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The sulfur controller-2 negative regulatory gene of *Neurospora crassa* encodes a protein with β-transducin repeats

(Gβ-homologous domain/WD-40 repeat/sulfur gene regulation/regulatory control loop/CYS3 protein)

ANUJ KUMAR AND JOHN V. PAIETTA

Department of Biochemistry and Molecular Biology, Wright State University, Dayton, OH 45435

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ABSTRACT **The sulfur regulatory system of *Neurospora crassa* is composed of a set of structural genes involved in sulfur catabolism controlled by a genetically defined set of trans-acting regulatory genes. These sulfur regulatory genes include *cys-3*$^+$, which encodes a basic region–leucine zipper transcriptional activator, and the negative regulatory gene *scon-2*$^+$. We report here that the *scon-2*$^+$ gene encodes a polypeptide of 650 amino acids belonging to the expanding β-transducin family of eukaryotic regulatory proteins. Specifically, SCON2 protein contains six repeated Gβ-homologous domains spanning the C-terminal half of the protein. SCON2 represents the initial filamentous fungal protein identified in the β-transducin group. Additionally, SCON2 exhibits a specific amino-terminal domain that potentially defines another subfamily of β-transducin homologs. Expression of the *scon-2*$^+$ gene has been examined using RNA hybridization and gel mobility-shift analysis. The dependence of *scon-2*$^+$ expression on CYS3 function and the binding of CYS3 to the *scon-2*$^+$ promoter indicate the presence of an important control loop within the *N. crassa* sulfur regulatory circuit involving CYS3 activation of *scon-2*$^+$ expression. On the basis of the presence of β-transducin repeats, the crucial role of SCON2 in the signal-response pathway triggered by sulfur limitation may be mediated by protein–protein interactions.**

Sulfur uptake and assimilation in *Neurospora crassa* are accomplished via a complex regulatory circuit encompassing a set of coordinately expressed structural genes involved in sulfur catabolism and a set of trans-acting regulatory genes (1, 2). These unlinked structural genes encode a variety of sulfur-related enzymes including arylsulfatase, sulfate permease I, and sulfate permease II (2, 3). The positive regulatory gene *cys-3*$^+$ encodes a basic region–leucine zipper protein that functions as a DNA-binding transcriptional activator (4, 5). *cys-3* mutants exhibit a pleiotropic loss of the entire set of sulfur-controlled enzymes as well as depressed *cys-3*$^+$ expression. The *cys-3*$^+$ gene is expressed only under low-sulfur derepressing conditions (5). *cys-3*$^+$ expression is further governed by the negative regulatory sulfur controller genes, *scon-1*$^+$ and *scon-2*$^+$ (6, 7). *scon-1* and *scon-2* mutants constitutively express the sulfur structural and *cys-3*$^+$ genes (6, 7). The *scon-2*$^+$ gene has been cloned and analyzed (7). *scon-2*$^+$ is expressed only under low sulfur conditions, as shown by increases in mRNA content. Furthermore, nuclear run-on assays show that *scon-2*$^+$ is subject to transcriptional control (7).

Here we report the complete nucleotide sequence of the *N. crassa scon-2*$^+$ gene.* Sequence analysis of the SCON2 gene product reveals the presence of six repeats homologous to those found within the β subunit of heterotrimeric G proteins (8). These β-transducin (or WD-40) repeats have been identified within a variety of proteins known to mediate a diverse array of cellular functions. Collectively, β-transducin homologs constitute an important family of eukaryotic regulatory proteins (9). Although β-transducin-like proteins have been found within most eukaryotes, SCON2 is the only reported filamentous fungal protein to date to display WD-40 repeats. Additionally, SCON2 exhibits a distinctive amino-terminal domain that defines a previously uncharacterized subset of β-transducin-like proteins. Finally, we have further characterized expression of the *scon-2*$^+$ gene with *in vitro* as well as *in vivo* evidence suggesting CYS3 transcriptional regulation of *scon-2*$^+$.

MATERIALS AND METHODS

Strains, Plasmids, and Culture Conditions. *N. crassa* Δ*cys-3*(18-4) was constructed by gene replacement as described (10). Strain 740R23-1a was used as the wild-type (WT) strain for these studies. pNH60 carried the *his-3*$^+$ gene on a 7.5-kb fragment in pRK9 (11). The plasmid pSCON2 contained the *scon-2*$^+$ gene as described (7). The T7 expression vector pET3a-CYS3, constructed in a previous study (12), was used as a source of *Escherichia coli*-expressed CYS3. *N. crassa* cultures were grown at 25°C on minimal Vogel medium (13) with supplements as required. Sulfur-repressing and -derepressing conditions were achieved with Vogel-minus-sulfur medium supplemented with 5.0 mM methionine and 0.25 mM methionine, respectively (3).

DNA Sequencing. A 4.4-kb *Sal* I–*Eco*RV fragment from pSCON2 containing the *scon-2*$^+$ gene was subcloned into M13mp18 and -mp19. ^{35}S-labeled dATP dideoxynucleotide sequencing of both DNA strands was done by using modified T7 bacteriophage DNA polymerase (14) and a combination of synthetic oligonucleotide primers derived from the sequence.

cDNA Cloning. The synthetic oligonucleotides 5′-CTCAAGCTTCCAAGTACTGCTATAGACACCA-3′ and 5′-CTCTCTAGAGATGTCAACATGGGTCGAGT-3′ were used to prime a PCR using a *N. crassa* cDNA library in λgt10 as a substrate. The resulting PCR product was cloned into M13mp18 and sequenced as described above.

Transcript Mapping. To locate the 5′ end of the *scon-2*$^+$ transcript by S1 nuclease analysis, the end-labeled oligonucleotide 5′-GGCGTAGATGCTGGGAATGTGTT-3′ (complementary to nt 47–69) was hybridized to M13mp18 containing the *scon-2*$^+$ gene under described conditions (15). The resulting hybrid was extended through the use of the Klenow fragment of *E. coli* DNA polymerase I. The extended primer was cut with *Bsa*HI, generating a 223-bp probe that was hybridized to 20 μg of mRNA isolated from wild-type *N. crassa* grown under low-sulfur-derepressing conditions. The probe/mRNA hybrids were then digested with 200 units of S1 nuclease at 30°C as described (15). To map the 5′ end of *scon-2*$^+$ mRNA by primer extension, the end-labeled oligonucleotide 5′-ACATGTTGGTGACGCTGTTGCGGGAGCG-CAACCTT-3′ (complementary to nt −31 to 4) was hybridized to 20 μg of mRNA isolated from wild-type *N. crassa* grown

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*The sequence reported in this paper has been deposited in the GenBank data base (accession no. U17251).

under derepressing conditions. The primer was then extended using 50 units of reverse transcriptase at 42°C for 2 hr.

Computer Analysis. The National Center for Biotechnology Information nonredundant and Swiss-Prot protein sequence data bases were searched using the BLAST (16) and BLITZ (17) e-mail servers respectively. The program MACAW (18) was used to identify ungapped similarity blocks in Figs. 3C and 4A. All gapped alignments were generated with the CLUSTALV program.

Northern Analysis. Poly(A)$^+$ mRNA was isolated as described (7). ^{32}P-labeled probes were prepared by oligolabeling of DNA fragments (19). Blots were hybridized and washed as outlined elsewhere (7).

Gel-Mobility Shifts. The following oligonucleotides and their complements were synthesized, each pair representing a single CYS3-binding site (or mutated CYS3-binding site) on the *scon-2*$^+$ promoter: (*i*) 5′-CGGATCGATGGCGACCAAGGTGCT-3′ and mutated 5′-CGGATCGATGGAGACCAAGGTGCT-3′, (*ii*) 5′-GCTGTGCATTGCGTGAGGTGCCAC-3′ and mutated 5′-GCTGTGCATTGAGTGAGGTGCCAC-3′, (*iii*) 5′-CCTGGTCAGCACGCCATCCATCGC-3′ and mutated 5′-CCTGGTCAGCACTCCATCCATCGC-3′, (*iv*) 5′-ACAATCGCAGCGTCATTCTGGGCC-3′ and mutated 5′-ACAATCGCAGCTTCATTCTGGGCC-3′. Mutated binding sites represent substitutions of pyrimidine for purine at base position 6 within the consensus CYS3-binding sequence (12). All binding sites and mutated binding sites were subjected to gel mobility-shift assays as described (10) using a crude bacterial extract prepared from *E. coli* overexpressing CYS3.

RESULTS

Sequence and Organization of the *scon-2*$^+$ Gene. The complete nucleotide sequence of the *scon-2*$^+$ gene and flanking 5′ region is presented in Fig. 1. The *scon-2*$^+$ gene is 2154 bp in length, encoding a polypeptide of 650 amino acids (molecular mass, 72.2 kDa). The *scon-2*$^+$ coding region is interrupted by

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-1147 AAAAGACCCTTTAAACACTTTGACTCGGATCGATGGCGACCAAGGTGCTGTGCATTGCGTGAGGTGCCACCTTGTCCCGTCCACATCCTCGCTGCTCCCGTCCTAGGTCATCCTCCAACA -1028
                                   └←—1——┘          └←—2——┘
-1027 GACATCGATTCCTCCTTAACGTGATTGCCCTCCAAACTGCAGCTATACTGCGCTGCACCAGATAAGAGTGCACTAACACTTACACCGCACCAATAACAACAGGTCCAACAACGTGCCCAC -908
 -907 CTCCACGGCAGGGAATCAATCCTTCAACGGTCCCTCTTTCATCTCAGCTGAGCTCTTCCTGATCGCCTCCAGTCCTCCTCCCTCCCCGGGGCTCCATCTCCAATCCTCCGCCAACAACAT -788
 -787 CCAGATCCCATCACGTGTCCATCCATCCAGAAACATCAAGATCGCCAGTTGGGCTGCCCGGCATCACATCGCTGCTGTTTGTTGTCTGCGGGACCCTGCCTTCCTTCTGCTCCTCCTCCA -668
 -667 GCTTCATCTAGACCTGCCCAACGGTGGAGCGAGTCTGGATTCCGCATTAACGAGATTGGACCTTTGAGACGGTAGACCGCCCAAACATCGACCACACCAGGGCATCCATCCATACACCTC -548
 -547 ACATCAACCAGCCCGATCACGCAAGACCACATACCCTTTCCCGCCCCAGCCTGACCGACGGGAGTACCTACTACTAGTACATACCTTGGACTGGACAGGACTGGACAGGCAGGACAGTAG -428
 -427 TGTACAGGACAGCACTGCGATACATACAGCAGCAATCTGCGTCCCCTGGTCAGCACGCCATCCATCGCCCTCTAGCCGACCAATGCTTGGTGTTGCCTCTTCCGCCATTCAACAACAGAA -308
                                                    └——3—→┘
 -307 CCACGCAACCGCAAACGCACTCTAAATTCCCCCTCAGAACGCACAATCGCAGCGTCATTCTGGGCCCGGCGGTCCTCGACTCCCCCTTCTCCCAATGGATTCCTACCACCTCGCCCCGTC -188
                                                └——4—→┘                   ↓  ↓  ↓    ↓
 -187 TCGTCCGACTGCGAGCTTCGGACATGATAATGACGCCCCGCTCCAACATGGGCCTCTAGTGAACTCCTACT[TCATCACC]ATAACAACCACTCGCGCGACACCGACTCCGATCCCACCGA -68
  -67 CCAAAACTGCGACCGCAAACGACCCGAGCAACAGGCAAGGTTGCGCTCCCGCAACAGCGTCACCAACATGTCGTCCGTCCTCATGAGCAAGACTGTCACGCCTTTCCTTCGGGAACACAT 53
    1                                                                         M  S  S  V  L  M  S  K  T  V  T  P  F  L  R  E  H  I 18
   54 TCCCAGCATCTACGCCCCCATCGGCAAGCCGGGGAACCAAGAGACGGCCAGAGCAGAGAACCCCAATTCCAAGTACTGCTATAGACACCACCCCGACTCAAAGTGCCGAAGGGCTGCCGA 173
   19 P  S  I  Y  A  P  I  G  K  P  G  N  Q  E  T  A  R  A  E  N  P  N  S  K  Y  C  Y  R  H  H  P  D  S  K  C  R  R  A  A  D 58
  174 CAAGGCCAAGATGGTCATGATTCAGAGCGAGCTAGACAAGCTCACATCAGCGGTGGGTCTTCATACAAAACATAAACATTGATTGCTTCTTGTCTCCAAGGTTTGGTGGACTGACTGGTA 293
   59 K  A  K  M  V  M  I  Q  S  E  L  D  K  L  T  S  A 75
  294 CCTTGCTTGAAATAATAGGATCAGCAAGCTGTGACCCACGTGTGGTCCCTGTTCTCGGCCGCCCCTGCCCGACATCGCGACCTGATGCTTCAGGGTATCCTCTCCCAGCTCTGCTTCCCC 413
   76                   D  Q  Q  A  V  T  H  V  W  S  L  F  S  A  A  P  A  R  H  R  D  L  M  L  Q  G  I  L  S  Q  L  C  F  P 109
  414 CAGCTCTCCTTCGTCTCGAGAGAAGTCAACGAAGCCCTCAAGATCGACTTCATCTCCGCCCTCCCCGTCGAGCTCGCCCAAAAGGTCTTATGCTATCTCGACACCGTCAGCTTGACCAAG 533
  110 Q  L  S  F  V  S  R  E  V  N  E  A  L  K  I  D  F  I  S  A  L  P  V  E  L  A  Q  K  V  L  C  Y  L  D  T  V  S  L  T  K 149
  534 GCCGCCCAGGTCAGCCAGAGGTGGCGAACCCTGGCCGACTCGGATGCCGTCTGGGTCCGCATGTGCGAGCAACACGTCAACAGGAAGTGCACAAAGTGTGGATGGGGATTGCCCTTGCTG 653
  150 A  A  Q  V  S  Q  R  W  R  T  L  A  D  S  D  A  V  W  V  R  M  C  E  Q  H  V  N  R  K  C  T  K  C  G  W  G  L  P  L  L 189
  654 GAACGCAAGAAGCTGCGCAACTACACCAGACAAAGACAGCTGGCCAAGGGTGGGCCACAAGGGAGGGTCACAGAACTGGCCGACTCCCACGACAGCCAGGATCGATCCGTCAATCAACAT 773
  190 E  R  K  K  L  R  N  Y  T  R  Q  R  Q  L  A  K  G  G  P  Q  G  R  V  T  E  L  A  D  S  H  D  S  Q  D  R  S  V  N  Q  H 229
  774 GGAAAGCGCCCGGCAGCAGAGGCGGAGGAAGAGGACCCCATCAAGAAGCGCCAGTGTATGGCTGCAGCAGAAGCCTCCAAGGCGGTAACGCAACCCAAGACCCGAAGCTGGAAGGCCGTC 893
  230 G  K  R  P  A  A  E  A  E  E  E  D  P  I  K  K  R  Q  C  M  A  A  A  E  A  S  K  A  V  T  Q  P  K  T  R  S  W  K  A  V 269
  894 TACCGGGATCGCTGGCAGGTCAGCTACAACTGGAAAAACAGCCGATACAAGTTGTCGGTTCTCAAGGGTCATGAGAATGGTGTCACATGTCTCCAGCTCGATGATAACATCCTTGCGACT 1013
  270 Y  R  D  R  W  Q  V  S  Y  N  W  K  N  S  R  Y  K  L  S  V  L  K  G  H  E  N  G  V  T  C  L  Q  L  D  D  N  I  L  A  T 309
 1014 GGATCGTACGACACCACTATCAAGATCTGTATGTGGTCTTGCTTCTTTAAACATTGGTGCTCGCTTTAAACTAACACATTGCTGACAGGGAACATTGAGACTGAGGAGTGCATTCGGACC 1133
  310 G  S  Y  D  T  T  I  K  I  W                                                          N  I  E  T  E  E  C  I  R  T 329
 1134 CTTGTCGGCCATACGGCTGGCATTCGTGCACTACAGTTTGACGATTCGAAGCTCATCAGTGGCAGTCTTGACCGTATGTTGCGCAACCCGCCTTCAATGATAGCGTGGCAATGTTGCTAA 1253
  330 L  V  G  H  T  A  G  I  R  A  L  Q  F  D  D  S  K  L  I  S  G  S  L  D  H 354
 1254 CTGATTCGTAGACACCATCAAGGTCTGGAACTGGCATACCGGCGAGTGCCTCTCCACGTTCGCTGCCCACACCGACTCAGTGATTAGCGTTCACTTCGATGGCCACCTACTCGCCAGTGG 1373
  355             T  I  K  V  W  N  W  H  T  G  E  C  L  S  T  F  A  A  H  T  D  S  V  I  S  V  H  F  D  G  H  L  L  A  S  G 390
 1374 GTCGAGCGACAAGACGGTCAAGATTTTCGACTTCAACTCCAAGGAGACATACTGCCTCAAGGGCCATAGCGATTGGGTCAACTCGACCCATGTTGACATCAAGAGCCGAACCGTGTTCTC 1493
  391 S  S  D  K  T  V  K  I  F  D  F  N  S  K  E  T  Y  C  L  K  G  H  S  D  W  V  N  S  T  H  V  D  I  K  S  R  T  V  F  S 430
 1494 CGCCTCGGATGACACGACCATCAAGCTGTGGGATCTGGACACGCGCCAGGTTATCCGGACCTACGAAGGACATGTCGGCCATGTGCAACAGGTCCTCATCCTCCCGCCCGAGTATGAGCC 1613
  431 A  S  D  D  T  T  I  K  L  W  D  L  D  T  R  Q  V  I  R  T  Y  E  G  H  V  G  H  V  Q  Q  V  L  I  L  P  P  E  Y  E  P 470
 1614 CGACGAGGAGGTGCTCAACGGTGCTTCGCAAGACAACCAAGACGCCATGTCAGTATCATCGGGCGGAAGCGGAAGCCCCTCCATGTCGCATGCACAAATCGAGCGCGCCGGCAGCCCTGG 1733
  471 D  E  E  V  L  N  G  A  S  Q  D  N  Q  D  A  M  S  V  S  S  G  G  S  G  S  P  S  M  S  H  A  Q  I  E  R  A  G  S  P  G 510
 1734 CAGCCATAGCTCCAGCCACAACCTGCTACCGAGCAGCCTGCCTAGTGGTGACGAGGACGTGCGCCATCTCTATGGATCCGCCTTTGTAGCCGACGAGTCACGGCCACTTCCCCCGCGCTA 1853
  511 S  H  S  S  S  H  N  L  L  P  S  S  L  P  S  G  D  E  D  V  R  H  L  Y  G  S  A  F  V  A  D  E  S  R  P  L  P  P  R  Y 550
 1854 TTTCATGACAGGCGGGCTCGACAGCACCATGCGTCTCTGGGACAGCGCGACCGGTCGGTGTTTGAGGACGCTGTTTGGCCACCTCGAGGGCGTTTGGTCTTTGGCGGGAGATACGATCCG 1973
  551 F  M  T  G  G  L  D  S  T  M  R  L  W  D  S  A  T  G  R  C  L  R  T  L  F  G  H  L  E  G  V  W  S  L  A  G  D  T  I  R 590
 1974 TGTGATCAGTGGAGCCAACGACGGCATGGTCAAGACGTGGGAGCCGCGCAGCGGAAAGTGCGATGCGACGTATACGGGCCACTGCGGACCCGTGACTTGTGTCGGGCTGAGCGATAGTCT 2093
  591 V  I  S  G  A  N  D  G  M  V  K  T  W  E  P  R  S  G  K  C  D  A  T  Y  T  G  H  C  G  P  V  T  C  V  G  L  S  D  S  L 630
 2094 AATGGCCAGTGGCAGTGAGGATGGAACGATTCGGTTACATTCCTTCAAGCCCTGTCGGCAGTGAGGTGGTGGCTGGGGTTTCCGTCACTTGGGAACGATGAGAGCATGTCGGGTTTCAAC 2213
  631 M  A  S  G  S  E  D  G  T  I  R  L  H  S  F  K  P  C  R  Q  * 650
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FIG. 1. Nucleotide and predicted amino acid sequence of the *scon-2*$^+$ gene. The sequence is shown from 1147 nt upstream of the translation start codon to 56 nt downstream of the stop codon (indicated with a star). The nucleotides are numbered relative to the initiator ATG codon. CYS3-binding sites within the *scon-2*$^+$ promoter are bracketed, with an arrow indicating orientation of the binding site with respect to the invariant (C/A)AT sequence. *scon-2*$^+$ transcriptional initiation sites are indicated with vertical arrows. The sequence corresponding to the *N. crassa* transcriptional initiation site consensus is boxed. The 5′ splice sites, 3′ splice sites, and internal lariat sequences within all *scon-2*$^+$ introns are underlined.

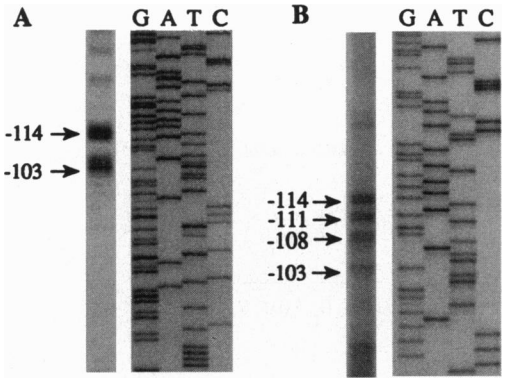

FIG. 2. Determination of transcriptional start sites within the *scon-2*$^+$ gene. (*A*) S1 nuclease analysis. The size of the S1 nuclease-protected fragment was determined by comparison with a DNA sequencing reaction generated with the same primer and template used to create the S1 probe. Arrows indicate protected fragments corresponding to start sites at nt −114 and −103 (Fig. 1). (*B*) Primer extension. Primer-extension products were sized by comparison against a DNA-sequencing reaction generated with the same primer. Arrows indicate primer-extension products corresponding to transcriptional initiation sites at nt −114, −111, −108, and −103 (Fig. 1).

three introns, as determined through comparison of cDNA and genomic *scon-2*$^+$ sequences. Each intron is small, ranging from 58 to 86 bp in length. Nucleotide sequences at all exon/intron boundaries conform exactly to an established *N. crassa* splice-site consensus sequence (20). Similarly, all *scon-2*$^+$ internal lariat sequences agree strongly with a *N. crassa* lariat consensus (20).

Transcriptional initiation sites within the *scon-2*$^+$ gene were mapped by S1 nuclease and primer-extension analysis (Fig. 2). S1 nuclease analysis indicated two transcriptional start sites 114 and 103 nt upstream of the initiator ATG codon. Primer-extension products were more heterogenous with strong bands corresponding to initiation sites at nt −114, −111, and −108 and a weaker band indicating a start site at nt −103. These initiation sites are clustered in and around the sequence TCATCACC, which exactly matches an established *N. crassa* transcription initiation site consensus of TCATCANC (20). Additionally, the *scon-2*$^+$ 5′-flanking region contains an appropriately positioned TATA-like sequence (ATGATAA) that bears partial resemblance to the *N. crassa* TATA box consensus (TATATAA) (20).

Sequence Analysis of SCON2. Inspection of the SCON2 amino acid sequence revealed six internal repeats of ≈40 amino acids in length spanning the carboxyl-terminal half of the protein (Fig. 3*A*). Each repeat exhibits a characteristic pattern of residues highlighted by strongly conserved Gly-His (GH) and Trp-Asp (WD) dipeptides (Fig. 3*B*). This repeated motif, referred to as the β-transducin, WD-40, or WD repeat, was first characterized in the β subunit of heterotrimeric G proteins (29). The WD-40 repeats within SCON2 are arranged tandemly, with the exception of repeats 4 and 5, which are separated by 83 amino acids.

The results of data-base searches using SCON2 as the query were dominated by highly significant matches to members of the β-transducin superfamily. The strong conservation of WD-40 repeats within this family is emphasized in Fig. 3*C*, which presents an alignment of the most significantly related repeat common to a diverse collection of β-transducin ho-

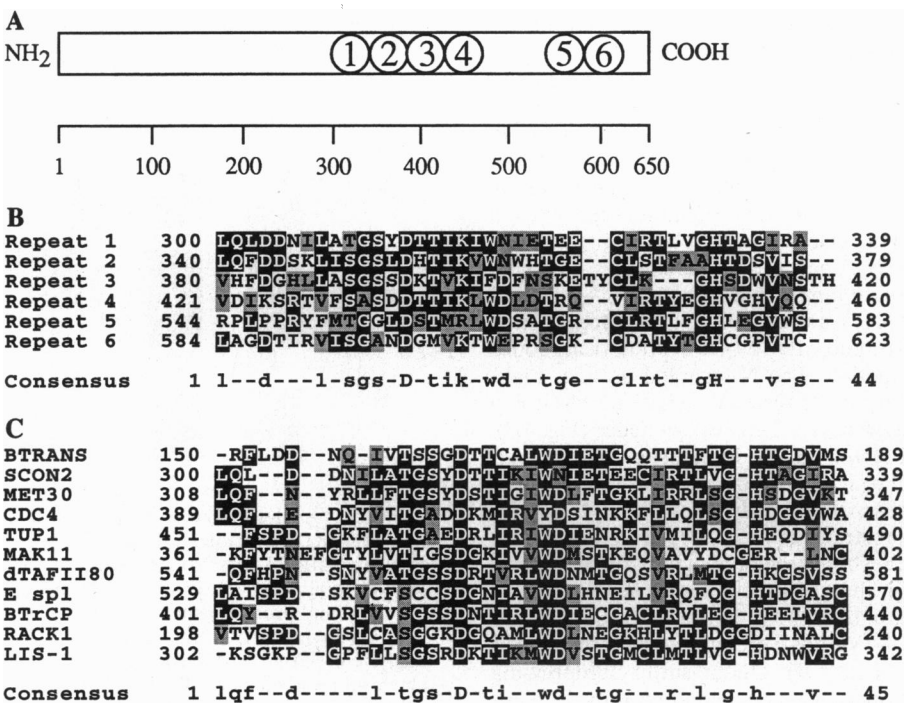

FIG. 3. WD-40 repeat domains in SCON2. (*A*) Schematic diagram of SCON2. The rectangle represents the complete SCON2 amino acid sequence with numbered circles indicating individual WD-40 repeats. Note the 83-amino acid spacer separating repeats 4 and 5. Similar gaps between WD-40 repeats have been found at various positions within a number of β-transducin homologs (9). (*B*) Alignment of the WD-40 repeats in SCON2. Complete sequences of the six SCON2 WD-40 repeats were aligned by using CLUSTALV. The resulting alignment was shaded using the BOXSHADE program. Identical residues are shown as white on black, while similar residues are represented as black on gray. The consensus line was generated by the PRETTY program of the Genetics Computer Group Package. Invariant residues are represented in the consensus sequence in capital letters. All WD-40 repeats are represented according to conventions established by Duronio *et al.* (21). (*C*) Sequence comparison of WD-40 repeats within various β-transducin-like proteins. MACAW was used to identify the most highly conserved internal repeat common to the following 11 β-transducin homologs: bovine BTRANS (8); *N. crassa* SCON2; yeast MET30 (GenBank:L26505); yeast CDC4 (8); yeast TUP1 (22); yeast MAK11 (23); *Drosophila melanogaster* TAFII80 (24); *D. melanogaster* E(spl) (25); *Xenopus* BTrCP (26); rat RACK1 (27); human LIS-1 (28). The resulting alignment was optimized using CLUSTALV, and residues were shaded as described above. Locations of these sequences within their parent proteins are indicated at the beginning and end of each line.

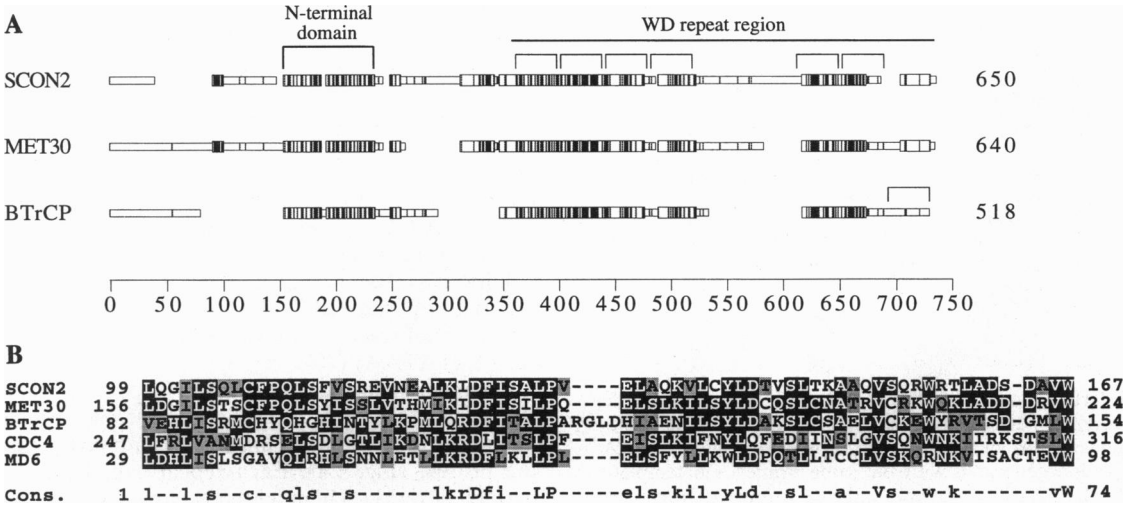

FIG. 4. SCON2 sequence homologies. (*A*) Schematic diagram illustrating conserved domains within SCON2, MET30, and BTrCP. Complete amino acid sequences were locally aligned using MACAW. Gaps were inserted to optimally align conserved sequence blocks. The resulting schematic was shaded by mean-pairwise score, so that strongly conserved regions appear black. WD-40 repeats are shown bracketed. Note the strongly conserved N-terminal domain within all proteins. (*B*) N-terminal domains within SCON2, MET30, BTrCP (26), CDC4 (8), and MD6 (Protein Identification Resource:S20665) were optimally aligned and shaded as described in Fig. 3. The numbers bracketing each line represent the position of each sequence within its parent protein. Within the N-terminal domain shown here, SCON2 exhibits 59% identity with MET30, 30% identity to BTrCP, 25% identity to CDC4, and 30% identity with MD6.

mologs. This alignment highlights the characteristic WD and GH dipeptides as well as an invariant aspartate residue 6 amino acids N-terminal to the WD.

The SCON2 protein shows strongest similarity to MET30 (GenBank:L26505), a *Saccharomyces cerevisiae* β-transducin-like protein of unknown function and to BTrCP (26), a *Xenopus* β-transducin homolog known to suppress mutations in CDC15 when expressed in yeast. Specifically, within WD-40 repeat regions, SCON2 shares 47% identity with MET30 and 36% identity with BTrCP. The complete amino acid sequences of SCON2, MET30, and BTrCP were locally aligned to identify conserved protein domains (Fig. 4*A*). In addition to the expected similarity among WD-40 repeats, this analysis unexpectedly revealed a strongly conserved domain N-terminal to the WD-40 repeat region. Data-base searches using only the N-terminal half of SCON2 uncovered several other members of the β-transducin protein family that shared this N-terminal domain, including CDC4 (8), a yeast cell-division-cycle protein required early in mitosis, and MD6 (9), a hypothetical mouse protein of unknown function. The N-terminal motif is highlighted by an invariant aspartate residue, an invariant Leu-Pro pair, and a strongly conserved Tyr-Leu-Asp sequence (Fig. 4*B*).

CYS3 Regulation of *scon-2*$^+$ Gene Expression. The role of CYS3 in the regulation of *scon-2*$^+$ gene expression was investigated by using Northern blot analysis of *scon-2*$^+$ mRNA levels in a *cys-3* deletion strain, Δ*cys-3*(18-4) (10). The Δ*cys-3* construct eliminated the basic region–leucine zipper region from the CYS3 protein, thereby precluding dimerization and DNA binding. Northern blots of poly(A)$^+$ mRNA isolated from wild-type and Δ*cys-3* strains were probed with the cloned *scon-2*$^+$ gene (Figs. 5 *A* and *B*). Under sulfur-derepressing conditions, *scon-2*$^+$ expression was detectable only in wild-type *N. crassa*. The *scon-2*$^+$ gene was not expressed in wild-type or Δ*cys-3* strains grown under sulfur-repressing conditions (data not shown).

To further examine CYS3 control of *scon-2*$^+$ expression, gel mobility-shift assays were used to scan the *scon-2*$^+$ promoter *in vitro* for CYS3-binding sites. Initially, 200- to 300-bp segments of the *scon-2*$^+$ promoter, generated by restriction enzyme digestion, were subjected to gel mobility-shift analysis. Fragments bound by CYS3 were further digested and analyzed for CYS3 binding until four distinct 24-bp sequences capable of binding CYS3 were identified (Fig. 5*C*). Each fragment contains a central 10-bp sequence (Fig. 1) that agrees strongly with a previously determined CYS3-binding site core consensus (12). A gradation of CYS3-binding affinity in the gel-mobility shifts is apparent, with CYS3 binding most strongly to binding site 3. To determine the specificity with which CYS3 binds to the *scon-2*$^+$ promoter, a single nucleotide at a critical position within each CYS3-binding site was mutated (see

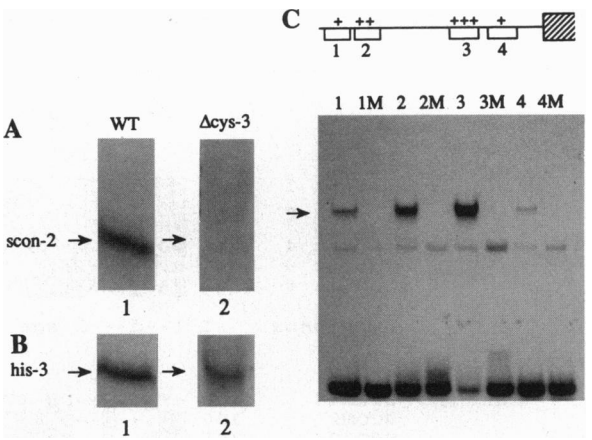

FIG. 5. Analysis of *cys-3*$^+$ regulation of *scon-2*$^+$ gene expression. (*A*) Northern hybridization analysis of *scon-2*$^+$ mRNA levels. Poly(A)$^+$ mRNA was isolated from *N. crassa* wild-type and Δ*cys-3*(18-4) strains grown on low-sulfur medium. Northern blots were prepared and probed with ^{32}P-labeled *scon-2*$^+$ DNA. Lanes: 1, wild-type (WT) mRNA; 2, Δ*cys-3* mRNA. (*B*) Northern hybridization analysis of *his-3*$^+$ mRNA levels. Northern blots were prepared as above and probed with ^{32}P-labeled *his-3*$^+$ DNA. Lanes 1 and 2 are as described above. The *his-3*$^+$ gene served as a control to ensure comparability between samples. (*C*) Mobility-shift analysis. Lanes: 1, CYS3-binding site 1 (nt −1115 to −1106); 1M, mutated CYS3-binding site 1; 2, CYS3-binding site 2 (nt −1094 to −1085); 2M, mutated CYS3-binding site 2; 3, CYS3-binding site 3 (nt −376 to −367); 3M, mutated CYS3-binding site 3; 4, CYS3-binding site 4 (nt −259 to −250); 4M, mutated CYS3-binding site 4. A single nucleotide within each binding site was mutated to assess the specificity of CYS3 binding. The arrow indicates DNA fragments exhibiting reduced electrophoretic mobility due to protein–DNA interactions. A nonspecific band due to an extract component appears in all lanes. The affinity of CYS3 for each binding site is depicted in the schematic above (+, weakest affinity; +++, strongest affinity).

Materials and Methods). This single base mutation eliminated CYS3 binding at each site (Fig. 5*C*).

DISCUSSION

We report here that the *N. crassa* sulfur controller-2 gene encodes a negative regulator belonging to the β-transducin family of proteins. Currently, the β-transducin protein superfamily encompasses over 30 eukaryotic regulatory proteins known to mediate a variety of cellular functions, ranging from signal transduction to vesicle fusion (9). In particular, several β-transducin homologs are known to regulate gene expression. In addition to SCON2, this subset of β-transducin-like proteins includes the yeast proteins TUP1 (22) and HIR1 (30), the *Arabidopsis* protein COP1 (31), and the *Drosophila* proteins GROUCHO (25) and $TAF_{II}80$ (24).

Each β-transducin-like protein is defined by the presence of four to eight highly conserved WD-40 repeat units (21). Although functions have been assigned to the majority of β-transducin homologs, the function of this WD-40 repeat itself remains unclear. Many β-transducin-like proteins form multiprotein complexes, suggesting a possible role for WD-40 repeats in mediating protein–protein interactions. For example, the β-transducin-like protein TUP1 is associated in a protein complex with the tetratricopeptide (TPR) repeat protein SSN6 (32). Similarly, an interaction between the yeast β-transducin homolog CDC20 and the yeast TPR proteins CDC16 and CDC23 is suspected (33). Aside from this WD-40/TPR association, the β-transducin-like protein RACK1 is known to bind activated protein kinase C through its WD-40 repeat region (27). The apparent ability of WD-40 repeats to mediate macromolecular assemblies is clearly relevant to any analysis of SCON2 function. SCON2 may interact with other proteins to form a multiprotein complex capable of repressing transcription. Alternatively, SCON2 may negatively regulate sulfur structural gene expression by binding and inactivating the transcriptional activator CYS3.

In addition to these WD-40 repeats, the SCON2 protein exhibits an interesting N-terminal domain that defines a subset of β-transducin-like proteins. This N-terminal sequence is characterized by several strongly conserved charged residues (Asp, Glu, Lys, Arg) and an invariant Leu-Pro dipeptide (Fig. 4*B*). This motif is shared by several other β-transducin homologs—including the mouse protein MD6 (9), the *Xenopus* protein BTrCP (26), and the yeast proteins MET30 and CDC4 (8). Collectively, these gene products represent a potential subfamily of β-transducin-like proteins. The extended sequence similarity within this subset suggests that these proteins may be functionally related, possibly acting as regulators of gene expression.

Analysis of the *scon-2*$^+$ promoter revealed a possible role for CYS3 in activation of *scon-2*$^+$ expression. Gel mobility-shift analysis uncovered four CYS3-binding sites within the *scon-2*$^+$ promoter, with CYS3 binding most strongly to site 3 *in vitro*. Moreover, Northern analysis of *scon-2*$^+$ mRNA levels indicated reduced expression of *scon-2*$^+$ in a *cys-3* deletion strain. Taken collectively, this *in vitro* and *in vivo* evidence strongly suggests CYS3 regulation of *scon-2*$^+$ expression. *cys-3*$^+$, therefore, may participate in a form of feedback inhibition, wherein *cys-3*$^+$ stimulates expression of the *scon-2*$^+$ negative regulator which, in turn, represses transcription of *cys-3*$^+$ when cells are subjected to high sulfur conditions. This control loop may be representative of more general regulatory mechanisms within eukaryotic multigene networks.

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1. Metzenberg, R. L. (1979) *Microbiol. Rev.* **43,** 361–383.
2. Marzluf, G. A. (1993) *Annu. Rev. Microbiol.* **47,** 31–55.
3. Paietta, J. V. (1989) *Mol. Cell. Biol.* **9,** 3630–3637.
4. Fu, Y-H., Paietta, J. V., Mannix, D. G. & Marzluf, G. A. (1989) *Mol. Cell. Biol.* **9,** 1120–1127.
5. Paietta, J. V., Akins, R. A., Lambowitz, A. M. & Marzluf, G. A. (1987) *Mol. Cell. Biol.* **7,** 2506–2511.
6. Burton, E. G. & Metzenberg, R. L. (1972) *J. Bacteriol.* **109,** 140–151.
7. Paietta, J. V. (1990) *Mol. Cell. Biol.* **10,** 5207–5214.
8. Fong, H. K. W., Hurley, J. B., Hopkins, R. S., Miake-Lye, R., Johnson, M. S., Doolittle, R. F. & Simon, M. I. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 2162–2166.
9. Neer, E. J., Schmidt, C. J., Nambudripad, R. & Smith, T. F. (1994) *Nature (London)* **371,** 297–300.
10. Paietta, J. V. (1992) *Mol. Cell. Biol.* **12,** 1568–1577.
11. Legerton, T. L. & Yanofsky, C. (1985) *Gene* **39,** 129–140.
12. Shuler, J. L. & Paietta, J. V. (1991) *FASEB J.* **5,** 1524 (abstr.).
13. Davis, R. H. & DeSerres, E. F. (1970) *Methods Enzymol.* **17,** 79–143.
14. Tabor, S. & Richardson, C. C. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 4767–4771.
15. Berk, A. J. & Sharp, P. A. (1977) *Cell* **12,** 721–732.
16. Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990) *J. Mol. Biol.* **215,** 403–410.
17. Rice, C. M., Fuchs, R., Higgins, D. G., Stoehr, P. J. & Cameron, G. N. (1993) *Nucleic Acids Res.* **21,** 2967–2971.
18. Schuler, G. D., Altschul, S. F. & Lipman, D. J. (1991) *Proteins* **9,** 180–190.
19. Feinberg, A. P. & Vogelstein, B. (1983) *Anal. Biochem.* **132,** 6–13.
20. Bruchez, J. J. P., Eberle, J. & Russo, V. E. A. (1993) *Fungal Genet. Newsl.* **40,** 89–96.
21. Duronio, R. J., Gordon, J. I. & Boguski, M. S. (1992) *Proteins* **13,** 41–56.
22. Williams, F. E. & Trumbly, R. J. (1990) *Mol. Cell. Biol.* **10,** 6500–6511.
23. Icho, T. & Wickner, R. B. (1988) *J. Biol. Chem.* **263,** 1467–1475.
24. Dynlacht, B. D., Weinzierl, R. O. J., Admon, A. & Tjian, R. (1993) *Nature (London)* **363,** 176–179.
25. Hartley, D. A., Preiss, A. & Artavanis-Tsakonas, S. (1988) *Cell* **55,** 785–795.
26. Spevak, W., Keiper, B. D., Stratowa, C. & Castanon, M. J. (1993) *Mol. Cell. Biol.* **13,** 4953–4966.
27. Ron, D., Chen, C.-H., Caldwell, J., Jamieson, L., Orr, E. & Mochly-Rosen, D. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 839–843.
28. Reiner, O., Carozzo, R., Shen, Y., Wehnert, M., Faustinella, F., Dobyns, W. B., Caskey, C. T. & Ledbetter, D. H. (1993) *Nature (London)* **364,** 717–721.
29. van der Voorn, L. & Ploegh, H. L. (1992) *FEBS Lett.* **307,** 131–134.
30. Sherwood, P. W., Tsang, S. V.-M. & Osley, M. A. (1993) *Mol. Cell. Biol.* **13,** 28–38.
31. Deng, X.-W., Matsui, M., Wei, N., Wagner, D., Chu, A. M., Feldmann, K. A. & Quail, P. H. (1992) *Cell* **71,** 791–801.
32. Keleher, C. A., Redd, M. J., Schultz, J., Carlson, M. & Johnson, A. D. (1992) *Cell* **68,** 709–719.
33. Sethi, N., Monteagudo, M. C., Koshland, D., Hogan, E. & Burke, D. J. (1991) *Mol. Cell. Biol.* **11,** 5592–5602.