding, and most substitutions for it eliminated or reduced DNA binding. In contrast, when tyrosine occupies this position (residue 116), it contributes extra binding strength to the DNA-protein complex. The -OH group introduced by tyrosine may allow the formation of a new H-bond that increases the strength of the protein-DNA interaction.

The in vivo function of the CYS3 proteins can be examined indirectly by the expression of the *cys-14* gene, which has an absolute requirement for activation by CYS3. Our results demonstrated that there is good agreement between the in vitro DNA-binding ability of wild-type and various mutant CYS3 proteins and the sulfate permease activity of the corresponding transformants.

Acknowledgments This research was supported by National Institutes of Health GM-23367. We thank Elena Storozhuk for assistance with site-directed mutagenesis and Tso-Yu Chiang for help with DNA-binding studies.

References

Akins RA, Lambowitz AM (1985) General method for cloning *Neurospora crassa* nuclear genes by complementation of mutants. Mol Cell Biol 5:227–2278

Brunelle A, Schleif RF (1987) Missing contact probing of DNA-protein interactions. Proc Natl Acad Sci USA 84:6673–6676

Cherniack AD, Garriga G, Kittle JD, Lambowitz AM (1990) Function of *Neurospora* mitochondrial tyrosyl tRNA-synthetase in RNA splicing requires an idiosyncratic domain not found in other synthetases. Cell 62:745–755

Ellenberger TE, Brandl CJ, Struhl K, Harrison SC (1992) The GCN4 basic-region leucine zipper binds DNA as a dimer of uninterrupted a helices: crystal structure of the protein-DNA complex. Cell 71:1223–1237

Fu YH, Marzluf GA (1990) cys-3, the positive-acting sulfur regulatory gene of *Neurospora crassa*, encodes a sequence-specific DNA-binding protein. J Biol Chem 265:11942–11947

Fu YH, Paietta JV, Mannix DG, Marzluf GA (1989) Cys-3, the positive-acting sulfur regulatory gene of *Neurospora crassa*, encodes a protein with a putative leucine zipper DANN-binding element. Mol Cell Biol 9:1120–1127

Gartenberg MR, Ampe C, Steitz TA, Crothers DM (1990) Molecular characterization of the GCN4-DNA complex. Proc Natl Acad Sci USA 87:6034–6038

Hai T, Curran T (1991) Fos/Jun and ATF/CREB cross-family dimerization a lters DNA-binding specificity. Proc Natl Acad Sci USA 88:3720–3724

Hanson MA, Marzluf GA (1975) Control of the synthesis of a single enzyme by multiple regulatory circuits in *Neurospora crassa*. Proc Natl Acad Sci USA 72:1240–1244

Kanaan M, Marzluf GA (1991) Mutational analysis of the DANN-binding domain of the CYS3 regulatory protein of *Neurospora crassa*. Mol Cell Biol 11:4356–4362

Kanaan MN, Marzluf GA (1993) The positive-acting sulfur regulatory protein CYS3 of *Neurospora crassa*: nuclear localization, autogenous control, and regions required for transcriptional activation. Mol Gen Genet 239:334–344

Kanaan M, Fu YH, Marzluf GA (1992) The DANN-binding domain of the CYS3 regulatory protein of *Neurospora crassa* is bipartite. Biochemistry 31:3197–3203

Kim J, Tzamarias D, Ellenberger TE, Harrison SC, Struhl K (1993) Adaptibility at the protein-DNA interface is an important aspect of sequence recognition by bZip proteins. Proc Natl Acad Sci USA 90:4513–4517

Kunkel TA (1985) Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc Natl Acad Sci USA 82:488–492

Marzluf GA (1970) Genetic and biochemical studies of distinct sulfate permease species in different developmental stages of *Neurospora crassa*. Arch Biochem Biophys 138:254–263

Marzluf GA (1994) Genetics and molecular genetics of sulfur assimilation in the fungi. Adv Genet 31:187–206

Marzluf GA, Li QH, Coulter K (1995) Global regulation of sulfur assimilation in *Neurospora*. Can J Bot 73: S167–S172

Maxam AM, Gilbert W (1980) Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol 65:499–558

Metzenberg RL, Parson JW (1966) Altered repression of some enzymes of sulfur utilization in a temperature-conditional lethal mutant of *Neurospora*. Proc Natl Acad Sci USA 55:629–635

Pall ML (1971) Amino acid transport in *Neurospora crassa*. II. Properties and regulation of a methionine transport system. Biochim Biophys Acta 233:201–214

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74: 5463–5467

Sellers JW, Vincent AC, Struhl K (1990) Mutations that define the optimal half-site for binding yeast GCN4 activator protein and identify an ATF/CREB-like repressor that recognizes similar DNA sites. Mol Cell Biol 10:5077–5086