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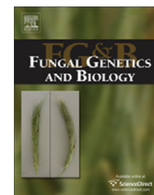

DNA-binding specificity of the CYS3 transcription factor of *Neurospora crassa* defined by binding-site selection

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ABSTRACT

The CYS3 transcription factor is a basic region-leucine zipper (bZIP) DNA-binding protein that is essential for the expression of a coordinately regulated group of genes involved in the acquisition and utilization of sulfur in *Neurospora crassa*. An approach of using binding-site selection from random-sequence oligonucleotides was used to define CYS3-binding specificity. The derived consensus-binding site of ATGGCGCC AT defines a symmetrical sequence (half-site A T G/t G/a C/t) that resembles that of other bZIP proteins such as CREB and C/EBP. By comparison, CYS3 shows a greater range of binding to a central core of varied Pur–Pyr–Pur–Pyr sequences than CREB as determined by gel shift assays. The derived CYS3 consensus binding sequence was further validated by demonstrating *in vivo* sulfur regulation using a heterologous promoter construct. The CYS3-binding site data will be useful for the genome-wide study of sulfur-regulated genes in *N. crassa*, which has served as a model fungal sulfur control system.

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1. Introduction

In the filamentous fungus *Neurospora crassa*, the synthesis of sets of enzymes in global carbon, nitrogen, phosphorus, and sulfur anabolism and catabolism are highly regulated (Davis, 2000). The sulfur-regulatory circuit of *N. crassa* provides a model fungal system for studying both coordinate gene regulation and how a cell regulates its sulfur status (i.e., intracellular sulfur levels) (reviewed by Paietta, 2004). An important part of the sulfur-regulatory system involves the positive regulatory gene, *cys-3⁺* (Paietta et al., 1987; Fu et al., 1989). *cys-3* mutants show a loss of expression of the entire set of sulfur-regulated enzymes (e.g., arylsulfatase) and are sulfur auxotrophs. *cys-3* mutants are also affected in a range of cellular properties such as nucleotide pools, energy charge, and ascospore viability (Pall and Robertson, 1988; reviewed by Paietta, 2004). Such pleiotropic effects have led to the suggestion of a broad regulatory role for CYS3. The *cys-3⁺* gene has been cloned (Paietta et al., 1987), and subsequent work has shown the encoded CYS3 product to be a 25.9 kDa basic region-leucine zipper (bZIP) protein that is functional as a homodimer *in vivo* (Paietta, 1995). CYS3 levels appear to be modulated by interaction with the SCON2 protein which allows for selective targeting of CYS3 for proteolytic destruction as directed by the N-terminal domain (NTD) of SCON2 (now termed the F-box) (Kumar and Paietta, 1998; Sizemore and Paietta, 2002).

Several genes under sulfur-regulated control (including the autoregulated *cys-3⁺* gene) have been cloned and characterized with respect to the binding of CYS3 to promoter elements. A consensus binding sequence of ATNPurPyrPurPyrCAT determined from DNase I footprinting studies of the *ars-1⁺* and *cys-3⁺* genes has been proposed (Shuler and Paietta, 1990; Shuler, 1993). Based on mutational studies of CYS3-binding sites within the *cys-3⁺* and *cys-14⁺* promoters a consensus-binding site of ATG-PurPyrPurPyrCAT was proposed (Li and Marzluf, 1996). Although useful, these proposed binding sites are based on a small number of DNA sequences from genes known to be regulated by CYS3 and have limited *in vivo* validation. Available data suggests that CYS3 binding may be tolerant of a number of sequence variations, particularly in the central core of the binding site (Paietta, 2004). By way of comparison, the homologous protein in *Aspergillus nidulans*, METR, may have a similar binding site based on the ability of a chimeric *metR* construct containing the CYS3 DNA-binding domain being able to transform a *metR* deletion strain to prototrophy (Natorff et al., 2003). In contrast, the binding site in *Saccharomyces cerevisiae* for the MET4 bZIP protein appears to be quite different (consensus sequence TCACGTG; reviewed by Thomas and Surdin-Kerjan, 1997).

An alternate method to determine the sequence requirements for a DNA-binding protein involves the *in vitro* selection of binding sites from random-sequence oligonucleotides by affinity chromatography (Oliphant and Struhl, 1987; Oliphant et al., 1989). An advantage of *in vitro* selection is that a large number of potential binding sites are present within the

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random-sequence DNA pool and; further, that a single protein–DNA interaction (e.g., CYS3) has specified the selected sequences. Using affinity binding or similar techniques, a number of DNA-binding proteins have had their sequence recognition properties defined (Oliphant et al., 1989; Merika and Orkin, 1993; Kojima et al., 2006; Jiang et al., 2007; Viola and Gonzalez, 2007). Current knowledge of the CYS3-binding site would be considerably expanded by the random-sequence approach and allow for new strategies in deciphering the sulfur-regulatory network. The value of such data is further enhanced by (i) the continuing expansion of the regulatory role and importance of CYS3 within sulfur metabolism (reviewed by Paietta, 2004); and (ii) the current availability of the Neurospora genomic sequence (Galagan et al., 2003) which will allow for genome-wide analysis of the sulfur control system (including combining gene expression profile data with binding site data for higher selectivity, as described by Fujibuchi et al., 2001).

In the study presented here, the *in vitro* selection of binding sites from random oligonucleotides has been used to more accurately define the CYS3 recognition sequence. In addition, the sulfur-regulatory function of the derived consensus CYS3-binding site has been demonstrated *in vivo* using a heterologous promoter system.

2. Materials and methods

2.1. Binding site selection from random-sequence oligonucleotides

The selection of CYS3-binding sites from random-sequence oligonucleotides followed the methodology of Oliphant et al. (1989). Basically, oligonucleotides containing 22 nucleotides of random-sequence DNA flanked by BamHI and PstI sites were synthesized on an Applied Biosystems 391EP DNA synthesizer and made double stranded by mutually primed synthesis. CYS3 protein was purified as previously outlined (Paietta, 1992) and was coupled to CNBr-activated Sepharose (Oliphant et al., 1989). A total of four cycles of affinity chromatographic purification were carried out as described for GCN4 (Oliphant et al., 1989), and the affinity-selected oligonucleotides concentrated by ethanol precipitation. Subsequently, the selected oligonucleotides were inserted into the pTZ18 vector. A total of 50 random clones were chosen for further study (25 were used from each of two repetitions of the selection protocol). Single-stranded DNA from each isolate was isolated following M13-K07 superinfection and sequenced by the dideoxy method. Sequencing was done in both directions for each clone; however, the sequence data are always shown aligned from the same orientation (as primed from the BamHI side) (Fig. 1).

2.2. Gel-mobility shifts

Oligonucleotides representing the binding sites to be tested were synthesized (Applied Biosystems 391EP synthesizer), labeled by T4 polynucleotide kinase with [γ-^{32}P]ATP, annealed, and gel purified. DNA-binding assays were carried out as we have described previously using *Escherichia coli* produced CYS3 protein (Paietta, 1992) or CREB protein (Williams et al., 1993) (generous gift of O. Andrisani, Purdue University). Specificity of binding was ensured by control experiments using competition by addition of excess unlabeled DNA. Four percent PAGE gels with a 50 mM Tris–80 mM glycine–2 mM EDTA (pH 8.5) running buffer were electrophoresed at 20 mA with the temperature maintained at 4 °C. Quantitation of gel shift assays was performed by using a Molecular Dynamics Phosphorimager.

2.3. Plasmid constructs

In order to construct the heterologous promoter used to test potential sequences for sulfur-regulated expression, the *qa-2*$^+$ (catabolic dehydroquinase) promoter from *N. crassa* carried in pVK57 (Alton et al., 1978) was fused to the *lacZ* gene in pDE1 (which also contains a truncated *his-3* gene for targeted integration at the *his-3* locus; Ebbole, 1990) which was modified to remove a BglII site. Site-directed mutagenesis by the phosphorothiolate method was used to introduce BglII and PpuMI sites flanking the binding site for the quinic acid transcriptional regulator (QA1F) in the *qa-2*$^+$ promoter to generate the pQZ-11 test vector. Introduction of selected binding site cassettes was carried out by synthesis of complementary oligonucleotides containing the desired CYS3 site and flanking restriction sites (BglII and PpuMI), annealing, digestion with BglII + PpuMI, and cloning into similarly digested pQZ-11 vector. Note that genes, including *qa-2*$^+$, regulated by the quinic acid control system are not responsive to changes in sulfur level in the medium (data not shown).

2.4. Transformation and lacZ assays

Neurospora crassa his-3 (Y155M261; Fungal Genetics Stock Center strain #462) was transformed with the pQZ-11 promoter constructs by the Novozyme 234 spheroplasting technique (Vollmer and Yanofsky, 1986). The isolation of prototrophs following transformation provides selection for insertions at a defined chromosomal location (i.e., the *his-3*) locus, as integration of pQZ-11 at *his-3* recreates an active *his-3*$^+$ gene. The targeted integration allows for accurate comparisons between constructs. Homokaryotic transformants were isolated by filtration of iodoacetate-grown conidial cultures (Ebbole and Sachs, 1990). Sulfur derepression and repression experiments were carried out using homokaryons grown with 0.25 mM methionine (low sulfur) or 5.0 mM methionine (high sulfur), respectively, as described (Paietta, 1992). The enzyme linked immunosorbent assay (ELISA) for bacterial β-galactosidase used a microtiter plate assay of crude *Neurospora* extracts incubated with primary antibody to bacterial β-galactosidase and secondary biotinylated antibody (to the anti-β-galactosidase antibody), incubation with streptavidin-conjugated alkaline phosphatase, and finally a *p*-nitrophenyl phosphate reaction was carried out as previously described (Paietta, 1992). The crude extracts from Neurospora were prepared by homogenization in extraction buffer (0.25 M Tris, pH 7.8, and 1.0 mM phenylmethylsulfonyl fluoride) and clarifying by centrifugation as described (Paietta, 1992). Values for promoter activity are set relative to the base construct which is not responsive to the level of sulfur in the medium and which contains the quinic acid transcriptional control site.

3. Results and discussion

3.1. Binding-site selection

In the work described here, *in vitro* selection by affinity chromatography was used to isolate CYS3-binding sites from random-sequence oligonucleotides. The random-sequence approach has allowed for assessment of the nature of the CYS3 DNA recognition sequence in an unbiased manner. A compilation of the sequences selected by the CYS3 protein is presented in Fig. 1. The sequences in Fig. 1 are all oriented as generated from sequencing using a primer near the BamHI site of the pTZ18 vector. The aligned 10 base boxed sequence in Fig. 1 highlights only those nucleotide positions showing a significant deviation from a random distribution based on the LOD score ($\log_{10}$ of the probability that the nucleotide pattern would be observed

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