MOLECULAR AND CELLULAR BIOLOGY, Apr. 1992, p. 1568–1577
0270-7306/92/041568-10$02.00/0

Vol. 12, No. 4

Production of the CYS3 Regulator, a bZIP DNA-Binding Protein, Is Sufficient To Induce Sulfur Gene Expression in *Neurospora crassa*

JOHN V. PAIETTA

Department of Biochemistry, Wright State University, Dayton, Ohio 45435

Received 1 October 1991/Accepted 9 January 1992

The *cys-3*$^+$ gene of *Neurospora crassa* encodes a bZIP (basic region-leucine zipper) regulatory protein that is essential for sulfur structural gene expression (e.g., *ars-1*$^+$). Nuclear transcription assays confirmed that *cys-3*$^+$ was under sulfur-regulated transcriptional control and that *cys-3*$^+$ transcription was constitutive in sulfur controller (*scon*)-negative regulator mutants. Given these results, I have tested whether expression of *cys-3*$^+$ under high-sulfur (repressing) conditions was sufficient to induce sulfur gene expression. The *N. crassa* β-tubulin (*tub*) promoter was fused to the *cys-3*$^+$ coding segment and used to transform a *cys-3* deletion mutant. Function of the *tub*::*cys-3* fusion in homokaryotic transformants grown under high-sulfur conditions was confirmed by Northern (RNA) and Western immunoblot analysis. The *tub*::*cys-3* transformants showed arylsulfatase gene expression under normally repressing high-sulfur conditions. A *tub*::*cys-3*^{ts} fusion encoding a temperature-sensitive CYS3 protein was used to confirm that the induced structural gene expression was due to CYS3 protein function. Constitutive CYS3 production did not induce *scon-2*$^+$ expression under repressing conditions. In addition, a *cys-3* promoter fusion to *lacZ* showed that CYS3 production was sufficient to induce its own expression and provides in vivo evidence for autoregulation. Finally, an apparent inhibitory effect observed with a strain carrying a point mutation at the *cys-3* locus was examined by in vitro heterodimerization studies. These results support an interpretation of CYS3 as a transcriptional activator whose regulation is a crucial control point in the signal response pathway triggered by sulfur limitation.

The sulfur regulatory system of *Neurospora crassa* is composed of a genetically defined set of *trans*-acting regulatory genes and a set of structural genes that encode enzymes used in the uptake and assimilation of a variety of sulfur compounds (36). When *N. crassa* is cultured under conditions of sulfur limitation (i.e., derepressing conditions), then the entire group of sulfur-related genes is coordinately expressed. The structural genes involved encode for arylsulfatase, choline sulfatase, choline sulfate permease, methionine permease, sulfate permeases I and II, and an extracellular protease (21, 27, 35, 40, 45). The system provides a useful model for the study of coordinate gene regulation in a multigene network and the cellular regulation of sulfur level.

The *cys-3*$^+$ positive regulatory gene is essential for sulfur structural gene expression. *cys-3* mutants typically have a pleiotropic loss of the entire set of sulfur-controlled enzymes and are sulfur-requiring auxotrophs. *cys-3*$^+$ was cloned by Paietta et al. (43), and further characterization has identified the encoded gene product to be a bZIP (basic region-leucine zipper) protein (17, 32). CYS3 has substantial homology to a number of bZIP proteins, including the oncogene *fos* product and the yeast regulator GCN4 (17, 29, 32). Studies have demonstrated that *Escherichia coli*-expressed CYS3 protein binds to its own promoter and to the sulfur structural gene promoters (16, 50). From this work and by analogy to similar proteins, the *CYS3* gene product appears to be a DNA-binding transcriptional activator. Analysis of the arylsulfatase structural gene using nuclear transcription assays indicate that transcriptional control of the sulfur structural genes is likely (40). In addition, the binding of CYS3 to its own promoter (16) and the depression of *cys-3*$^+$ expression observed in *cys-3* mutants (43) suggest that autoregulation of *cys-3*$^+$ expression may occur.

The sulfur controller genes, *scon-1*$^+$ and *scon-2*$^+$, are negative regulators in the control system (4, 41). *scon-1* and *scon-2* mutants show constitutive derepression of sulfur structural gene expression (e.g., arylsulfatase) and of *cys-3*$^+$, rather than sulfur-regulated expression. An epistatic hierarchy of *scon-1*$^+$ → *scon-2*$^+$ → *cys-3*$^+$ has been proposed on the basis of available data (41). By this model, *cys-3*$^+$ is the most proximal control point relative to the sulfur structural genes in the signal response pathway triggered by sulfur limitation. *cys-3*$^+$ may also be involved in a feedback control loop, along with *scon-1*$^+$, in influencing *scon-2*$^+$ expression (41). The sulfur controller genes may operate by modulating the function and level of CYS3. Upon release from sulfur repression, presumably involving negative regulator function and the sulfur metabolic signal, there is an increase in expression of *cys-3*$^+$, and consequently sulfur structural gene transcription occurs. Experimentally increasing only the expression of *cys-3*$^+$ under high-sulfur conditions may result in induction of sulfur gene expression, possibly by titration of negative factors, and the release of CYS3 function from control. I use the approach here of constructing a gene fusion to alter the expression pattern of CYS3 and to test the organizational model for the sulfur control system. The transcription of *cys-3*$^+$ directed by an appropriate promoter under high sulfur would simulate the effect of the *scon* mutants on *cys-3*$^+$ but would leave the regulatory system otherwise intact. Thus, the important question of whether induction of CYS3 is a sufficient signal in itself for sulfur gene expression can be examined. Such an approach with other regulators has been a useful probe of regulatory mechanisms. For example, the *GAL4* and *STE12* gene products, among others, have been studied in this regard (9, 19, 25). The approach also provides a means of obtaining substantial quantities of in vivo-produced protein which would otherwise be difficult to isolate.

I report here that the in vivo constitutive production of CYS3 protein by use of a gene fusion leads to both sulfur structural (*ars-1*$^+$) and *cys-3*$^+$ regulatory gene expression under high-sulfur (repressing) conditions. The direct role of CYS3 protein in the induction was demonstrated by use of a gene fusion encoding temperature-sensitive CYS3 protein. Essential to demonstrating the sulfur gene induction was the construction of a Δ*cys-3* strain which had the basic region-leucine zipper coding segment from *cys-3*$^+$ deleted. An inhibitory effect seen with a *cys-3* point mutant was examined in vitro with respect to the formation of non-DNA-binding heterodimers of CYS3 protein. The results presented provide further insight into the organization of the sulfur regulatory system.

MATERIALS AND METHODS

Strains. *N. crassa scon-1*(36-18), *scon-2*(PSD272), and *cys-3*(p22) were described previously (40, 41). Δ*cys-3*(18-4) was constructed as described in this report. 740R23-1a was used as the wild-type (WT) strain for these studies. *his-3* (FGSC 462) was obtained from the Fungal Genetics Stock Center (Kansas City, Kans.).

Media, crosses, and culture conditions. Vogel minimal medium (8), with supplements as required, was used. Crosses were carried out according to standard techniques, using cornmeal agar or Westergard-Mitchell medium (8). *N. crassa* cultures were grown at 25°C except for experiments with temperature-sensitive *cys-3* mutants. Sulfur derepression and repression experiments were done by filtration harvesting of mycelia and transfer to Vogel-minus-sulfur medium plus high-sulfur (5.0 mM methionine) and low-sulfur (0.25 mM methionine) medium, respectively (40). Alternately, experiments involved inoculation of spores directly into Vogel-minus-sulfur medium with high or low sulfur concentrations (as noted above).

Plasmid constructs and in vitro mutagenesis. Site-directed mutagenesis was carried out by the phosphorothioate method (54). Oligonucleotides were synthesized on an Applied Biosystems model 391 synthesizer. All constructs that involved site-directed mutagenesis were completely sequenced by the dideoxy method (48). Briefly, the *tub*::*cys-3*$^+$ fusion was constructed as follows. The β-tubulin (*tub*) promoter (39) on a *Sal*I-*Eco*RI fragment was cloned into M13mp18, and a *Nde*I site was introduced at the start codon by site-directed mutagenesis with the oligonucleotide 5′-CACCAAACCGTCCATATGCGTGAAATTGTAAG-3′ (alterations underlined). *cys-3*$^+$, cloned into M13mp18 in a previous study (17), was similarly mutagenized to introduce a *Nde*I site with the oligonucleotide 5′-CCGCTGACCATG GCCATATGTCTTCAGCCGAC-3′. The *Sal*I-*Nde*I *tub* promoter fragment and *Nde*I-*Eco*RI *cys-3*$^+$ coding fragment were then ligated into *Sal*I- and *Eco*RI-digested M13mp18. *tub*::*cys-3*^{ts} was similarly constructed, using a temperature-sensitive *cys-3* mutant clone (Gln-for-Arg-106 substitution) (17). The *Nde*I site was later removed by site-directed mutagenesis to restore the original *tub* sequence and optimal translational context (carried out after initial construct testing). The *tub*::*cys-3* fusion constructs were isolated from M13 replicative-form DNA following *Eco*RI and *Sal*I digestion by gel purification and then used for transformation. pJP40, used for in vitro transcription, contained the *cys-3*$^+$ coding region on a *Nru*I-*Bam*HI fragment, cloned into *Sma*I-and-*Bam*HI-digested pGEM4Z. Basic region (Gln substitutions for Lys-105 and Arg-106) and leucine zipper (Val substitutions for Leu-141 and Leu-148) mutations were generated in a 4.4-kb *Bgl*II *cys-3*$^+$ insert in M13 with the oligonucleotides 5′-GCCTTGCCGCCGAGGAAGACCAG CAAAAGCGCAACACCGCAGCTAGC-3′ and 5′-GCGAG AAGGTCACCCAAGTTGAGGGACGCATCCAGGCTGT CGAGACGGAGAACAAG-3′, respectively. The mutagenized segments were then cloned into pGEM4Z to give pCYS105 (Gln-105, Gln-106) and pCYS148 (Val-141, Val-148). All clones to be transcribed in vitro had an additional *Ppu*MI cleavage site introduced for producing a truncated *cys-3* transcript which still encoded the basic region-leucine zipper region. All constructs could then be transcribed either truncated or full length, depending on the restriction enzyme used (i.e., *Ppu*MI or *Bam*HI, respectively). A *cys-3*::*lacZ* fusion was generated by cloning an *Eco*RV-*Stu*I fragment carrying the *cys-3*$^+$ promoter along with N-terminal sequence into *Sma*I-digested pDE1 (12). For use in making the *N. crassa* construct with a *cys-3* deletion, plasmid pJP10 was digested with *Stu*I and *Sac*I, the *Sac*I site was blunt ended, and the ends were ligated to generate pJP30. Plasmids with the *scon-2*$^+$ (pSCON2), *cys-3*$^+$ (pJP10), *am*$^+$ (pJP11), and *ars-1*$^+$ (pJP18) genes were as described previously (41). pDE1 (12) was obtained from the Fungal Genetics Stock Center.

Transformation and homokaryon isolation. *N. crassa* was transformed by the Novozyme 234 spheroplasting technique of Vollmer and Yanofsky (58). DNA fragments to be used for transformation were gel purified and electroluted before use. Homokaryons were isolated from heterokaryotic primary transformants by growth on Westergard and Mitchell medium with 1 mM iodoacetate, harvesting of the microconidia, and filtration through 5-μm-pore-size Millex filters as outlined by Ebbole and Sachs (13). Homokaryon isolation was confirmed by Southern blot analysis as well as growth tests of sample reisolates. Selection for chromate resistance in construction of the Δ*cys-3*(18-4) strain was by overlay of 10 mM potassium chromate in 1.5% agar of transformation plates as described previously (41). *E. coli* transformation was performed with competent cells prepared by calcium chloride treatment (7).

Arylsulfatase assays. Arylsulfatase was assayed by incubation of mycelial extracts with *p*-nitrophenol sulfate according to standard methods (38, 40). *p*-Nitrophenol liberation was monitored at 405 nm. Total protein in extracts was measured by the Bradford assay (3).

DNA isolation and Southern blots. *N. crassa* chromosomal DNA was isolated by extraction in pronase–lithium EDTA–Triton X-100 and precipitation in ethanolic perchlorate, followed by DNA spooling as described by Metzenberg and Baisch (37). Plasmid DNA was isolated from *E. coli* by the alkaline extraction method (2). Southern blot transfers of DNA to nitrocellulose from 0.9% agarose gels were hybridized with [^{32}P]dCTP-oligolabeled probes and washed as described previously (40, 53).

RNA isolation and analysis. Total RNA was isolated by the phenol extraction procedure of Reinert et al. (47) as modified (40). Briefly, mycelial samples were harvested, frozen in liquid nitrogen, and homogenized in a 1:1 mixture of phenol-chloroform-isoamyl alcohol (49:49:2) and extraction buffer (1% Sarkosyl, 100 mM sodium acetate, 1 mM EDTA [pH 5.0]). After phenol-chloroform extractions, precipitation, and sodium acetate washes, the poly(A)$^+$ mRNA was isolated by oligo(dT)-cellulose chromatography (1). Poly(A)$^+$ mRNA levels were quantified in the samples by spectrophotometry and in subsequent Northern (RNA) blot experiments by probing with the constitutively expressed *am*$^+$ (28) gene. ^{32}P-labeled probes were prepared by oligolabeling of

DNA fragments (15). Blots were hybridized at 42°C in 50% formamide–5× SSPE (SSPE is 0.18 M sodium chloride, 1 mM EDTA, and 10 mM sodium phosphate [pH 7.7]) for 24 h and washed as described previously (41). Primer extension analysis was done by using a 5′-end-labeled 18-mer oligonucleotide that hybridized at +75 of the *tub*::*cys-3* sequence with poly(A)$^+$ mRNA, isolated from cells grown on high sulfur, and Moloney murine leukemia virus reverse transcriptase. The mRNA transcribed from the resident *cys-3* locus (partially deleted) was present only at a low level and did not interfere significantly with transcript analysis of the gene fusion. Nuclear transcription analysis using Percoll gradient-isolated nuclei was done as described previously (41) except that the transcription buffer of Loros and Dunlap (34) was used. Hybridization of synthesized RNA to slot blots was done as outlined elsewhere (41).

In vitro transcription and translation. Plasmids pJP40 (WT), pCYS105, and pCYS148 were linearized with *Bam*HI or *Ppu*MI at the 3′ end of the *cys3* insert. The linearized DNA was then transcribed by SP6 RNA polymerase as follows: 5 μg of DNA was typically added to transcription buffer (40 mM Tris [pH 7.5], 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl), 0.5 mM ribonucleoside triphosphates, 10 mM dithiothreitol, 100 U of RNasin, and 30 U of SP6 RNA polymerase (Promega); after a 2-h incubation at 37°C, the sample was digested with 5 U of RQ1 DNase, phenol extracted, and ethanol precipitated.

For in vitro translation, 1 μg of transcript was added to rabbit reticulocyte lysate (Stratagene) with 4 U of RNasin and 25 μCi of [^{35}S]methionine. For nonlabeling assays, 20 μM methionine was substituted for [^{35}S]methionine. As a control, identical reactions were run with water rather than RNA. Reaction mixtures were incubated for 60 min at 30°C; aliquots were subsequently mixed with sodium dodecyl sulfate (SDS)-Laemmli loading buffer, heated, and subjected to SDS-polyacrylamide gel electrophoresis (PAGE) (31) for analysis.

Immunological procedures. Polyclonal antibodies were prepared against a CYS3 peptide (pep a) of sequence CK HGSKEDILK-COOH (synthesized by the Indiana University Biotechnology Center, Indianapolis). The peptide was cross-linked via an N-terminal cysteine by maleimide to keyhole limpet hemocyanin (Pierce) and used to immunize rabbits with Freund's adjuvant. The resulting antibody was immunoreactive to in vitro-translated CYS3 and to *N. crassa* CYS3 that was isolated from nuclei by heparin-agarose and oligonucleotide affinity purification (40, 42). Anti-CYS3 (pep a) antibody was affinity purified with immobilized antigen on nitrocellulose (14, 52). Western immunoblots were processed by using anti-CYS3 (pep a) antibody and goat anti-rabbit antibody conjugated with alkaline phosphatase and finally were stained with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate according to standard techniques (22).

Immunoprecipitation was done with the anti-CYS3 (pep a) antibody and Affinica protein A-agarose (Schleicher & Schuell) as outlined by Kouzarides and Ziff (29) for immunoprecipitation studies of *jun* and *fos*. Briefly, the procedure involved incubation of the anti-CYS3 (pep a) antibody with the translation mixture, addition of protein A-agarose (10% [vol/vol] in RIPA buffer), and three washes in RIPA. The sample was suspended in Laemmli sample buffer, heated, and subjected to SDS-PAGE. Proteins were ^{35}S labeled as described above for the in vitro translations.

The enzyme linked immunosorbent assay (ELISA) for bacterial β-galactosidase used a microtiter plate assay of crude extracts incubated with primary antibody to bacterial β-galactosidase (5 Prime, 3 Prime, Inc., or Boehringer Mannheim) and secondary biotinylated antibody to β-galactosidase, incubation with streptavidin-conjugated alkaline phosphatase, and finally a *p*-nitrophenyl phosphate color reaction carried out according to the manufacturer's specification. The sensitivity of the test was 20 pg of β-galactosidase protein per 0.2 ml of crude extract. The extracts were prepared by grinding *N. crassa* in extraction buffer (0.25 M Tris [pH 7.8] with 1.0 mM phenymethylsulfonyl fluoride) and clarifying the sample by centrifugation. Total protein was determined with the Bio-Rad protein assay. After binding steps, washes, and color development for 30 min, the optical density at 405 nm was determined. An expression unit, as defined here, was 100 pg of β-galactosidase protein per mg of total protein. Dilution curves of β-galactosidase standards and experimental samples were carried out to calculate β-galactosidase levels present.

Gel mobility shifts. An oligonucleotide of the sequence 5′-GACAACGCTCCCCGAGAATGGTGTCATTCTCGT GACTTT-3′ and its complement, representing a single CYS3 binding site on the *cys-3*$^+$ promoter (16), were labeled by T4 polynucleotide kinase with [γ-^{32}P]ATP, annealed, and gel purified (6). DNA binding was carried out in 1× binding buffer [20 mM *N*-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.9), 60 mM KCl, 2 mM dithiothreitol, 10% glycerol, 2 μg of poly(dI-dC), ~5,000 cpm of DNA end labeled with ^{32}P] in a final volume of 20 μl. In vitro-synthesized or in vivo-isolated CYS3 was added, and the reaction mixture was incubated at room temperature for 30 min. Specificity of CYS3 binding was ensured by control experiments using competition by addition of excess unlabeled DNA. Four percent polyacrylamide gels with a 50 mM Tris–380 mM glycine–2 mM EDTA (pH 8.5) running buffer (6) were electrophoresed at 20 mA with the temperature maintained at 4°C. The gels were dried and autoradiographed with Kodak XAR-5 film and Amersham Hyperscreens at −70°C.

RESULTS

Transcriptional control of *cys-3*$^+$. Nuclear run-on transcription assays were used to demonstrate the presence of regulation at the transcriptional level for the *cys-3*$^+$ gene. Such assays allow for assessment of RNA polymerase II loading levels for a gene (20, 55). Sulfur-derepressed and sulfur-repressed nuclei were isolated from wild-type *N. crassa* cells; RNA was synthesized with [^{32}P]UTP as a label and was quantitated by hybridization to DNA slot blots. RNA synthesized by derepressed nuclei hybridized to *cys-3*$^+$ and *am*$^+$ DNA but not to pBR322 (control) DNA. Conversely, RNA that was synthesized by repressed nuclei hybridized only to *am*$^+$ DNA, not to *cys-3*$^+$ DNA or to the control pBR322 DNA (Fig. 1). The result demonstrates that the sulfur-regulated *cys-3*$^+$ steady-state mRNA levels seen by Northern blot analysis (43) were primarily due to transcriptional control.

In addition, nuclear transcription assays were done with nuclei isolated from the *scon-1* and *scon-2* strains grown under sulfur-repressing and -derepressing conditions. In *scon* nuclei from both growth conditions, RNA was synthesized that hybridized to *am*$^+$ and *cys-3*$^+$ DNA but not to the control (pBR322) DNA (data not presented). The *scon-1* and *scon-2* mutations, therefore, resulted in constitutive transcription of *cys-3*$^+$ (as well as constitutive sulfur structural gene [*ars-1*$^+$] transcription as shown previously [40]).

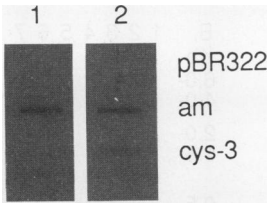

FIG. 1. Nuclear transcription assay of the *cys-3*$^+$ gene of *N. crassa*. Nuclei were isolated from mycelia of WT 74-OR23-1a grown under repressing (high-sulfur) or derepressing (low-sulfur) conditions. In vitro transcription was performed, and the synthesized RNA was hybridized to slot blots of nitrocellulose bound *am*$^+$, *cys-3*$^+$, and pBR322 DNAs. The bound *cys-3*$^+$ and *am*$^+$ DNAs (from pJP10 and PJP18, respectively) had no vector sequence. Nonspecific background hybridization was assessed with pBR322 DNA. Lanes: 1, blot hybridized with labeled RNA from sulfur-repressed nuclei; 2, blot hybridized with labeled RNA from derepressed nuclei.

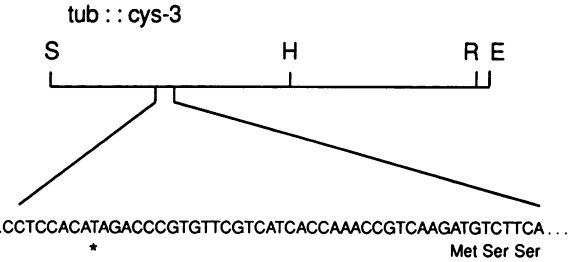

FIG. 2. Schematic diagram of the *tub* promoter to a *cys-3*$^+$ coding segment gene fusion. The fusion was constructed as described in Materials and Methods following site-directed mutagenesis. Sequence from the ATG codon upstream to the *Sal*I site is that of the *tub* promoter. The proper translational context has been maintained in the construct. The asterisk indicates the major transcriptional start site (−33) as indicated by primer extension analysis. The construct as a *Sal*I-*Eco*RI (within the polylinker of pJP40) fragment was used for transformation. Two versions of *tub*::*cys-3* were made, one with the WT coding sequence and one with a mutation resulting in a temperature-sensitive protein (Gln substitution for Arg-106). A restriction map of the construct is shown (E, *Eco*RI; H, *Hin*dIII; S, *Sal*I; R, *Eco*RV).

***tub*::*cys-3* gene fusion induces arylsulfatase gene expression.** The strategy chosen was to simulate the constitutive expression of *cys-3*$^+$ seen in sulfur controller mutants but to do it by affecting only the level of *cys-3*$^+$ mRNA rather than by a control system defect. This approach allowed for a test of whether *cys3*$^+$ induction can itself lead to sulfur structural gene expression under usually repressing conditions. To produce substantial levels of CYS3 under repressing conditions, a gene fusion was constructed by using the constitutive promoter from the *tub* locus (39) and the coding segment of the *cys-3*$^+$ locus of *N. crassa*. A precise fusion using 360 bp of *tub* upstream sequence was constructed as shown in Fig. 2. The upstream segment of *tub* to the start ATG codon has been exactly maintained, as has the coding sequence of *cys-3*$^+$. Results with other promoters (e.g., *am*, *cpc-1*, and *his-3*) tested were unsatisfactory as to the level of expression obtainable. The direct role of the CYS3 protein in these studies was shown by the use of a temperature-sensitive mutant of CYS3 (17) for which the coding segment was similarly fused to the constitutive promoter.

The construction of a *cys-3* deletion strain was also a key element in the experiments. The deletion strain constructed, Δ*cys-3*(18-4), eliminated the basic region and leucine zipper region from the CYS3 protein, which should preclude dimerization (Fig. 3). The possible inhibitory effect of heterodimerization of point mutant and normal CYS3 subunits was examined in experiments described below. The general approach used to construct the deletion was that used previously to generate gene disruptions at the *am* locus (44) in *N. crassa*. The *Eco*RV fragment from pJP30 carrying a *Stu*I-to-*Sac*I deletion in the *cys-3* coding sequence was used to transform *scon-2*, with subsequent plating on methionine supplemented medium with chromate. The *scon-2* strain constitutively produces sulfate permease, transports in toxic levels of chromate, and is killed (41). If the *cys-3*$^+$ gene was inactivated by an insertion event, then sulfate permease expression would be blocked and no transport of chromate would occur. The supplemented methionine provides the sulfur requirement for the newly generated auxotroph (i.e., generated by the disruption of *cys-3*). Twenty chromate-resistant isolates produced in this way were analyzed, and several represented exact replacement events. Subsequent backcrosses provided an isolate, Δ*cys-3*(18-4), without the *scon-2* mutant locus; this was shown by transforming Δ*cys-3*(18-4) with pJP10 (*cys-3*$^+$) DNA and demonstrating normal regulation in transformants for both *cys-3*$^+$ and sulfur structural gene activity.

The Δ*cys-3*(18-4) strain was transformed with the *tub*::*cys-3* gene fusion. Homokaryotic transformants were generated from microconidia as described in Materials and Methods without crossing to avoid a repeat-induced point mutation (RIP) effect (5) due to the presence of duplicate sequences. Transformants generally have integrations at a number of different chromosomal sites and are known to give variation in expression with an identical transforming construct (42). Ten homokaryons with single-copy ectopic integrations were screened by RNA dot blot analysis to select for an isolate with the highest level of *cys-3*$^+$ transcript present. First, the nature and level of *cys-3* transcript produced by the selected *tub*::*cys-3*(2-8) transformant were examined. Northern blots of mRNA from *tub*::*cys-3*(2-8) and WT grown under high and low sulfur were prepared and probed with the segment of *cys-3* deleted in the Δ*cys-3*(18-4) strain (Fig. 4A). A fusion transcript intermediate in size between the two transcripts normally produced by the *cys-3*$^+$ locus (1.3 and 1.6 kb) was observed at about 1.5 kb. Primer extension analysis showed a start site at −33 for the fusion gene. The dual transcripts from the *cys-3*$^+$ locus (1.3 and 1.6 kb) did not prove to be essential for growth, as the *tub*::*cys-3* gene fusion was able to complement Δ*cys-3*(18-4). Marked differences in the gene fusion transcript between high- and low-sulfur growth conditions were not observed for the *tub*::*cys-3*(2-8) transformant (Fig. 4A). The WT pattern of *cys-3*$^+$ induction and dual transcripts under low-sulfur conditions are shown in Fig. 4A for comparison.

That CYS3 protein was actually being produced by translation of the fusion transcript under high-sulfur (repressing) conditions was demonstrated by Western blot analysis using an affinity-purified anti-CYS3 (pep a) antibody (Fig. 5A). No CYS3 protein is detectable in the Δ*cys-3*(18-4) strain under the same growth conditions. Note that anti-CYS3 (pep a) antibody was prepared against a peptide whose coding sequence in *cys-3*$^+$ was deleted in the Δ*cys-3*(18-4) construct. The CYS3 protein was also detectable under low-sulfur conditions but not under high-sulfur conditions in the WT sample (data not shown). Typical band shifting by CYS3 of an oligonucleotide containing a CYS3 binding site can be

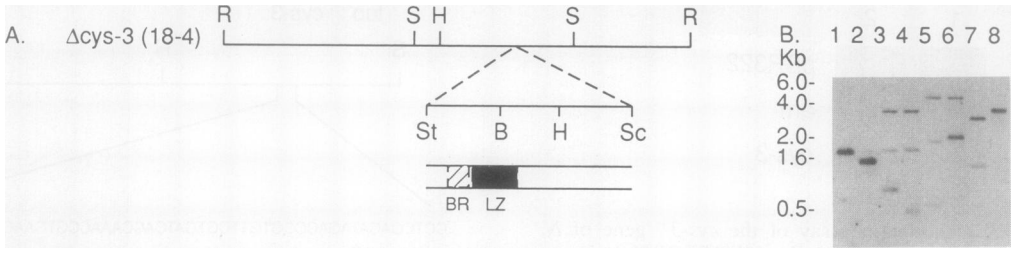

FIG. 3. (A) Restriction map of the *cys-3* region in the Δ*cys-3*(18-4) construct. Δ*cys-3*(18-4) was isolated by positive selection for *cys-3*$^+$ inactivation by transformation with a *cys-3* DNA fragment with a *Stu*I-to-*Sac*I deletion. The expanded region shows the extent of the deletion which removed the sequences coding for the basic region and leucine zipper of *cys-3* (B, *Bst*EII; H, *Hin*dIII; R, *Eco*RV; S, *Sal*I; Sc, *Sac*I; St, *Stu*I). (B) Southern blot of *N. crassa* genomic DNA probed with a ^{32}P-labeled 1.8-kb *cys-3*$^+$ fragment from pJP10. Lanes: 1, 3, 5, and 7, WT DNA; 2, 4, 6, and 8, Δ*cys-3*(18-4) DNA. Lanes 1 and 2 are *Eco*RV digests. Note in lane 2 the deleted *cys-3* fragment at 1.5 kb compared with that of the WT DNA at 1.8 kb (lane 1). Lanes 3 and 4 are *Sal*I digests; *Sal*I sites closely flank the deleted region (see panel A) and therefore yield a smaller fragment at 500 bp for Δ*cys-3*(18-4) than for WT DNA (800 bp). Lanes 5 and 6 (*Hin*dIII digest) and lanes 7 and 8 (*Bst*EII digest) demonstrate that the restriction sites as shown in the expanded region in panel A are absent in Δ*cys-3*(18-4) compared with the WT DNA. Note the missing band and larger fragment size for each case (lanes 6 and 8) for Δ*cys-3*(18-4).

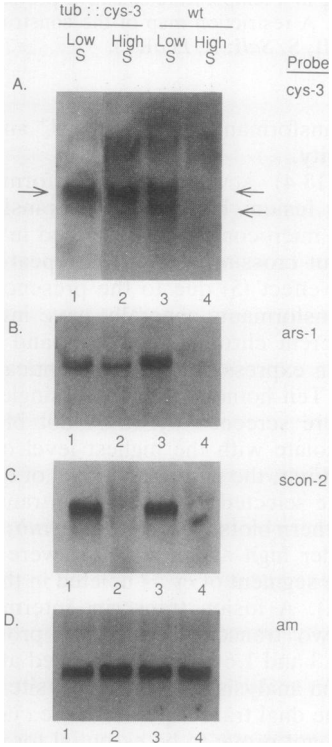

FIG. 4. Northern blot analysis of sulfur gene mRNA levels in *tub*::*cys-3*(2-8) transformant and WT strains. The strains were grown under high- and low-sulfur conditions as described in the text. Poly(A)$^+$ mRNA was isolated, and replicate blots were prepared and probed with ^{32}P-labeled DNA as indicated. (A) *cys-3* probe. Northern blots of *tub*::*cys-3* and WT mRNA were probed with the *Stu*I-*Sac*I fragment of *cys-3*$^+$. Note the presence of a single transcript in lanes 1 and 2 representing a 1.5-kb fusion transcript. No transcript is detectable in Δ*cys-3*(18-4) (data not shown). Lanes 3 and 4 show typical sulfur regulation in the WT mRNA and the presence of two transcripts (1.3 and 1.6 kb) under low-sulfur conditions. (B) *ars-1* probe. Northern blots as in panel A were probed with pJP18 *ars-1*$^+$ DNA. Note that *ars-1*$^+$ mRNA was present in lane 2 under repressing conditions for *tub*::*cys-3*$^+$ and absent for the WT sample (lane 4) under the same condition. (C) *scon-2* probe. Northern blots as in panel A were probed with pSCON2 DNA. Note that the band pattern in lanes 1 and 2 was similar to that in 3 and 4. (D) *am* probe. The constitutively expressed *am*$^+$ gene in pJP11 was used as a control to ensure comparability between samples.

observed with the *N. crassa* CYS3 protein isolated from a *tub*::*cys-3* transformant (Fig. 5B). A complete analysis of CYS3 isolated from *N. crassa* will be presented elsewhere (42). I note here only evidence that CYS3 protein was actually produced by the fusion construct under high-sulfur growth.

The key result comes with the analysis of arylsulfatase mRNA levels and enzyme levels. Normally, arylsulfatase activity and mRNA are undetectable under high-sulfur conditions. In the gene fusion transformants, there was *ars-1*$^+$ mRNA detectable under high-sulfur conditions (Fig. 4B), and substantial arylsulfatase enzyme activity was also present (Table 1). Therefore, expression of *cys-3*$^+$ under high-sulfur conditions was a sufficient signal for induction of arylsulfatase gene expression. The overall levels of arylsulfatase expression obtained were about 60% of that seen in the WT (Table 1). Slightly higher levels were seen under low-sulfur growth conditions for the gene fusion construct.

A further important step was to confirm that a functional CYS3 protein was itself responsible for the induction of *ars-1*$^+$ in the gene fusion. A construct of the *tub* promoter with a *cys-3* coding segment carrying a mutation resulting in

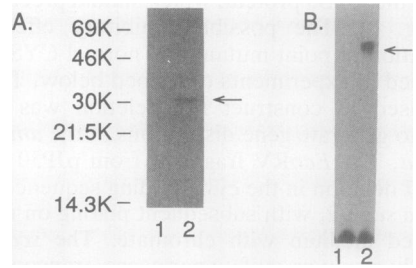

FIG. 5. Confirmation that CYS3 protein was produced under high-sulfur growth in the *tub*::*cys-3* transformant. (A) Immunoblot analysis. CYS3 extracts were prepared from Δ*cys-3*(18-4) (lane 1) and Δ*cys-3*(18-4) *tub*::*cys-3* (lane 2), both grown on high sulfur. Samples of the extracts were subjected to SDS-PAGE, transferred to nitrocellulose, and probed with anti-CYS3 (pep a) antibody as described in Materials and Methods. The arrow indicates the expected band at 30 kDa for CYS3 based on mobility seen with in vitro-produced CYS3. (B) Mobility shift analysis. Samples of the extracts were used in a mobility shift assay with ^{32}P-labeled DNA containing a CYS3 binding site (see Materials and Methods). Lanes are as for panel A.

TABLE 1. Arylsulfatase activity of WT, sulfur mutant, and *tub*::*cys-3* transformant strains

Strain	Arylsulfatase sp act[a]	
	High S[b]	Low S[c]
WT	<0.05	7.9
Δ*cys-3*	<0.05	<0.05
scon-1	7.2	7.7
Δ*cys-3 tub*::*cys-3*	4.9	5.8

[a] Expressed as nanomoles per minute per milligram of total protein (38).
[b] High-sulfur medium with 5.0 mM methionine.
[c] Low-sulfur medium with 0.25 mM methionine.

temperature-sensitive CYS3 protein was tested as with the *tub*::*cys-3* construct. In this case, with the *tub*::*cys-3*^{ts}(4-5) transformant under permissive growth conditions, arylsulfatase activity and mRNA were present under both high and low sulfur (Fig. 6B) as with *tub*::*cys-3* (Fig. 4B). Arysulfatase enzyme activity in *tub*::*cys-3*^{ts} at 25°C was at essentially the same level (~90%) as seen for *tub*::*cys-3* (data not shown). However, under restrictive conditions (i.e., upon raising the temperature), arylsulfatase mRNA (Fig. 6B) and

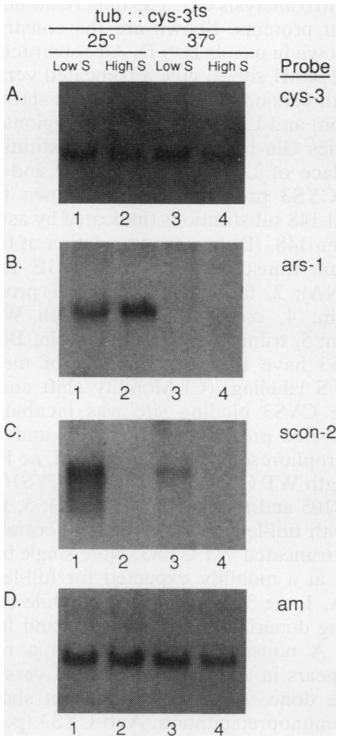

FIG. 6. Northern blot analysis of sulfur gene mRNA levels in a *tub*::*cys-3*^{ts}(4-5) transformant under permissive and restrictive growth conditions. Poly(A)$^+$ mRNA was isolated; replicate blots were prepared and probed. The probes are the same as in Fig. 4. (A) *cys-3* probe. Similar levels of the gene fusion transcript are present in lanes 1 to 4. (B) *ars-1* probe. *ars-1*$^+$ mRNA was present constitutively (lanes 1 and 2) under the lower permissive temperature but absent at the restrictive temperature (lanes 3 and 4). (C) *scon-2* probe. Lanes 1 and 2 show the WT expression pattern with *scon-2*$^+$ mRNA present only under low-sulfur conditions. Note that expression of *scon-2*$^+$ was markedly reduced in lane 3 at the restrictive temperature. (D) *am* probe. The constitutively expressed *am*$^+$ gene was used to ensure comparability between samples.

TABLE 2. β-Galactosidase ELISA assay for *cys-3*::*lacZ* expression

Construct	β-Galactosidase level[a] ± SD			
	High S[b]		Low S[c]	
	25°C	37°C	25°C	37°C
Δ*cys-3 tub*::*cys-3*^{ts} *lacZ*[d]	2.3 ± 0.3	2.8 ± 0.5	2.4 ± 0.7	3.0 ± 0.6
Δ*cys-3 tub*::*cys-3*^{ts} *cys-3*::*lacZ*	9.5 ± 0.9	2.2 ± 0.7	10.8 ± 1.2	2.8 ± 0.9

[a] Relative expression units as 100 pg of β-galactosidase protein per mg of total protein per unit (triplicate assays).
[b] High-sulfur medium with 5.0 mM methionine.
[c] Low-sulfur medium with 0.25 mM methionine.
[d] Promoterless *lacZ* construct.

enzyme activity were absent. The *tub*::*cys-3*^{ts} fusion transcript was present at similar levels under all conditions (Fig. 6B).

scon-2$^+$ mRNA level was also examined in the fusion constructs. Previously it was postulated that *scon-2*$^+$ expression was affected in a positive manner by both the *cys-3*$^+$ and *scon-1*$^+$ gene products. I examined whether overexpression of *cys-3*$^+$ alone had any effect on *scon-2*$^+$ under high-sulfur conditions. *scon-2*$^+$ mRNA normally increases in level only as the cells are under sulfur limitation. No increase in *scon-2*$^+$ level was seen under conditions of *cys-3*$^+$ expression under repressing conditions in the *tub*::*cys-3* transformant (Fig. 4C). The depressing effect of a *cys-3* mutation on *scon-2*$^+$ mRNA level seen in a previous study was seen with the temperature-sensitive construct (Fig. 6C), thus confirming the role for CYS3 protein function in this effect.

CYS3 production and autoregulatory effects. To examine the effects of CYS3 production on its own expression, I chose to use a gene fusion of the *cys-3* promoter to the β-galactosidase (*lacZ*) coding segment in pDE1. pDE1 also carries a truncated *his-3* gene, allowing for targeted integration at the *his-3* locus (12). First, as a control, it was confirmed that the *cys-3*::*lacZ* fusion showed typical sulfur-controlled regulation in that the level of β-galactosidase was responsive to the sulfur level. *cys-3*$^+$ *his-3* strains were transformed with pDE1 (promoterless *lacZ*) and the pDE1 *cys-3*::*lacZ* construct. A threefold increase in β-galactosidase protein was observed under derepressing conditions in a comparison of the *cys-3*::*lacZ* strain (5.6 U) with the promoterless *lacZ* strain (1.7 U) (see Materials and Methods). Under repressing conditions or in a Δ*cys-3*(18-4) background, the increase was not present (data not shown). The effect was comparable to that seen in Northern blot experiments showing a lack of *cys-3* mRNA in a *cys-3* mutant or under repressing growth conditions (43). The critical experiment was to test whether CYS3 was sufficient to stimulate expression of *cys-3*::*lacZ*. The *cys-3*::*lacZ* fusion was transformed into a *his-3* Δ*cys-3*(18-4) strain, a resulting homokaryon was then transformed with *tub*::*cys-3*^{ts}, and a final homokaryon was isolated. I found an approximately fourfold increase in β-galactosidase protein level over the control background level under repressing conditions, which was eliminated by growth at the restrictive temperature (Table 2). As seen previously with *ars-1*$^+$ expression, constitutive CYS3 production can lead to stimulation of expression of *cys-3*$^+$. This was consistent with the finding that the CYS3 protein can bind to its own promoter (16) and previous

evidence that a defect in CYS3 essentially blocks its own production (43).

Dimerization analysis of mutant and normal CYS3 protein. Given the stimulation of *cys-3*$^+$ expression by the CYS3 protein itself, it seemed possible that an inhibitory effect on CYS3 function might occur in certain constructs due to dimerization with defective subunits (23). Thus, constitutive production of CYS3 would promote transcription of a resident *cys-3* mutant gene.

The initial overexpression experiments were carried out with the *cys-3*(p22) mutant. The *cys-3*(p22) strain contains a mutated basic region (17) which results in a loss of DNA binding activity (16). *tub*::*cys-3* transformants of *cys-3*(p22) showed only marginal induction of arylsulfatase expression (20% of that later observed in the Δ*cys-3* strain). To test the model that defective heterodimers could be causing the effect with *cys-3*(p22), in vitro transcription and translation were used to produce CYS3 protein for DNA binding and immunoprecipitation analysis.

WT and mutant CYS3 proteins were generated as either full-length or truncated versions by in vitro transcription with SP6 RNA polymerase followed by translation in a rabbit reticulocyte system (Fig. 7A and B). In each case, the expected-size CYS3 protein was produced with the transcripts translated separately (Fig. 7B, lanes 2, 3, and 5) or in cotranslations (Fig. 7B, lane 4). The CYS3 WT and mutant proteins were then tested in a band shift assay for DNA binding. Cotranslated full-length and truncated WT CYS3 gave a characteristic band shift pattern (Fig. 7C, lane 6). Three bands corresponding to full-length (top band) and truncated (bottom band) homodimers as well as a heterodimer of full-length and truncated subunits (middle band) are seen (Fig. 7C, lane 6). A control experiment, using instead ^{35}S-labeled protein and SDS-PAGE in a second dimension (not shown) in the approach that Hope and Struhl (24) used for the dimerization analysis of GCN4, confirmed band identity. When, however, cotranslated full-length WT CYS3 and truncated mutant CYS105 were tested, then only a single band running at a position corresponding to a full-length homodimer was seen (Fig. 7C, lane 4). The CYS105 construct carries the same basic region alteration as in the *cys-3*(p22) mutant (Gln for Lys-105 and Arg-106) (17). A comparable shift of only a single band was seen with cotranslated full-length WT CYS3 and a truncated leucine zipper mutant construct (CYS148) expected to be defective in dimerization on the basis of other bZIP protein studies (18, 30, 49, 56) and as independently confirmed for CYS3 (26). That is, there was no indication of dimerization of WT CYS3 and CYS148 with the corresponding appearance of additional bands in the gel shift assay (Fig. 7C, lane 5). Reciprocal experiments carried out with truncated WT CYS3 and full-length mutant CYS105 and CYS148 gave comparable results, with only a single band present at a position corresponding to a homodimer of truncated subunits (not shown).

Although cotranslated full-length WT CYS3 and mutant CYS105 gave a single band shift, evidence from immunoprecipitation shows that heterodimers were formed. The anti-CYS3 (pep a) antibody recognized full-length protein but not truncated versions, because the antibody was produced by using a peptide that was coded for 3′ to the *Ppu*MI site used for producing truncated template. The experiment in Fig. 7D shows that the truncated CYS105 protein alone was not immunoprecipitated (Fig. 7D, lane 3) but that CYS105 protein could be immunoprecipitated from a cotranslation with full-length WT CYS3 (Fig. 7D, lane 4). A control immuno-

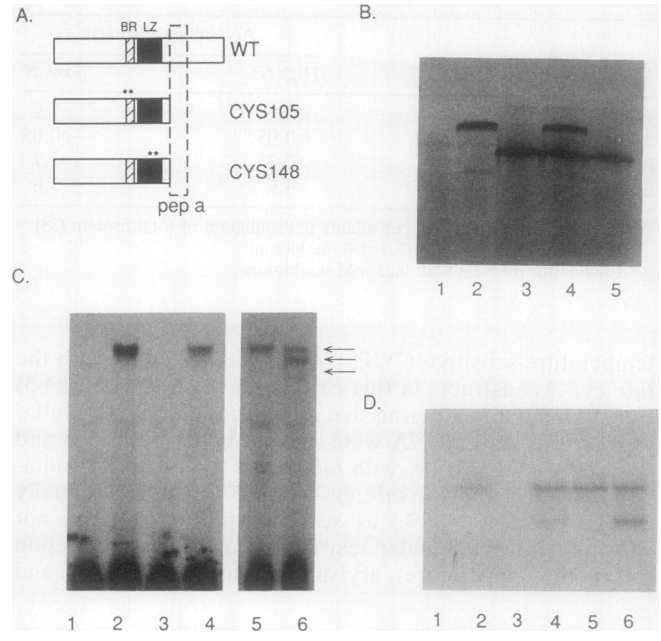

FIG. 7. In vitro analysis of CYS3 dimerization. (A) Structures of WT and mutant proteins. Shown are the constructs used for the experiments shown in panels B to D. All constructs were digested at an introduced *Ppu*MI site to give a truncated version or *Bam*HI to give a full-length version. The WT protein is shown full length with BR (basic region) and LZ (leucine zipper) regions. CYS105, shown truncated, carries Gln-105 and Gln-106 substitutions (indicated by asterisks) in place of Lys-105 and Arg-106 and codes for a non-DNA-binding CYS3 protein. CYS148, shown truncated, carries Val-141 and Val-148 substitutions (indicated by asterisks) in place of Leu-141 and Leu-148. (B) In vitro translation of full-length WT and truncated mutant transcripts and SDS-PAGE analysis. Lanes: 1, control (no RNA); 2, full-length CYS3 WT protein; 3, truncated CYS105 protein; 4, cotranslated full-length WT and truncated CYS105 protein; 5, truncated CYS148 protein. Both full-length and truncated CYS3 have the same number of methionine residues available for ^{35}S labeling. (C) Mobility shift analysis. ^{32}P-labeled DNA with the CYS3 binding site was incubated with in vitro-translated unlabeled protein (see Materials and Methods) as indicated and electrophoresed. Lanes: 1, control, no RNA in translation mix; 2, full-length WT CYS3; 3, truncated CYS105; 4, cotranslated truncated CYS105 and full-length WT CYS3; 5, truncated CYS148 cotranslated with full-length WT CYS3; 6, cotranslated full-length WT CYS3 and truncated WT CYS3. Note single bands in lanes 2, 4, and 5 that are at a mobility expected for full-length homodimers bound to DNA. Lane 3 shows no band, while lane 6 shows three bands indicating dimerization of truncated and full-length proteins (see Results). A nonspecific band due to a reticulocyte lysate component appears in all lanes. Truncation versus full-length protein tests were done reciprocally (data not shown) for the constructs. (D) Immunoprecipitation. Anti-CYS3 (pep a) antibody was used to immunoprecipitate, by protein A agarose, ^{35}S-labeled in vitro-translated protein. Note that the antibody was specific for the full-length protein and was not immunoreactive to the truncated version (region represented by the boxed area shown in panel A). Lanes: 1, control, no RNA in translation mix; 2, full-length WT CYS3; 3, truncated CYS105; 4, cotranslated full-length WT and truncated CYS105 (note that truncated CYS105 was immunoprecipitated along with full-length WT protein); 5, full-length WT CYS3 cotranslated with CYS148 (note that only full-length protein was immunoprecipitated); 6, full-length WT CYS3 cotranslated with truncated WT CYS3. In this case, two bands corresponding to full-length and truncated CYS3 are immunoprecipitated.

precipitation with full-length and truncated WT CYS3 shows a pattern (Fig. 7D, lanes 6) similar to that for full-length WT and CYS105 (Fig. 7D, lane 4). That the immunoprecipitation was less for CYS105 than for the WT protein (Fig. 7D, lane 4 versus lane 6) may reflect some effect of the mutated basic region on heterodimerization. The specificity of the immunoprecipitation was shown in that truncated CYS148 protein (with a mutated leucine zipper) was not immunoprecipitated from a cotranslation with full-length WT CYS3; only the full-length WT CYS3 can be observed (Fig. 7D, lane 5). The immunoprecipitation experiments indicated that non-DNA-binding heterodimers were present in cotranslated WT CYS3 and CYS105 preparations.

DISCUSSION

In this report, I have tested the role of the CYS3 positive regulator in the signal response pathway triggered by sulfur limitation. The current model of the sulfur regulatory system suggests that CYS3 is the direct mediator of transcriptional activation of the genes under its control (e.g., the arylsulfatase gene). In WT *N. crassa*, *cys-3*$^+$ is expressed in parallel with the target structural genes and only under conditions of sulfur limitation. I first examined whether overproduction of CYS3 under repressing conditions (high sulfur) would be sufficient to activate transcription of the sulfur structural genes. A heterologous promoter fusion (*tub*::*cys-3*) was used to demonstrate that the expression of CYS3 under repressing conditions was sufficient to stimulate transcription of the arylsulfatase gene. In *tub*::*cys-3* transformants, both arylsulfatase mRNA and enzyme activity were clearly detected under normally repressing conditions. A novel temperature-sensitive construct of CYS3 was used to confirm that the induction was due to CYS3 protein function (Fig. 6). The *tub*::*cys-3*^{ts} construct had a Gln-for-Arg-106 substitution (17) in the basic region of CYS3 which resulted in temperature-sensitive DNA binding. The basic region represents the DNA-binding domain of bZIP proteins (26, 33, 57).

The production of CYS3 was therefore necessary and sufficient to lead to the stimulation of sulfur structural gene expression. The fact that CYS3 expression under high sulfur can induce the system suggests that an additional sulfur regulatory component, one that would be induced or activated by sulfur limitation, is not necessary for transcriptional induction. It is possible, however, that *tub*::*cys-3*-directed CYS3 production may stimulate another component under high-sulfur growth or that the component could be produced constitutively. In addition, the results suggest that no negative factor must be inactivated or repressed for CYS3 protein, once present, to induce transcription of the sulfur genes. The results indicate that the crucial control point in the regulatory system is whether or not *cys-3*$^+$ is transcribed.

Of interest are the *scon* negative regulatory mutants and their phenotype of constitutive sulfur gene expression (4). Nuclear run-on assays with the *scon* mutants showed constitutive, rather than regulated, transcription of *cys-3*$^+$. Further, the *tub*::*cys-3* transformants are similar in phenotype to the *scon* mutants, since in both cases there is constitutive sulfur gene expression. These results suggest that the *scon* regulators exert their effect by influencing the level of *cys-3*$^+$ expression.

A further aspect of the control model concerns the autoregulation of *cys-3*$^+$ expression. The *cys3*::*lacZ* constructs provide direct in vivo evidence for the role of CYS3 in its own expression (Table 2). The use of the *tub*::*cys*^{ts} *cys-3*::*lacZ* Δ*cys-3* transformant confirmed that CYS3 protein function was necessary and sufficient for induction of *cys-3*$^+$ expression. In regard to autoregulation, the *scon* regulators role may be to directly modulate CYS3 function and hence the level of transcription for the *cys-3*$^+$ gene.

scon-2$^+$ mRNA level was not affected by the production of CYS3 under high-sulfur (repressing) growth, as indicated by Northern blot analysis (Fig. 4C). In *cys-3* mutants, it has been shown that there are depressed levels of *scon-2*$^+$ mRNA present, and a possible feedback loop has been proposed (41). In that model, both *scon-1*$^+$ and *cys-3*$^+$ would have an effect on *scon-2*$^+$ expression. A positive interaction with the *scon-1*$^+$ regulator, as well as *cys-3*$^+$, was suggested (41). The lack of effect on *scon-2*$^+$ mRNA level in *tub*::*cys-3* transformants is consistent with that model. An important mechanistic point is that although *scon-2*$^+$ and *cys-3*$^+$ expression normally parallel one another, the overexpression of *cys-3*$^+$ was in itself sufficient for transcriptional activation. Another implication is that *scon-1*$^+$ may not be present or functional under high-sulfur conditions. The cloning of *scon-1*$^+$, which is in progress (42), will help resolve this matter.

Critical to a demonstration of the effects reported was the construction of a *cys-3* deletion strain [Δ*cys-3*(18-4)]. Normally, homologous integrations are relatively rare in *N. crassa*, and therefore a novel positive selection scheme was devised to select for a deletion in the *cys-3* gene. The approach has been most successful in the production of relatively short deletions (42). The deletion constructed had both the basic region and leucine zipper deleted. The *cys-3* deletion strain, Δ*cys-3*(18-4), would therefore not produce a protein able to interact and dimerize with an introduced *cys-3* construct. The positive selection for *cys-3*$^+$ disruption or inactivation may have a general usefulness for mutational and other types of study. Of additional importance was the use of homokaryotic isolates from primary transformants. Arylsulfatase gene expression under high-sulfur conditions could be detected at only a low level in primary heterokaryotic transformants (data not shown) but was clearly detected in homokaryons (Table 1).

Initially, experiments yielded only low levels of induced expression of *ars-1*$^+$ under repressing conditions in homokaryotic *tub*::*cys-3* transformants in a standard *cys-3* (i.e., allele p22) mutant background [~20% of that seen in the *tub*::*cys-3* Δ*cys-3*(18-4) strain; Table 1]. The mutation in *cys-3*(p22) results in a CYS3 protein with defective DNA-binding (17). That mutant CYS3(p22) protein, as well as normal CYS3 protein, was produced in *tub*::*cys-3* *cys-3*(p22) transformants seemed likely, since *cys-3*(p22) mRNA was detectable in such constructs (data not shown). Heterodimerization of normal and mutant CYS3 subunits would then be possible. If the heterodimer was nonfunctional, then there may be an inhibitory effect due to a reduction in the effective concentration of active CYS3 homodimer.

The current isolation of CYS3 via binding affinity precludes direct in vivo proof of non-DNA-binding heterodimers. An initial investigation of dimerization by in vitro modeling was carried out. The validity of an in vitro approach with CYS3 was supported by Western blot and band shift experiments that indicated that CYS3 protein isolated from *N. crassa* has the same properties (Fig. 5) as that produced in vitro. The in vitro experiments suggest that non-DNA-binding CYS3 heterodimers can be formed.

CYS3 dimerization was demonstrated by immunoprecipitation as with Jun and Fos as well as in other cases (10, 29). The experiments showed that cotranslated normal and trun-

cated WT CYS3 could produce homodimeric and heterodimeric complexes that bound DNA in a sequence-specific manner (Fig. 7C). In contrast, a mixture of cotranslated full-length WT CYS3 and truncated basic-region-mutated CYS105 showed only full-length homodimers that bound DNA. However, immunoprecipitation experiments with anti-CYS3 (pep a) antibody indicated that non-DNA-binding heterodimers were present in such mixtures of WT CYS3 and truncated CYS105 protein (Fig. 7C and D). Mutations in the leucine zipper blocked such heterodimeric associations and consequently any immunoprecipitation (Fig. 7D). This finding demonstrated, as well, the importance of the leucine zipper for dimerization in CYS3 and confirmed its similarity to other bZIP proteins. The apparent inhibitory effect of the *cys-3*(p22) allele may therefore be due to a dominant negative (23) effect. Dominant negative interactions have been observed in other bZIP proteins such as CREB, Jun, and Fos (11, 51). A more pronounced effect might be produced in vivo by production of the mutant protein at a higher level. The findings with CYS3 should be of general interest with regard to the organization of gene regulation systems involving a diversity of known bZIP proteins (32, 46, 57).

ACKNOWLEDGMENTS

I thank Melinda Knudson-Stanley for assistance with mRNA isolation.

This work was supported by Public Health Service grant GM-38671 from the National Institutes of Health.

REFERENCES

1. **Aviv, H., and P. Leder.** 1972. Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid-cellulose. Proc. Natl. Acad. Sci. USA **69:**1408–1412.
2. **Birnboim, H. D., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7:**1513–1523.
3. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
4. **Burton, E. G., and R. L. Metzenberg.** 1972. Novel mutation causing derepression of several enzymes of sulfur metabolism in *Neurospora crassa*. J. Bacteriol. **109:**140–151.
5. **Cambareri, E. B., B. C. Jensen, E. Schabtach, and E. U. Selker.** 1989. Repeat-induced G-C to A-T mutations in *Neurospora*. Science **244:**1571–1575.
6. **Chodosh, L. A.** 1989. Mobility shift DNA-binding assay using gel electrophoresis, p. 12.2.6 *In* F. M. Ausubel, R. Brent, R. I. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.), Current protocols in molecular biology. John Wiley & Sons, New York.
7. **Dagert, M., and S. D. Ehrlich.** 1979. Prolonged incubation in calcium chloride improves the competence of *Escherichia coli* cells. Gene **6:**23–28.
8. **Davis, R. H., and E. F. DeSerres.** 1970. Genetic and microbiological research techniques for *Neurospora crassa*. Methods Enzymol. **17A:**79–143.
9. **Dolan, J. W., and S. Fields.** 1990. Overproduction of the yeast STE12 protein leads to constitutive transcriptional induction. Genes Dev. **4:**492–502.
10. **Draginis, A. M.** Binding of yeast a1 and α2 as a heterodimer to the operator DNA of a haploid-specific gene. Nature (London) **347:**682–685.
11. **Dwarki, V. J., M. Montminy, and I. M. Verma.** 1990. Both the basic region and the 'leucine zipper' domain of the cyclic AMP response element binding (CREB) protein are essential for transcriptional activation. EMBO J. **9:**225–232.
12. **Ebbole, D.** 1990. Vectors for construction of translational fusions to β-galactosidase. Fungal Genet. Newsl. **37:**15–16.
13. **Ebbole, D., and M. S. Sachs.** 1990. A rapid and simple method for isolation of *Neurospora crassa* homokaryons using microconidia. Fungal Genet. Newsl. **37:**17–18.
14. **Ebbole, D. J., J. L. Paluh, M. Plamann, M. S. Sachs, and C. Yanofsky.** 1991. *cpc-1*, the general regulatory gene for genes of amino acid biosynthesis in *Neurospora crassa*, is differentially expressed during the asexual life cycle. Mol. Cell. Biol. **11:**928–934.
15. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132:**6–13.
16. **Fu, Y. H., and G. A. Marzluf.** 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.
17. **Fu, Y. H., J. V. Paietta, D. G. Mannix, and G. A. Marzluf.** 1989. *cys-3*, the positive-acting sulfur regulatory gene of *Neurospora crassa*, encodes a protein with a putative leucine zipper DNA-binding element. Mol. Cell. Biol. **9:**1120–1127.
18. **Gentz, R., F. J. Rauscher III, C. Abate, and T. Curran.** 1989. Parallel association of fos and jun leucine zippers juxtaposes DNA binding domains. Science **243:**1695–1699.
19. **Gill, G., and M. Ptashne.** 1988. Negative effect of the transcriptional activator GAL4. Nature (London) **334:**721–724.
20. **Greenberg, M. E., and E. B. Ziff.** 1984. Stimulation of 3T3 cells induces transcription of the c-*fos* proto-oncogene. Nature (London) **311:**433–438.
21. **Hanson, M. A., and G. A. Marzluf.** 1973. Regulation of a sulfur-controlled protease in *Neurospora crassa*. J. Bacteriol. **116:**785–789.
22. **Harlow, E., and D. Lane (ed.).** 1988. Antibodies: a laboratory manual, p. 480–504. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
23. **Herskowitz, I.** 1987. Functional inactivation of genes by dominant negative mutations. Nature (London) **329:**219–222.
24. **Hope, I. A., and K. Struhl.** 1987. GCN4, a eukaryotic transcriptional activator protein, binds as a dimer to target DNA. EMBO J. **6:**2781–2784.
25. **Johnston, S. A., and J. Hopper.** 1982. Isolation of the yeast regulatory gene GAL4 and analysis of its dosage effects on the galactose/melibiose regulon. Proc. Natl. Acad. Sci. USA **79:**6971–6975.
26. **Kanaan, M., and G. A. Marzluf.** 1991. Mutational analysis of the DNA-binding domain of the CYS3 regulatory protein of *Neurospora crassa*. Mol. Cell. Biol. **11:**4356–4362.
27. **Ketter, J. S., and G. A. Marzluf.** 1988. Molecular cloning and analysis of the regulation of *cys-14*, a structural gene of the sulfur regulatory circuit of *Neurospora crassa*. Mol. Cell. Biol. **8:**1504–1508.
28. **Kinnaird, J. H., M. A. Keighren, J. A. Kinsey, M. Eaton, and J. R. S. Fincham.** 1982. Cloning of the *am* (glutamate dehydrogenase) gene of *Neurospora crassa* through the use of a synthetic DNA probe. Gene **20:**387–396.
29. **Kouzarides, T., and E. Ziff.** 1988. The role of the leucine zipper in the fos-jun interaction. Nature (London) **336:**646–651.
30. **Kouzarides, T., and E. Ziff.** 1989. Leucine zippers of fos, jun and GCN4 dictate dimerization specificity and thereby control DNA binding. Nature (London) **340:**568–571.
31. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
32. **Landschulz, W. H., P. F. Johnson, and S. L. McKnight.** 1988. The leucine zipper: a hypothetical structure common to a new class of DNA binding proteins. Science **240:**1759–1764.
33. **Landschulz, W. H., P. F. Johnson, and S. L. McKnight.** 1989. The DNA binding domain of the rat liver nuclear protein C/EBP is bipartite. Science **243:**1681–1688.
34. **Loros, J. J., and J. C. Dunlap.** 1991. *Neurospora crassa* clock-controlled genes are regulated at the level of transcription. Mol. Cell. Biol. **11:**558–563.
35. **Marzluf, G. A.** 1970. Genetic and metabolic controls for sulfate metabolism in *Neurospora crassa*: isolation and study of chromate-resistant and sulfate transport-negative mutants. J. Bacteriol. **102:**716–721.

36. **Metzenberg, R. L.** 1979. Implications of some genetic control mechanisms in *Neurospora*. Microbiol. Rev. **43**:361–383.
37. **Metzenberg, R. L., and T. J. Baisch.** 1981. An easy method for preparing *Neurospora* DNA. Neurospora Newsl. **28**:20–21.
38. **Metzenberg, R. L., and J. W. Parson.** 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.
39. **Orbach, M. J., E. B. Porro, and C. Yanofsky.** 1986. Cloning and characterization of the gene for beta-tubulin from a benomyl-resistant mutant of *Neurospora crassa* and its use as a dominant selectable marker. Mol. Cell. Biol. **6**:2452–2461.
40. **Paietta, J. V.** 1989. Molecular cloning and regulatory analysis of the arylsulfatase structural gene of *Neurospora crassa*. Mol. Cell. Biol. **9**:3630–3637.
41. **Paietta, J. V.** 1990. Molecular cloning and analysis of the *scon-2* negative regulatory gene of *Neurospora crassa*. Mol. Cell. Biol. **10**:5207–5214.
42. **Paietta, J. V.** Unpublished data.
43. **Paietta, J. V., R. A. Akins, A. M. Lambowitz, and G. A. Marzluf.** 1987. Molecular cloning and characterization of the *cys-3* regulatory gene of *Neurospora crassa*. Mol. Cell. Biol. **7**:2506–2511.
44. **Paietta, J. V., and G. A. Marzluf.** 1985. Gene disruption by transformation in *Neurospora crassa*. Mol. Cell. Biol. **5**:1554–1559.
45. **Pall, M. L.** 1971. Amino acid transport in *Neurospora crassa* II: properties and regulation of a methionine transport system. Biochim. Biophys. Acta **233**:201–214.
46. **Paluh, J. L., and C. Yanofsky.** 1991. Characterization of *Neurospora* CPC1, a bZIP DNA-binding protein that does not require aligned heptad leucines for dimerization. Mol. Cell. Biol. **11**:935–944.
47. **Reinert, W. R., V. B. Patel, and N. H. Giles.** 1981. Genetic regulation of the *qa* gene cluster in *Neurospora crassa*: induction of *qa* messenger ribonucleic acid and dependency on *qa-1* function. Mol. Cell. Biol. **1**:829–835.
48. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
49. **Sassone-Corsi, P., L. J. Ransone, W. W. Lamph, and I. M. Verma.** 1988. Direct interaction between fos and jun nuclear oncoproteins: role of the 'leucine zipper' domain. Nature (London) **336**:692–695.
50. **Shuler, J. L., and J. V. Paietta.** 1990. CYS3 regulatory protein expressed in *E. coli* binds the promoter of the arylsulfatase gene of *Neurospora crassa*. FASEB J. **4**:A2131.
51. **Smeal, T., P. Angel, J. Meek, and M. Karin.** 1989. Different requirements for formation of jun:jun and jun:fos complexes. Genes Dev. **3**:2091–2100.
52. **Smith, D. E., and P. A. Fisher.** 1984. Identification, developmental regulation, and response to heat shock of two antigenically related forms of a major nuclear envelope protein in Drosophila embryos: application of an improved method of affinity purification of antibodies using polypeptides immobilized on nitrocellulose blots. J. Cell Biol. **99**:20–28.
53. **Southern, E.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
54. **Taylor, J. W., J. Ott, and F. Eckstein.** 1985. The rapid generation of oligonucleotide-directed mutations at high frequency using phosphorothioate-modified DNA. Nucleic Acids Res. **13**:8765–8785.
55. **Treisman, R., and T. Maniatis.** 1985. Simian virus 40 enhancer increases number of RNA polymerase II molecules on linked DNA. Nature (London) **315**:72–75.
56. **Turner, R., and R. Tjian.** 1989. Leucine repeats and an adjacent DNA binding domain mediate the formation of functional cfos-cjun heterodimers. Science **243**:1689–1694.
57. **Vinson, C. R., P. B. Sigler, and S. L. McKnight.** 1989. Scissors-grip model for DNA recognition by a family of leucine zipper proteins. Science **246**:911–946.
58. **Vollmer, S. J., and C. Yanofsky.** 1986. Efficient cloning of genes of *Neurospora crassa*. Proc. Natl. Acad. Sci. USA **83**:4869–4873.