Nucleotide Sequence, Messenger RNA Stability, and DNA Recognition Elements of *cys*-14, the Structural Gene for Sulfate Permease II in *Neurospora crassa*[†,‡]

James S. Ketter,[§] Gabor Jarai, Ying-Hui Fu, and George A. Marzluf*
Departments of Biochemistry and Molecular Genetics, The Ohio State University, Columbus, Ohio 43210
Received September 19, 1990; Revised Manuscript Received November 9, 1990

ABSTRACT: The complete nucleotide sequence of the *cys*-14 gene which encodes sulfate permease II, a member of the sulfur regulatory circuit, is presented. The *cys*-14 gene contains four introns with consensus splice site sequences and is transcribed from four closely spaced initiation sites located approximately 20 bp upstream of the ATG initiation condon. The translated CYS14 protein is composed of 781 amino acids with a molecular weight of 87 037 and contains 12 potential hydrophobic membrane-spanning domains. *cys*-4 mRNA was found to turn over with a half-life of approximately 15 min, which presumably contributes to the regulation of sulfate permease II function. The *cys*-14 gene is highly expressed, but only in cells subject to sulfur limitation, and is turned on by the positive-acting CYS3 sulfur regulatory protein. Results are presented which show that CYS3 protein binds with higher affinity to DNA fragments which contain two or three tandem copies of a binding site sequence. Analyses of binding site specificity via mutated binding site elements showed that different regions of the partially symmetrical CYS3 binding site are important for recognition by the CYS3 regulatory protein.

The sulfur regulatory circuit of the filamentous fungus *Neurospora crassa* is composed of a set of genes which encode enzymes of sulfur metabolism that are only expressed when cellular levels of sulfur become limited. These sulfur catabolic enzymes include aryl sulfatase, choline sulfatase, an extracellular protease, and various permeases which are responsible for the uptake of sulfur metabolites from the environment (Metzenberg & Parson, 1966; Marzluf, 1970, 1972a; Pall, 1971; Hanson & Marzluf, 1975).

Sulfur catabolite repression exerts a global, parallel control over the entire set of sulfur catabolic enzymes, and is mediated by at least two control genes. A major positive-acting control genes, *cys*-3, encodes a regulatory protein which is required to activate the structural genes of the circuit (Marzluf & Metzenberg, 1968; Paietta et al., 1987). The CYS3 protein possesses a leucine zipper and an adjacent basic region, which together constitute a DNA binding domain (Fu et al., 1989; Fu & Marzluf, 1990). A distinct negative-acting control gene appears to prevent *cys*-3 expression when cellular levels of sulfur are high (Burton & Metzenberg, 1972). Thus, a cascade of regulatory elements act within the sulfur circuit and include sulfur repression mediated by *scon* that controls the expression of the *cys*-3 control gene, which in turn regulates the various structural genes of the entire circuit.

N. crassa possesses two distinct sulfate transport systems, which can readily be distinguished by simple biochemical properties, and which appear to be encoded by separate, unlinked genes (Marzluf, 1970). The dual sulfate transport systems are both members of the sulfur circuit and are controlled by sulfur repression and by both the *cys*-3 and *scon* control genes. Both sulfate permease species are also developmentally regulated. Sulfate permease I, encoded by the *cys*-13 gene, occurs primarily in conidiospores, whereas permease II, encoded by *cys*-14, is mainly found in mycelia (Marzluf, 1970). Interestingly, the sulfate transport systems turn over with a half-life of approximately 2 h, whereas aryl sulfatase, one of the cytoplasmic sulfur catabolic enzymes, appears to be very stable (Marzluf, 1972b).

The structural gene for sulfate permease II, *cys*-14, has been cloned, and its expressison has been shown to be highly regulated at the level of mRNA accumulation by sulfur repression and by the *cys*-3 and *scon* genes (Ketter & Marzluf, 1988). The expression of the related enzyme, aryl sulfatase, has also been found to occur at the level of mRNA accumulation (Paietta, 1989), thus suggesting that the entire set of structural genes of the sulfur circuit is controlled at the transcription level. The *cys*-3 regulatory protein has been expressed in *Escherichia coli* and shown to be a sequence-specific DNA binding protein which binds at three distinct sites in the promoter region of *cys*-14 (Fu & Marzluf, 1990).

It is apparent that a detailed characterization of representative structural genes will contribute to our understanding of the operation of the sulfur regulatory circuit. We present here the entire nucleotide sequence of the *cys*-14 gene and its flanking regions. The *cys*-14 gene appears to contain 4 introns and to encode a large protein which contains 12 potential helical membrane spanning domains, suggesting that it may reside in the cell membrane and act as a sulfate ion transporter. Studies of the expression and turnover of *cys*-14 mRNA are also presented which imply that the synthesis of sulfate permease II is rapidly modulated when environmental circumstances change. Finally, the results of experiments to define the specificity of CYS-3 binding to the *cys*-14 promoter DNA recognition elements are reported.

Materials and Methods

Nested Deletion Subclones. Three DNA fragments of the *cys*-14[+] gene region were subcloned into pBLUESCRIPT KS(+), and nested deletions were created by first treating the plasmid DNA with two restriction enzymes, one of which leaves a 3′ overhang. This DNA was then digested in a controlled fashion using exonuclease III, followed by treatment with mung bean nuclease, prior to closure with T4 DNA ligase

[†] This research was supported by U.S. Public Health Service Grant GM-23367 from the National Institutes of Health.
[‡] The nucleic acid sequence in this paper has been submitted to GenBank under Accession Number J05321.
* Address correspondence to this author of the Department of Biochemistry, The Ohio State University, 484 West 12th Ave., Columbus, OH 43210.
[§] Present address: National Institutes of Health, Building 37, Bethesda, MD 20892.

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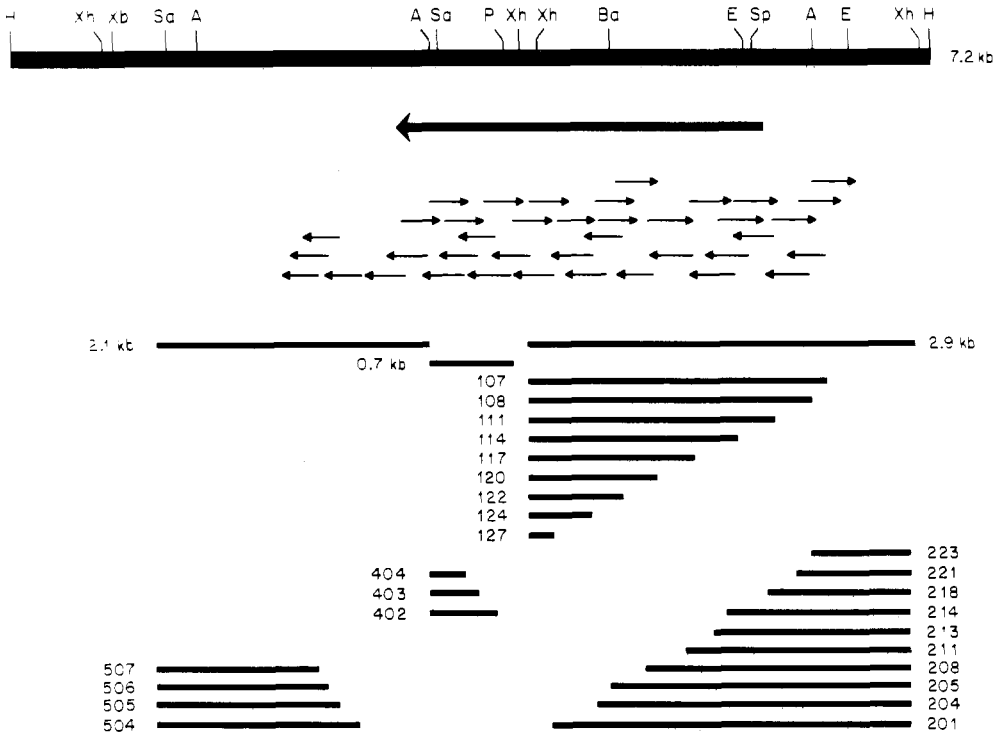


FIGURE 1: Physical map and DNA sequencing strategy for the *cys*-14 gene. The boldface arrow under the restriction map of the gene region displays the direction of transcription. The thin arrows reveal the sequencing strategy which employed deletion clones and oligonucleotide primers. At the botton are displayed the three subcloned fragments (2.1, 0.7, and 2.9 kb) and deletion subclones derived from them, each with its numerical designation. The bars represent the DNA segment which is present in each of the deletions. Restriction sites: H, *Hin*dIII; Xh, *Xho*I; Xb, *Xba*I; Sa, *Sal*I; A, *Aaa*I; P, *Pst*I; Ba, *Bam*HI; e, *Eco*RI; and Sp, *Sph*I.

and subsequent transformation into competent *E. coli* cells.

DNA Sequencing and Primer Extension Mapping. DNA sequencing was accomplished by the dideoxy chain termination method (Sanger et al., 1977) using [α-^{32}P]dATP and a modified T7 bacteriophage DNA polymerase, "Sequenase" (U.S. Biochemical Corp.). Nucleotide sequences were read from autoradiograms using a digitizer ("Gel reader") and analyzed by Pustell software (International Biotechnologies). Oligonucleotide primers were synthesized on an Applied Biosystems Model 380B DNA synthesizer.

S1 nuclease mapping was used to map the 3′ end of the *cys*-14 transcript. A 2.1-kb band-isolated *Sal*I fragment was labeled by fill-in with the Klenow fragment of DNA polymerase and hybridized with mRNA at 55 °C, and mapping was conducted with the procedure of Birk and Sharp (1977).

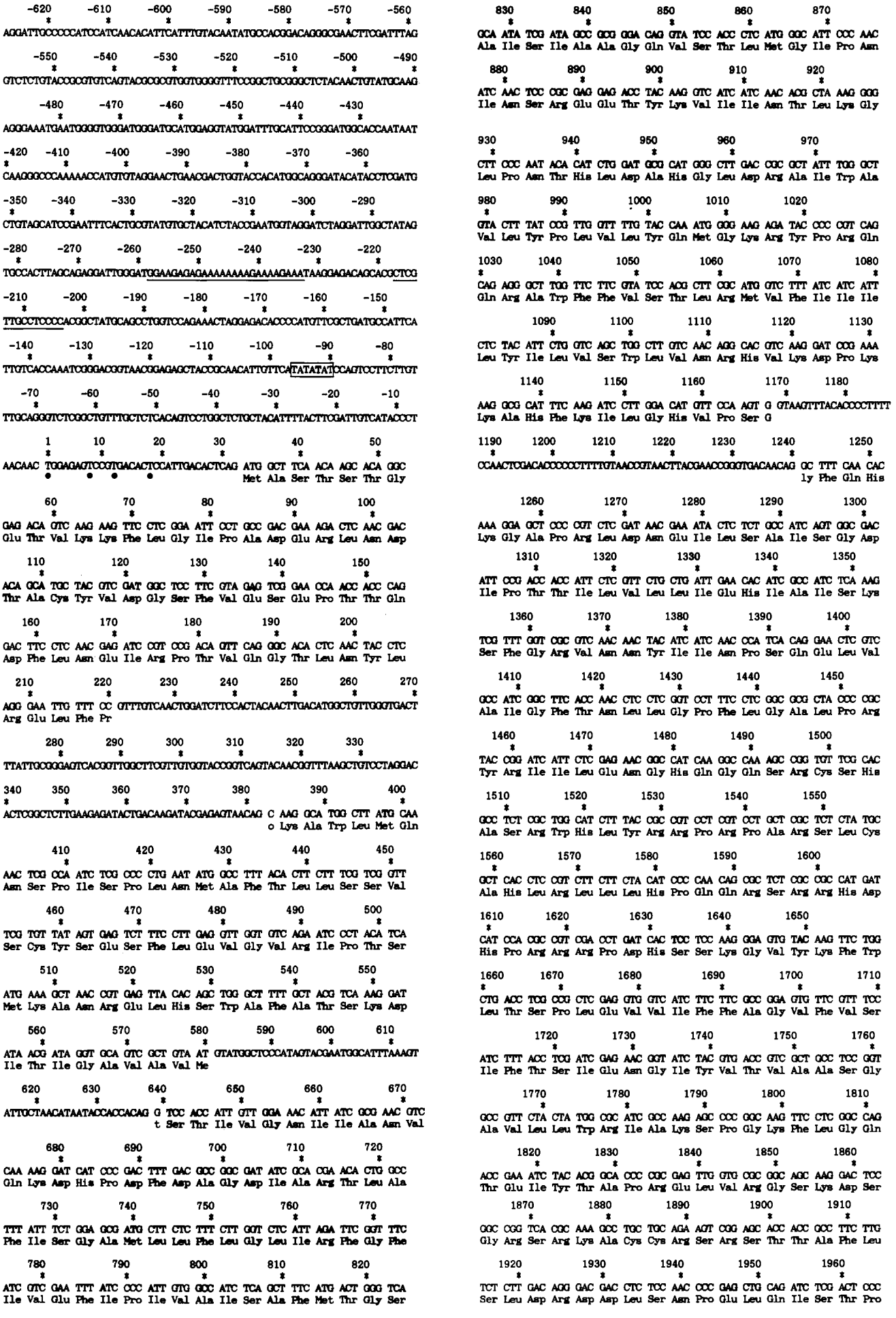
The 5′ end of the *cys*-14 transcript was mapped by primer extension. One hundred nanograms of a primer with the sequence 5′-TTCGTCGGCAGGAATTCC-3′ was added to 10 μg of poly(A+) RNA in 45 μL of 1.1× M-MLV reverse transcriptase buffer [1× buffer consists of 50 mM Tris (pH 7.5)/75 mM potassium chloride/10 mM dithiothreitol/3 mM magnesium chloride]. This mixture was heated to 65 °C for 2 min and then cooled slowly (over 30–60 min) to 30 °C. The mixture was then incubated for another 15 min at 30 °C, followed by the addition of 0.5 μL each of 10 mM dCTP, dGTP, and dTTP, 2.5 μL of [α-^{32}P]dATP, and 200 units of M-MLV reverse transcriptase. The reaction was allowed to proceed at 37 °C for 25–30 min. The extended primer products were recovered by the addition of 2.5 μL of 1 M NaCl and 130 μL of ethanol, followed by freezing in liquid nitrogen and pelleting in a microfuge. The pellet was redissolved in 10 μL of water, and 2 μL was mixed with 2 μL of DNA sequencing loading buffer and analyzed on a 6% denaturing polyacrylamide gel.

Screening a λgt10 cDNA Library. Poly(A+) RNA from cells grown under low sulfur conditions (for the derepression of sulfur circuit genes) was isolated and used for the preparation of a cDNA library in the *Eco*RI site of the λgt10 vector. This library represented approximately 10^6 clones after amplification, and had a phage titer of approximately 5×10^9 pfu/mL. Approximately 750 000 plaques were screened at high density. After three rounds of screening, pure plaques of the desired recombinant phage clones were isolated. The cDNA inserts were cloned into the *Eco*RI site of pBLUESCRIPT and analyzed by DNA sequencing.

Dot Blot Analysis. *Neurospora crassa* total RNA was isolated by the method of Reinert et al. (1981) from wild-type cells grown on media containing either 5 mM methionine (sulfur repression) or 0.25 mM methionine (sulfur derepression), and the poly(A+) RNA was then purified as described (Aviv & Leder, 1972). The blotting of the RNA samples to nitrocellulose membrane and subsequent hybridization were carried out according to Thomas (1980) and White and Bancroft (1982).

In Vitro Mutagenesis. In vitro mutagenesis was conducted with the Muta-Gene kit as described by the manufacturer (Bio-Rad, Richmond, CA). Approximately 400 ng (0.4 pmol) of uracil-containing single-stranded DNA was annealed to 5 pmol of phosphorylated mutagenic primer and 2.5 pmol of phosphorylated M13 universal primer in 20 mM Tris-HCl, (pH 7.4)/2 mM magnesium chloride/50 mM sodium chloride, at a final volume of 10 μL. The mixture was heated to 70 °C for 2 min and then incubated at 37 °C for 10 min. The universal primer anneals upstream from the mutagenic primer, and helps to stabilize it when the extending DNA reaches it. The room temperature extension step was increased from 5 to 30 min to allow this stabilization to occur.

DNA Mobility Shift Experiments. The CYS3 protein was expressed in *E. coli*, and gel mobility shift experiments were performed as previously described (Fu & Marzluf, 1990). The DNA fragments were radioactively labeled by filling in recessed ends with the Klenow fragment of DNA polymerase



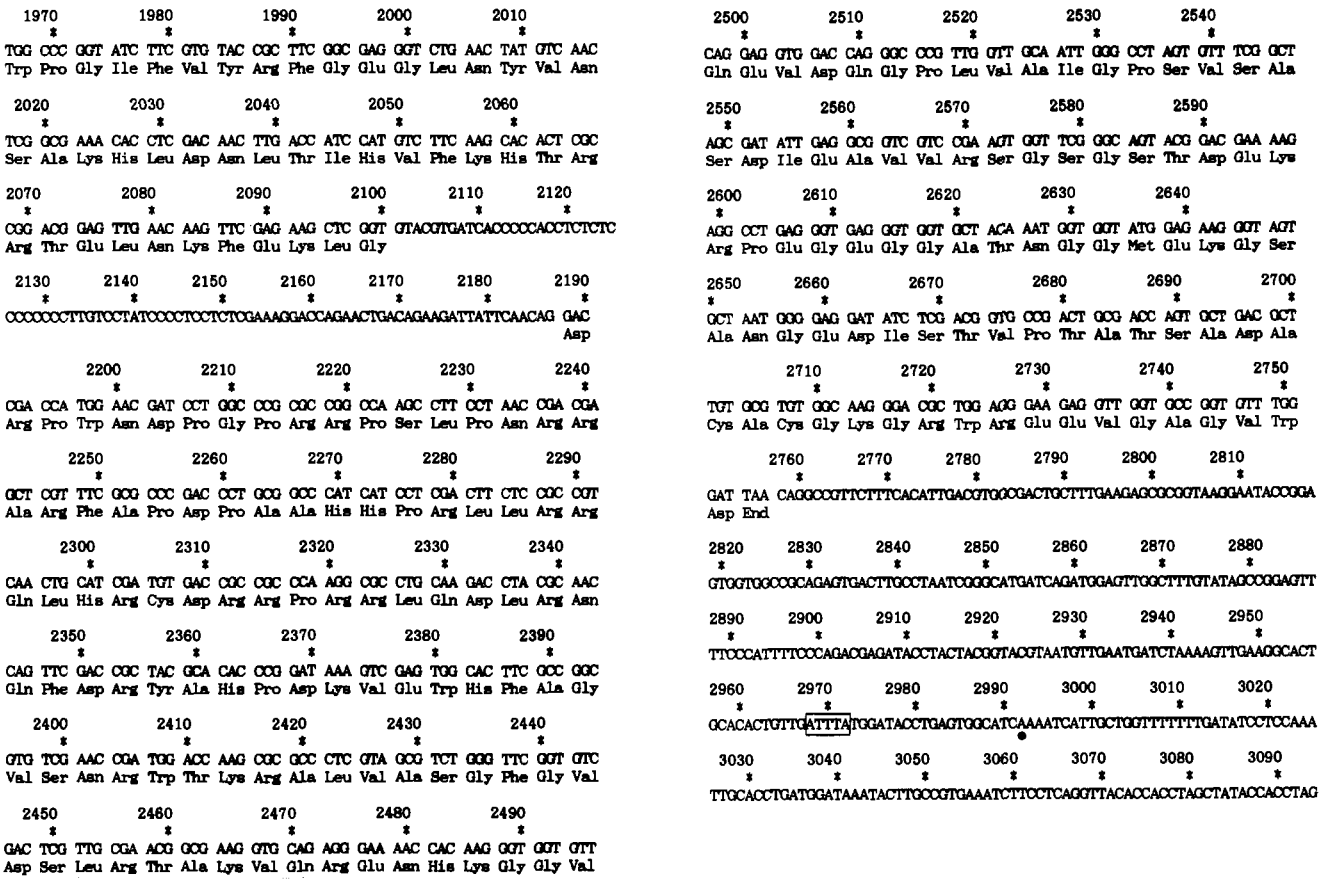


FIGURE 2: Nucleotide sequence of *cys*-14 and flanking DNA. The translated CYS14 protein, consisting of 781 amino acids, is shown directly below the nucleotide sequence. The polypurine stretch and pyrimidine-rich stretch in the 5′-flanking region are underlined, and the putative TATA box at −90 is boxed. The four transcription start sites (at +1, 8, 12, and 18) and the 3′-termination site at 2992 are indicated by closed circles. An ATTTA motif implicated in mRNA stability at 2970 is boxed. The four introns begin at nucleotides +221, +581, +1169, and +2101.

using one [^{32}P]dNTP in the first or second position. Gels were wrapped in cellophane and exposed to Kodak XAR-5 film with the use of an intensifying screen.

RESULTS

Nucleotide Sequence of the cys-14$^+$ Gene. Because of the relatively large size of the *cys*-14$^+$ gene region and the particular arrangement of restriction sites within it, three separate fragments of this gene were subcloned for use in creating deletions (Figure 1). Deletion clones were generated in both orientations with the 2.9-kb fragment, and in one direction for the 0.7-kb middle fragment (Figure 1) and for the 2.1-kb fragment (which was subsequently found to contain only 500 bp of the transcribed region). The initial sequencing was performed by using either the M13 "Universal Primer" or "Reverse Primer", depending on the orientation of a particular clone. A series of primers was synthesized by using these sequence data, corresponding to the nucleotide sequence at various intervals along the DNA. By use of both the deletion subclones and specific primers, the entire sequence was determined twice for each DNA strand. A restriction map for *cys*-14 and the location of the deletion subclones and oligonucleotide primers, as well as the overall sequencing strategy, are presented in Figure 1. The entire nucleotide sequence of the *cys*-14$^+$ gene and its flanking regions is presented in Figure 2.

Localization and Transcript Mapping of cys-14. Selected nested deletion subclones were employed as radiolabeled probes of a set of identical Northern blots of derepressed poly(A+) RNA. The failure of a particular clone to hybridize to the *cys-14*$^+$ message indicated that it lay entirely outside the transcribed region. This method allowed a rapid approximate localization of the gene. In this assay, deletion 506 gave a negative result whereas deletion 507 was positive, indicating that the 3′ end of the *cys*-14 transcript lies between these two deletions. Similarly, DNA of deletion 218 failed to hybridize with *cys*-14 mRNA whereas deletion 214 was positive, indicating that the 5′ end of the transcript occurs in the region between them (results not shown). These results indicated that the transcribed region was approximately 3 kb in length, which was in good agreement with the size estimated for the *cys*-14 transcript on Northern blots.

The 3′ end of the transcript was determined by S1 nuclease mapping using the 2.1-kb *SalI* fragment of the gene region as a probe. Poly(A+) RNA was isolated from wild-type cells grown under either sulfur-repressed or -derepressed conditions. As evident in Figure 3, one intense band of approximately 500 bp is visible in the derepressed RNA lane. A second band of approximately 600 bp is also visible in this lane, and may represent a second, less frequently used polyadenylation site. The probe is visible as a 2.1-kb band.

The 5′ end of the *cys*-14 transcript was determined by a primer extension assay. An oligodeoxynucleotide (5′-TTCGTCGGCAGGAATTCC) specific to the 5′ region of the *cys*-14 transcript was used as a primer with template RNA from either sulfur-repressed or -derepressed wild-type cells, since only the latter should possess *cys*-14 mRNA. Figure 3 shows that four strong bands were present only in the lane representing derepressed RNA. All four potential start sites occur at T residues, clustered together 15, 21, 25, and 32 nucleotide bases upstream of the ATG initiation codon (Figure 2).

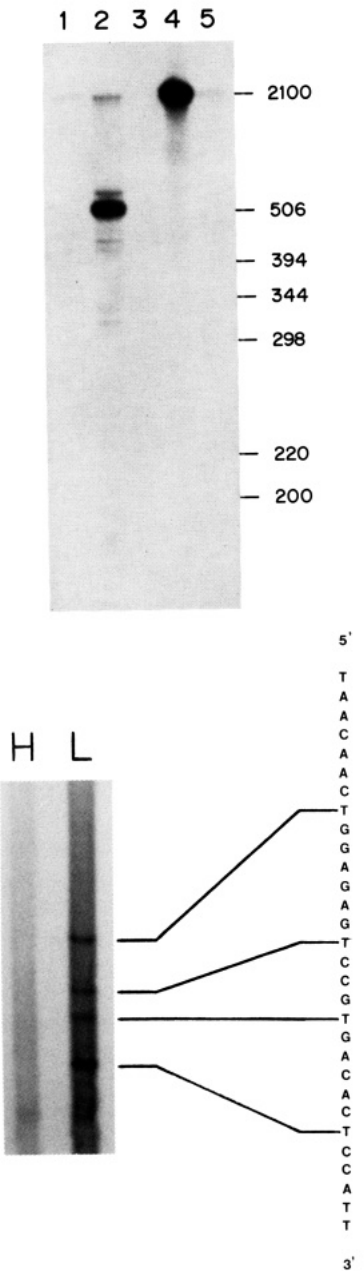


FIGURE 3: Transcript mapping. (Left panel) S1 nuclease mapping of the 3′ end of the *cys*-14 transcript. Lanes 1 and 2 show S1 mapping with poly(A+) RNA from sulfur-repressed and -derepressed cells, respectively, using an end-labeled 2.1-kb *Sal*I fragment as the probe; lanes 3 and 5, negative controls in which the labeled DNA probe alone was denatured and treated with S1 nuclease; lane 4, probe alone (not denatured) treated with S1 nuclease. The position of molecular weight markers is shown to the right. (Right panel) primer extension mapping of the 5′ end of the *cys*-14 transcript. Poly(A+) RNA from sulfur-repressed (H) and derepressed cells (L) was used as template for primer extension as described under Materials and Methods. The four start sites, each corresponding to a T residue, are shown.

Isolation and Analysis of cDNA Clones. Approximately 150 000 plaques from a λgt10 cDNA library were screened with pJSK-1A′; 12 plaques hybridized to this *cys*-14-specific probe. Three rounds of screening produced a number of pure phage clones. Purified phage DNAs were digested with *Eco*RI (to excise the insert DNA) and analyzed on an agarose gel. The clone which possessed the largest insert, 2.4 kb, was selected for subcloning and further analysis. This insert was cloned into pBLUESCRIPT and sequenced. This clone possessed a stretch of approximately 85 deoxyadenylate residues corresponding to a very substantial poly(A) tail. The

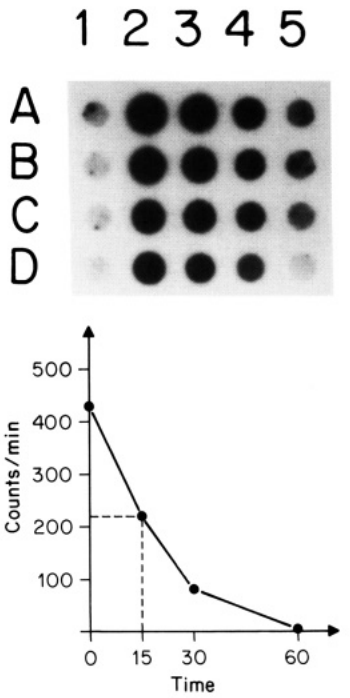


FIGURE 4: Dot blot analysis of *cys*-14 mRNA stability. (Upper panel) RNA samples were blotted to nitrocellulose using a Minifold apparatus. Equal amounts of RNA were loaded in all dots within a row; 8, 4, 2, or 1 μg in rows A, B, C, and D, respectively. To verify that equal amounts of RNA were loaded, the blot was hybridized with a β-tubulin probe in control experiments (not shown). Lane 1, RNA from uninduced cells served as a negative control; lanes 2–5, RNA isolated from induced cells transferred to S-repression medium for 0, 15, 30, and 60 min, respectively. (Lower panel) Kinetics of *cys*-14 mRNA turnover. Radioactivity within each dot was measured in a scintillation counter, and the average value of the four different RNA amounts for each time point was plotted, yielding a half-time estimated to be 15 min.

3′ end identified by the cDNA corresponds to the region predicted by the 3′ S1 nuclease mapping results. The locations of three introns (2, 3, and 4) were positively established by directly comparing the cDNA and genomic sequences. The presence of nonsense codons in all three reading frames as well as an analysis of codon usage bias (International Biotechnologies, Inc./Pustell; data not shown) in the upstream portion of the *cys*-14$^+$ gene indicated the presence of one more intron 5′ to the region present in this cDNA clone. We were unable to find a 5′ cDNA clone. The position of the putative intron 1 was therefore deduced from a combination of reading frame and splice site consensus data. The positions of the *cys*-14$^+$ introns and the predicted amino acid sequence of the sulfate permease II protein are given in Figure 2.

Stability of cys-14 mRNA. It has been known for some time that the activity of the sulfate transport systems displays turn over, but until now it has not been possible to determine the stability of mRNAs which correspond to the genetically defined permease genes.

To examine the stability of the *cys*-14 mRNA, a dot blot analysis was carried out. Wild-type cells were grown overnight under sulfur-derepressing conditions (low methionine) and then transferred to medium containing high methionine (sulfur repression). Cultures were quickly cooled and harvested at different time points, and mRNA was isolated. In a preliminary experiment, we used hourly intervals; however, no *cys*-14 message could be detected even after 1 h (data not shown). Then mRNA was isolated from cells that were growing for 0–60 min after transfer. RNA samples were blotted and hybridized with the cDNA of *cys*-14 (Figure 4). After au-

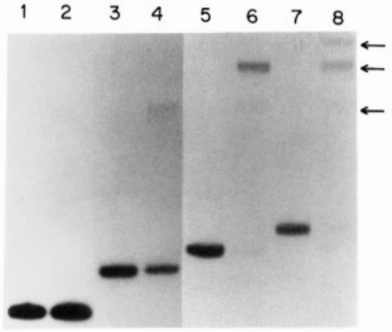


FIGURE 5: CYS3 protein binding to DNA fragments with a variable number of binding sites. Mobility shift experiments were conducted with CYS3 protein and labeled DNA fragments as described under Materials and Methods. Lanes 1 and 2, DNA fragment with zero binding sites; lanes 3 and 4, with one binding site; lanes 5 and 6, two binding sites; lanes 7 and 8, three binding sites. Samples in lanes 1, 3, 5, and 7 lacked CYS3 protein and thus showed the mobility of the free DNA probes. Samples of lanes 2, 4, 6, and 8 contained CYS3 protein (0.6 μg).

toradiography, the nitrocellulose was cut into pieces, and the activity of the individual dots was measured in a liquid scintillation counter. The counts obtained were used to calculate the half-life of the mRNA, estimated to be 15 min (Figure 4).

Binding of CYS3 Protein to Promoter Elements. The CYS3 protein has been shown to bind to three sites upstream of *cys*-14 and to a single site upstream of the *cys*-3 gene itself (Fu & Marzluf, 1990). Two of the binding sites are approximately 20 bp in length, whereas the other two are twice this length, suggesting that these latter sites actually each constitute two adjacent binding sites. A synthetic 27 bp double-stranded oligonucleotide was synthesized which contains a 19 bp region identical with the proximal CYS3 binding site in the *cys*-14 promoter; this 19 bp sequence is protected by the CYS3 protein from DNase I cleavage in vitro (Fu & Marzluf, 1990).

It was of interest to determine whether DNA fragments carrying varying numbers of this specific binding site could bind a corresponding number of CYS3 protein molecules and whether fragments having a greater number of binding sites would appear to have a higher affinity for CYS3. The 27 bp oligonucleotide was cloned into the *Cla*I site of pBLUESCRIPT, and clones were isolated which had zero, one, two, and three tandem copies of this target sequence. Treating these DNAs with *Bam*HI and *Kpn*I yielded fragments carrying different numbers of binding sites.

Figure 5 shows the results of mobility shift experiments designed to examine the binding of CYS3 protein to DNA fragments containing different numbers (0, 1, 2, or 3) of binding sites. DNA binding was specific, and clear mobility shifts of these DNA fragments were observed. The number of shifted bands in each case corresponded to the number of binding sites. The control DNA fragment lacking a CYS3 binding site was not shifted. Moreover, the affinity of the CYS3 protein for DNA fragments with either two or three tandem binding sites was considerably greater than with the fragment containing only a single site (Figure 5).

Analysis of Mutant Binding Sites. The 19 bp binding site was mutagenized in vitro as described under Materials and Methods to determine which regions of the binding site are important for interaction with the CYS3 protein. Four mutant oligonucleotides were employed, each containing three adjacent bases altered from the sequence of the wild-type binding site. Figure 6 shows the sequences of the four mutant binding sites, compared with the wild-type site.

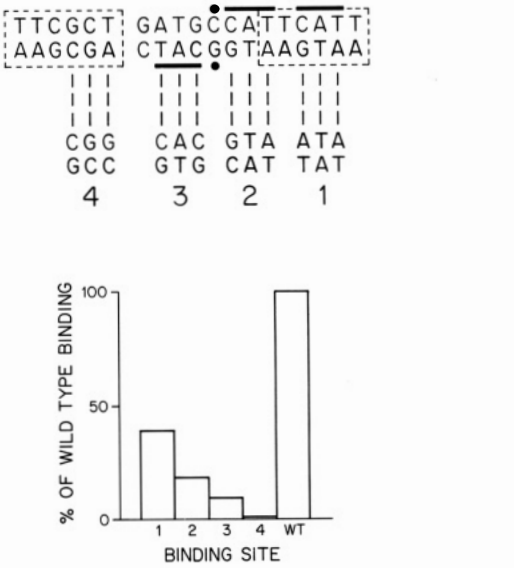


FIGURE 6: Mutant CYS3 binding sites. (Upper panel) The wild-type 19 bp CYS3 binding site is displayed above the substituted bases for each of the four mutated binding sites. CAT motifs are underlined or overlined, and 6 bp homology blocks at each end are enclosed by dashed lines. Solid circles indicate the presumed center of symmetry. (Lower panel) The binding of CYS3 protein to DNA fragments containing each of the binding sites is displayed as a percentage of that observed with the wild-type site. These results were obtained by densitometric analysis of the fraction of the DNA fragment which was shifted in each case, normalized to that obtained with the wild-type binding site.

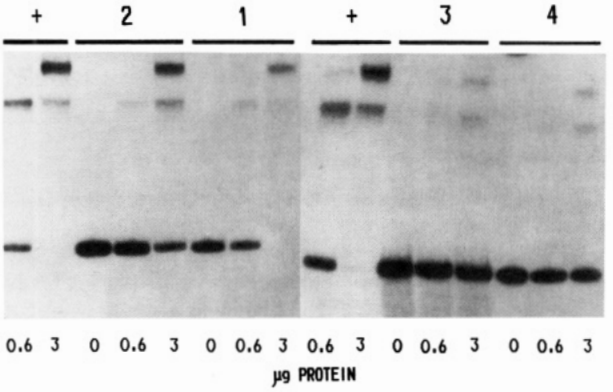


FIGURE 7: Mobility shift assays with CYS3 protein and mutated binding sites. Standard shift assays were conducted by incubating CYS3 protein with DNA fragments containing a single copy of the wild-type and each of the four mutant binding sites shown in Figure 6. The identity of the binding sites is shown above each lane, and the amount of CYS3 protein used in each sample is shown below each lane. Mutants 1 and 2 and a wild-type standard were run in one gel, and a separate gel was utilized for mutants 3 and 4 and a wild-type standard. The same amount of each probe was loaded in each lane, and the (+) probe without *cys*-3 protein runs at the same position as found with the four mutant probes (not shown).

The results of CYS3 protein binding assays with the wild-type and mutant DNA binding sites are shown in Figure 7. It is important not to conduct binding assays with too high a protein concentration, because even a weak binding site could become fully occupied and thus appear to be nearly equivalent to the wild-type site, e.g., which did occur to some extent when 3 μg of CYS3 protein was used (Figure 7). The CYS3 protein concentration used (0.6 μg) for comparison of the binding sites is within the most sensitive range of the saturation curve (not shown). It is obvious that the CYS3 protein has considerably less affinity for DNA fragments containing mutant binding sites than those with a wild-type site. The mobility shift experiments shown in Figure 7 were quantitated by densito-

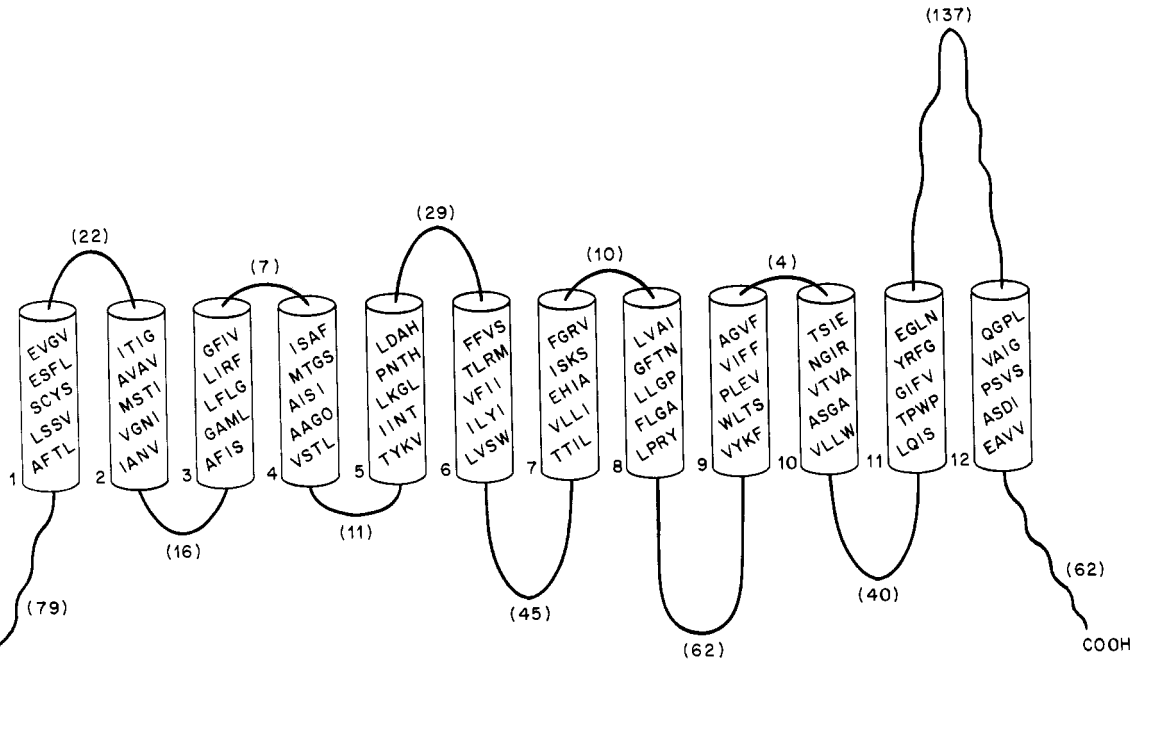


FIGURE 8: Postulated secondary structure and membrane localization of the CYS14 protein. The 12 putative membrane-spanning helices are represented as cylinders. The number of amino acid residues in other segments of the protein is shown in parentheses. The CYS14 protein has been demonstrated by immunochemical studies to be localized in the membrane fraction of the cells (Jarai and Marzluf, unpublished results).

metric scanning. These data are displayed in Figure 6 and reveal that all four mutant sites show reduced binding by CYS3 in comparison to a wild-type site, although the mutant binding sites clearly differ substantially from each other in binding affinity.

Discussion

We report here the complete nucleotide sequence of the *cys*-14 gene. The *cys*-14 gene appears to be transcribed from 4 closely spaced start sites located 15–32 nucleotide bases upstream of an ATG initiation codon. A possible TATA box (TATATAT) is situated 90 bp upstream of the first start site, a distance typical of fungal genes (Hahn et al., 1985). The *cys*-14 gene possesses 4 introns of 162, 87, 72, and 58 bases, each with a *Neurospora* consensus 5′ and 3′ splice site and internal branch point sequences. The size and distribution of these introns resemble those of the β-tubulin gene of *N. crassa*, which contains 6 introns ranging from 57 to 240 bases in length (Orbach et al., 1986). A number of potentially significant features are found in the nucleotide sequence of *cys*-14. A stretch of 26 bases consisting entirely of purine residues occurs from nucleotides −258 to −233, and a pyrimidine-rich stretch occurs at −217 to −204. Such features are found in other *Neurospora* genes, but their function, if any, has not been established, although they may play a role in producing bent DNA structures (Satchwell et al., 1986) or in excluding nucleosomes (Morse & Simpson, 1988), thus possibly affecting *cys*-14 expression. A possible CAAT box is found at −140 and could contribute to the high level of expression of *cys*-14 which occurs during sulfur derepression. The *cys*-14 mRNA is approximately 3 kB in length and contains untranslated regions of approximately 30 and 234 bases at its 5′ and 3′ end, respectively.

Mutants of *cys*-14 gene are deficient in sulfate permease II, but it has remained unclear whether this gene encoded a membrane transporter protein or in some other way contributed to the sulfate transport system. The *cys*-14 gene appears to encode a large protein comprised of 781 amino acids with a molecular weight of 87037. Analysis of the translated CYS14 protein indicated that it contains 10–12 hydrophobic regions, each with a length of at least 20 amino acids, which may constitute helical membrane-spanning domains (Figure 8). In this respect, it is similar to other carrier proteins (Bell et al., 1986; Kaback, 1987; Maiden et al., 1987). The sequences of 2 other carrier proteins of *Neurospora* have recently been reported, a 590-residue phosphate permease (Mann et al., 1989) and a putative quinic acid permease of 537 amino acids (Geever et al., 1989). Both of these carrier proteins also appear to possess a secondary structure with 12 membrane-spanning helices (Mann et al., 1989; Geever et al., 1989). The CYS14 protein is larger than these other two carrier proteins and appears to have longer amino- and carboxy-terminal segments that extend beyond the portions of the protein anchored within the membrane. A relatively long hydrophilic domain occurs between the last two membrane domains, similiar to an even longer domain between the eighth and ninth membrane-spanning helices of the phosphate carrier protein (Mann et al., 1989).

Recent immunochemical studies have demonstrated that the CYS14 protein is indeed highly localized within the membrane fraction of *N. crassa* cells (Jarai and Marzluf, unpublished results). The CYS14 protein contains a number of possible phosphorylation sites and at least potential glycosylation sites. A search of the NBRF data base failed to locate any proteins with extensive homology to CYS14, although the ribose transporter protein of *E. coli* (Bell et al., 1986) has a stretch of 13 amino acids with 70% homology; these homologous regions occur in the amino terminus of each protein, although their function, if any, is unknown.

The sulfate transport systems of *N. crassa* turn over with a functional half-life of approximately 2 h. In contrast, aryl sulfatase, a sulfur metabolic enzyme, whose synthesis is regulated in a parallel fashion to that of the sulfate permeases, appears to be very stable (Marzluf, 1972b). Uptake of me-

tabolites represents a first point at which control of an entire metabolic pathway could be exerted, and thus permease species may be highly regulated at several levels, e.g., synthesis, function, and stability. We showed here that the *cys*-14 mRNA, which encodes sulfate permease II, turns over with a half-life of approximately 15 min. This relatively rapid turnover of mRNA ensures that synthesis of the permease stops quickly when sulfur repression is established. Mammalian messenger RNAs which exhibit rapid turnover frequently contain one or more copies of an AUUUA motif which mediates mRNA instability (Shaw & Kamen, 1986). The nontranslated 3′ region of *cys*-14 mRNA contains 1 copy of this motif approximately 25 nucleotides upstream from the polyadenylation site, although it is unknown whether such an element confers messenger instability in filamentous fungi.

The *cys*-3 regulatory gene controls the expression of the entire set of structural genes of the sulfur circuit. The CYS3 protein, which contains a DNA binding domain composed of a leucine zipper and an adjacent basic region, binds to three sites upstream of the *cys*-14 gene and to a single site upstream of its own structural gene (Fu & Marzluf, 1990). Two of these binding sites are approximately 20 bp in length whereas the other two are twice as long, perhaps comprising two adjacent binding sites. We showed here that DNA fragments which contain two or three of the short binding elements in tandem repeats (whose sequence was dictated by the proximal site upstream of *cys*-14) appear to bind a corresponding number of CYS3 molecules as revealed by multiple shifted bands. The DNA fragments with two or three tandemly repeated elements also clearly possess a greater affinity for CYS3 than does a fragment with a single binding site. These results may thus explain the clear size differences observed for natural CYS3 binding sites and the fact that a DNA with one of the longer binding elements showed greater affinity for CYS3 than did a fragment with a short element (Fu & Marzluf, 1990).

In order to examine what regions of a binding site were important for binding of the CYS3 regulatory protein, we compared four mutant binding sites with a wild-type site. Each mutated site had three adjacent bases altered with purines replaced by pyrimidines, and vice-versa, with the expectation that such changes might have an obvious effect upon CYS3 binding. The substituted bases span most of the binding site, as defined by DNase I protection studies, and were designed to examine elements of the apparent imperfect dyad (Figure 6). It is clear that the two halves of the partial dyad within the binding site are not equivalent. Mutants 1 and 2 occur on the right side of the center of symmetry and cause a relatively small decrease in binding by CYS3. Mutants 3 and 4, on the other hand, show stronger effects, particularly mutant 4, with which more than 99% of the binding by CYS3 has been lost. Thus, the nucleotide bases in the homology block which were altered in binding site mutant 4 are very important for CYS3 binding to the proximal element upstream of *cys*-14. Each of the CYS3 binding sites contains at least one and usually several CAT sequences, which were suggested to comprise a core binding sequence (Fu & Marzluf, 1990). Mutants 2 and 3 each alter a single CAT sequence, but on opposite sides of the apparent center of symmetry, and they retain 10% (mutant 3) or 18% (mutant 2) of the wild-type level of CYS3 binding. Thus, these two regions are nearly equivalent and are important for CYS3 binding although a change in either CAT motif does not lead to a complete loss of Cys3 protein binding. It should be noted that mutant 2 resulted in generation of a CAT motif in the lower strand but caused the loss of symmetry. The various binding elements recognized by CYS3 have different nucleotide sequences, perhaps not surprisingly since CYS3 is a global regulatory protein which turns on different structural genes to different extents. The results presented here represent only a beginning in understanding the precise manner by which the CYS3 protein activates gene expression.

Acknowledgments

We thank Richard Swenson and Jane Tolley, Ohio State Biochemical Instrument Center, who prepared the oligonucleotides used in this work.

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