Cloning and Characterization of scon-3\(^{+}\), a New Member of the *Neurospora crassa* Sulfur Regulatory System

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The sulfur regulatory system of *Neurospora crassa* consists of a group of sulfur-regulated structural genes (e.g., arylsulfatase) that are under coordinate control of the CYS3 positive regulator and sulfur controller (SCON) negative regulators. Here we report on the cloning of scon-3\(^{+}\), which encodes a polypeptide of 171 amino acids and is a Skp1 family homolog. Repeat-induced point mutation of scon-3\(^{+}\) resulted in a phenotype of constitutive expression of arylsulfatase, a phenotype consistent with other sulfur controller mutants. Northern analysis indicated that, unlike other members of the sulfur regulatory system, expression of scon-3\(^{+}\) is not under the direct control of the CYS3 transcriptional activator. In particular, scon-3\(^{+}\) mRNA was detectable under sulfur repressing or derepressing conditions in a Δcys-3 mutant. In yeast, Skp1p and an F-box protein binding partner are core constituents of a class of E3 ubiquitin ligases known as SCF complexes. The *N. crassa* negative regulator SCON2 contains an F-box motif essential for the operation of the sulfur regulatory system and suggests a role for an SCF complex in the *N. crassa* sulfur regulatory system. A crucial set of experiments, by using a yeast two-hybrid approach with confirming coimmunoprecipitation assays, demonstrated that SCON3 interacts with SCON2 in a manner dependent upon the F-box motif of SCON2. The protein-protein interaction detected between SCON2 and SCON3 represents the initial demonstration in a filamentous fungus of functional interaction between putative core components of a SCF complex.

The sulfur regulatory system of *Neurospora crassa* is composed of a set of *trans*-acting regulatory genes and a group of structural genes that encode enzymes used in the uptake and assimilation of a variety of sulfur compounds (20, 27). When *N. crassa* cultures are grown under conditions of sulfur limitation (i.e., derepressing conditions), then the entire group of sulfur-related genes is coordinately expressed (20). The structural genes involved encode for arylsulfatase, choline sulfatase, choline sulfate permease, methionine permease, sulfate permeases I and II, and an extracellular protease (20, 27). Essentially, the sulfur regulatory system monitors the cellular sulfur status and expresses the sulfur structural genes as needed to ensure an adequate internal supply of sulfur, while subjecting the sulfur-related genes to repression under conditions of sulfur sufficiency.

The positive regulator of sulfur-controlled gene expression in *N. crassa* is CYS3, a basic region-leucine zipper protein that functions as a DNA-binding transcriptional activator (20, 24, 25). cys-3\(^{+}\) gene expression is controlled in part by a positive feedback loop involving the CYS3 protein and in part by the negative regulatory sulfur controller genes, scon-1\(^{-}\) and scon-2\(^{-}\) (25, 26). scon-1 and scon-2 mutants show constitutive expression of cys-3\(^{+}\) and the sulfur structural genes (26). scon-2\(^{-}\) has been cloned and found to be expressed only under conditions of sulfur limitation (16, 26). The SCON2 protein contains two domains implicated in protein-protein interactions: (i) a region of six WD40 (or β-transducin) repeats and (ii) a motif that we originally termed the “N-terminal domain” (16). The N-terminal domain is now referred to as the F-box motif (1, 12).

F-box motifs are found in a large number regulatory proteins (12, 14, 28). In *Saccharomyces cerevisiae*, F-box proteins have been shown to assemble with Skp1p,Cdc53p, and Rbx1p to form a complex known as the SCF (for Skp1p/Cdc53p/F-box) (28, 38). SCF complexes act as E3 ubiquitin ligases to target proteins for ubiquitin-mediated proteolysis. SCF complexes have been implicated in diverse processes, including cell cycle progression, glucose sensing, and developmental processes (7, 10, 12, 14, 28, 43). The F-box protein component is responsible for SCF complex target specificity, with the F-box motif thought to be responsible for the interaction with Skp1p (1, 29, 38). In yeast, Met30p is the F-box protein that is homologous to SCON2 (41).

Previous work has demonstrated that the F-box domain of SCON2 is required for SCON2’s role as a regulator within the *N. crassa* sulfur control system (17). The ability of SCON2 to function as a negative regulator of sulfur-related gene expression may be due to the ability of SCON2 to form a functional SCF complex in *N. crassa* via protein-protein interactions between the F-box motif of SCON2 and the *N. crassa* homolog of yeast Skp1p. Skp1 homologs, both designated sconC, have been found in *Aspergillus nidulans* (31) and *Microsporum canis* (GenBank AF408428) but have not been examined for possible molecular interactions with other putative SCF complex constituents.

We report here the isolation and analysis of scon-3\(^{+}\), a new member of the *N. crassa* sulfur regulatory system. scon-3\(^{+}\) encodes a protein showing strong homology to the Skp1p family of proteins. Repeat-induced point mutation (RIP) of the scon-3\(^{+}\) locus resulted in the production of mutants having a phenotype of constitutive expression of arylsulfatase, confirm-
ing the putative role of scon-3 in sulfur gene regulation. Importantly, we found that SCON3 interacted with SCON2 in vivo as detected by two-hybrid assays and in vitro by using coimmunoprecipitation. Further, the interaction between SCON3 and SCON2 was found to be dependent upon the F-box motif of SCON2. The data presented supports a role for an SCF complex partially comprised of SCON2 and SCON3 as an crucial component required for the regulated expression of sulfur-related genes in N. crassa.

MATERIALS AND METHODS

Strains and culture conditions. The N. crassa strains DeltaS-3 (18-4) and scon-2 (PSD272) have been described previously (25, 26). 74OR23-1a was used as the wild-type strain for these studies. N. crassa cultures were grown at 25°C on minimal Vogel medium (6) with supplements as required. Cells were grown under sulfur-repressing and sulfur-derepressing conditions by using Vogel’s minimal medium supplemented with 5.0 mM methionine and 0.25 mM methionine, respectively (25). The assay of arylsulfatase activity, which is normally derepressed under sulfur-limiting growth conditions (27), was used to confirm the presence of sulfur derepression or repression. Arylsulfatase assays were performed by monitoring p-nitrophenyl sulfate at 405 nm from p-nitrophenol liberated by standard methods (21, 27).

Crosses were carried out with cornmeal agar or Westergard-Mitchell medium (6). Homokaryons were isolated by growth on Westergard-Mitchell medium with 1 mM iodacetate, harvesting of the microconidia, and filtration through 5-μm (pore-size) Millex filters (8). For S. cerevisiae, each strain was subjected to PCR with the primers 5'-AAGAATTC TTCATGTGAGCAGGC-3' and 5'-ACGGAATTCT CGTTCTCGGCGGC-3' to amplify a 300-bp portion of the S. cerevisiae Fbox as a template in a PCR amplification. The resulting PCR products were used, with pAD-scon2, pAD-scon2 WD40, or pAD-scon2ΔFbox, as templates for the in vitro transcription and translation reactions. The resulting ribosome-protein fusions were then used as substrates for coupled in vitro transcription and translation with a T7 RNA polymerase and rabbit reticulocyte lysate mixture (Promega) with [35S]methionine as a radiolabel. In vitro coimmunoprecipitation of SCON2 and SCON3. Duplicate samples containing 10 μl of in vitro-translated 35S-Myc-3CON3, HA-SCON2, HA-SCON2ΔFbox, or HA-SCON2ΔFbox either alone or 10 μl of c-myc-3CON3 plus 10 μl of HA-SCON2, HA-SCON2ΔFbox, or HA-SCON2ΔFbox were incubated at 4°C for 1 h. After a 1-h incubation at 4°C, the M-tag polyclonal antibody was then added to one tube of each sample pair, and the samples were incubated for an further hour at 25°C. The samples were then crossed to wild-type 74-OR23 (4, 36), and the progeny analyzed for sulfur regulatory or other potential defects. Of the RIP progeny isolated, a phytotypically representative isolate Z1-1b was studied more extensively. The specificity of the RIP events to the scon-3 locus was confirmed by restoration of a wild-type phenotype by transformation with the wild-type scon-3 gene. The RIP allele 12b-1 was subsequently cloned by PCR and sequenced by the dideoxy method.

mRNA isolation and Northern blot analysis. Total RNA was isolated by the phenol extraction procedure of Reinert et al. (32) as modified by Paitetta (26). Briefly, mycelial samples were harvested, frozen in liquid nitrogen, and homogenized in a 1:1 mixture of phenol-chloroform-isomyl alcohol (49:49:2) and Sarkosyl extraction buffer. After phenol-chloroform and chloroform extractions, precipitation, and sodium acetate washes, the poly(A)+ mRNA was isolated by oligo(DT)-cellulose chromatography. Northern blot and hybridization conditions were as described previously (25). 32P-labeled probes were prepared by random priming (9). Northern blots were probed with the the constitutively expressed ame gene (11) of N. crassa to confirm that comparable amounts of poly(A)+ mRNA were loaded for each sample. In addition, the scon-2 gene was used as a control to demonstrate the typical sulfur-regulated expression of genes in the sulfur control system. Quantitation of Northern blots was accomplished by a Molecular Dynamics PhosphorImager.

Transcription start site determination. The 5′ end of the scon-3 transcript was mapped by primer extension and S1 nuclease analysis. For primer extension, the 5′-end oligonucleotide RGGRGTTGACGACGAGGACGGACGACGACGGACTACGTGACCAGACGGG-3′ was used to position the transcription start site, which was confirmed by sequencing.

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In vitro transcription and translation. PCR was used to produce templates for transcription that carried a T7 promoter and fused coding region specifying an epitope for later use in coimmunoprecipitation experiments. The oligonucleotide primers 5′-AAATTGTAATACGACTC TTGTTGTGTGGACTACGTGACCAGACGGG-3′ and 5′-GTCTTGGGACGGAGGAGGACGGAGGAGGAGG-3′ were used to amplify a 200-bp fragment from the ame gene. The resulting PCR product was digested with EcoRV and inserted into pSPORT1 to generate pSC3.

pSC3 was used to probe a X71 genomic library (obtained from the Fungal Genetics Stock Center, Kansas City, Kans.) by plaque hybridization and a hybriding clone, designated AC21, was isolated. Subsequently, AC21 was sequenced by the dideoxy method (8). For S. cerevisiae, the 5′-end oligonucleotide 5′-CCGGGTAATTACGACTCTTGCTTCTG-3′ was used to amplify a 300-bp portion of the S. cerevisiae Fbox as a template in a PCR amplification. The resulting PCR products were used, with pAD-scon2, pAD-scon2 WD40, or pAD-scon2ΔFbox, as templates for the in vitro transcription and translation reactions. The resulting ribosome-protein fusions were initially grown on minimal media (SD-Leu-Trp) to select for expression of TTR1 and LEU2 (2). Double transformants were then isolated and screened for induction of HIS3 expression, as measured by growth on minimal media (SD-Leu-Trp-His) containing 20 mM 3-amino-1,2,4-triazole. Double transformants were also assayed for expression of lacZ by growth on minimal medium (SD-Leu-Trp) containing X-Gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside) with sucrose as the sole carbon source.

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precipitated, washed, electrophoresed, and fixed according to the manufacturer’s specification (Clontech).

Nucleotide sequence accession number. The genomic DNA sequence of scon-3/H11001 has been deposited in GenBank (AF402682). The nucleotide sequence of the RIP-generated 12b-1 allele of scon-3/H11001 has also been deposited as AY135642.

RESULTS

Sequence and organization of the scon-3+ gene. The scon-3+ gene was isolated by using mixed oligonucleotide primed PCR amplification to generate a partial gene segment, followed by hybridization screening of a C. gloeosporioides genomic library to identify a clone containing the entire gene. The predicted polypeptide sequence is given below the nucleotide sequence in single-letter code. The 5' splice sites, the 3' splice sites, and the internal lariat sequences within the introns are underlined. The sequence corresponding to the N. crassa transcriptional initiation site consensus is boxed. A vertical arrow indicates the major transcriptional initiation site at -72. A potential CYS3 binding site is bracketed.

FIG. 1. Nucleotide and predicted amino acid sequence of the scon-3+ gene. The sequence is shown from 1,547 nucleotides upstream of the translation start codon to 339 nucleotides downstream of the stop codon (indicated by an asterisk). The nucleotides are numbered relative to the initiator ATG codon. The predicted polypeptide sequence is given below the nucleotide sequence in single-letter code. The 5' splice sites, the 3' splice sites, and the internal lariat sequences within the introns are underlined. The sequence corresponding to the N. crassa transcriptional initiation site consensus is boxed. A vertical arrow indicates the major transcriptional initiation site at -72. A potential CYS3 binding site is bracketed.

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A single, strong transcriptional start site at nucleotide −72 was identified by both primer extension and S1 nuclease transcript analysis (Fig. 2). The initiation site is found immediately adjacent to the sequence TCACATAG, which closely matches the transcription initiation start site consensus determined for N. crassa (3). The promoter region does not contain any sequences exactly matching the established consensus binding site for the sulfur regulatory system transcription activator, CY3S (19, 37). A single site, at −1100, has a two-base mismatch versus the consensus sequence (ATGTRYRYCAT) and represents, based on our prior binding site studies (37), a weak potential site for CY3S. In comparison, the N. crassa scon-2 regulatory gene, which is clearly under CY3S control based on Northern blot analysis, has four putative CY3S binding sites (17).

**Sequence analysis of SCON3.** Database searches with SCON3 revealed highly significant matches to the members of the Skp1 protein family. An alignment of SCON3 with the M. canis and A. nidulans SCONC proteins, as well as other Skp1 family homologs, is shown in Fig. 3. SCON3 shows the highest homology (87%) to SCONC of M. canis (GenBank no. AF408428). The conservation of residue identity relative to N. crassa for the Skp1 family proteins presented in Fig. 3 ranges from 70 to 87%, excluding S. cer...
 Components of the sulfur regulatory system that have been examined (e.g., *ars-1*/*H11001*, *cys-3*/*H11001*, and *scon-2*/*H11001*) (26). The *scon-2*/*H11001* gene included as a control and used to reprobe the blot in Fig. 4 demonstrates the expected high expression under low-sulfur conditions and no expression under high-sulfur conditions. A further experiment examined the level of *scon-3*/*H11001* transcript in the *cys-3* mutant grown under sulfur derepressing and repressing conditions. The *scon-3*/*H11001* transcript was detectable under derepressing conditions in *cys-3* (as lane 3 in Fig. 4 demonstrates), but the level of *scon-3*/*H11001* was twofold lower under derepressing conditions compared to wild-type based on the phosphorimager data. In sharp contrast, the level of *scon-3*/*H11001* transcript was highly elevated in *cys-3* grown under repressing conditions compared to wild-type (an approximate twofold increase). The unusual pattern of expression for *scon-3*/*H11001* transcript levels in *cys-3* was consistently observed between mRNA preparations, and the high sulfur response is unlike that observed for other known genes in the *N. crassa* sulfur regulatory system (i.e., *cys-3* mutants show highly reduced transcript levels of sulfur-related genes under both derepressing and repressing conditions). *scon-2*/*H11001*, again acting as a control, demonstrates no expression in *cys-3* as expected (Fig. 4).

**TABLE 1. Arylsulfatase activity of wild-type, sulfur mutant, and scon-3* RIP strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Arylsulfatase sp act* on:</th>
<th></th>
<th>High-sulfur mediuma</th>
<th>Low-sulfur mediumb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td></td>
<td></td>
<td>0.05</td>
<td>8.9</td>
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<tr>
<td><em>cys-3</em> (<em>P22)</em></td>
<td></td>
<td></td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
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<tr>
<td><em>scon-1</em> (36-18)</td>
<td></td>
<td></td>
<td>9.4</td>
<td>9.9</td>
</tr>
<tr>
<td><em>scon-2</em> (PSD272)</td>
<td></td>
<td></td>
<td>10.2</td>
<td>11.7</td>
</tr>
<tr>
<td><em>scon-3</em> (12b-1)</td>
<td></td>
<td></td>
<td>8.1</td>
<td>8.3</td>
</tr>
<tr>
<td><em>scon-3</em> (12b-1) <em>scon-3</em></td>
<td></td>
<td></td>
<td>&lt;0.05</td>
<td>8.5</td>
</tr>
</tbody>
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*a* Expressed as nanomoles per minute per milligram of total protein.

*b* High-sulfur medium with 5.0 mM methionine.

**FIG. 3. SCON3 sequence homologies.** The deduced amino acid sequences of *N. crassa* (Nc) SCON3 (AF402682), *M. canis* (Mc) SCONC (AF40848), *A. nidulans* (An) SCONC (AAB18274), and the Skp1 proteins from *H. sapiens* (Hs) (AAH25073), *A. thaliana* (At) (NP_565123), *D. melanogaster* (Dm) (NP_477340), *S. cerevisiae* (Sc) (AAC49492), and *S. pombe* (Sp) (NP_595455) were optimally aligned by using CLUSTALW. The resulting alignment was shaded by using the BOXSHADE program. Sequences, other than SCON3, were obtained from BLASTP searches. Identical residues are shown as white on black, whereas similar residues are shown as black on gray. Brackets indicate PEST and P-loop sequences. Invariant residues are represented in the consensus line as capital letters, whereas conserved residues are represented as lowercase letters.
Northern blots to ensure that the bulk mRNA levels in the samples were comparable.

**Protein-protein interaction between SCON2 and SCON3.** The SCON2 and SCON3 proteins represent major constituents of a hypothetical SCF complex involved in sulfur-related gene regulation in *N. crassa*. Initial screening for in vivo interactions between SCON2 and SCON3 were carried out by using the yeast two-hybrid system with BD and AD derived from the GAL4 system (2, 30). Full-length SCON3 and full-length SCON2, as well as truncated versions of SCON2 with either a deletion of the F-box domain or WD-40 domain, were assayed for interactions. A vector, pBDscon3, encoding the wild-type SCON3 protein was tested against pAD vectors containing three versions of SCON2 (wild-type, ∆F-box, and ∆WD-40).

Two significant interactions, detectable by the expression of lacZ activity, were observed: (i) pBDscon3 (wild-type) showed interaction with pADscon2 (wild-type), and (ii) pBDscon3 (wild-type) showed interaction with pADscon2∆WD40 (Fig. 5). In demonstration of the importance of the F-box domain for SCON2-SCON3 interactions, the experiments showed no interaction present between pBDscon3(wild-type) and pADscon2∆F-box. Although deletion of the WD-40 domain in SCON2 had no detectable effect on the interaction present, the deletion of the SCON2 F-box eliminated the interaction between SCON2 and SCON3. The result is consistent with the putative role of the F-box to interact with Skp1 homologs, whereas the WD-40 domain is thought to be involved in the protein-protein interaction with the particular target protein of the SCF complex.

A second set of assays was done by using in vitro coimmunoprecipitation (30) to provide confirmation of the interactions observed between SCON2 and SCON3 in the yeast two-hybrid system. For these experiments, a T7 promoter and tag sequence encoding an epitope (either Myc or HA) were added to the SCON2 and SCON3 coding sequence by PCR with appropriately designed primers. The PCRs produced the templates used for coupled transcription and translation. SCON3 was generated in its full-length with a HA tag, whereas SCON2 was generated in full-length, ∆F-box, and ∆WD-40 forms all with a Myc tag. In each case, proteins of the expected sizes were produced with the transcripts translated separately and examined by polyacrylamide gel electrophoresis. After transcription and translation, the SCON2 and SCON3 products were incubated in various combinations, and antibodies to the HA and Myc epitopes were used to assay whether the proteins were interacting and could be coimmunoprecipitated or not. In the experiments shown in Fig. 6, coimmunoprecipitation was seen only in the following combinations: (i) full-length SCON3 plus full-length SCON2 and (ii) full-length SCON3 plus SCON2 with WD-40 region deleted. No coimmunoprecipitation was seen when the F-box was deleted from SCON2 and tested against SCON3. In addition, all control combinations were negative for coimmunoprecipitation demonstrating the specificity of the the assay. The protein-protein interactions detected by the coimmunoprecipitation assays agreed exactly with the yeast two-hybrid assays.

**DISCUSSION**

We report here on the cloning and characterization of *sulfur controller-3*, which encodes a protein that acts as a negative regulator in the *N. crassa* sulfur regulatory system and belongs to the Skp1 gene family. The Skp1 grouping, including SCON3,
of Skp1 are known and result in cell cycle arrest in G1 phase, whereas other alleles result in cell cycle arrest in G2 phase (1, 5, 29). The conditional yeast Skp1 mutants, skp1-11 and skp1-12, show a range of pleiotropic effects, including defective sulfur gene regulation (e.g., MET25) (29). Within the Skp1 family, however, the only currently known mutations that result solely in a phenotype of altered expression of sulfur-related genes, as represented here by arylsulfatase activity levels, are at the sconC locus of A. nidulans (31). Extensive searches of the recently available N. crassa genome (release 3; http://www-genome.wi.mit.edu) have revealed, however, only the presence of the Skp1 homolog reported in the present study.

In A. nidulans, sconC transcript levels are not significantly affected by sulfur concentration in the wild type (31). A. nidulans metR1 mutants show an increased level of sconC transcript under sulfur derepressing conditions (31). Based upon these data it has been suggested that, in A. nidulans, METR may act as a negative regulator of sconC expression (31). Given the fact that the sulfur regulatory systems of N. crassa and A. nidulans show many similarities (22), including homology between the positive regulators CY3S and METR, we investigated the possibility that CY3S might influence the expression of SCON3 in N. crassa. We observed detectable expression of scon-3+ under repressing and derepressing conditions in both wild-type and Δcys-3 strains. In wild-type N. crassa the steady-state level of scon-3+ transcript was substantially higher under sulfur derepressing conditions, an expression pattern similar to what has been observed for other components of the N. crassa sulfur regulatory system and perhaps suggesting positive transcriptional control of scon-3+ by CY3S. However, in repeated experiments, the Δcys-3 strain demonstrated an unusual pattern of scon-3+ expression. Under low-sulfur conditions, expression
was severely reduced, whereas under high-sulfur conditions expression was elevated. As a control, probing the blots with scon-2-1 expression (Fig. 4) demonstrated the expected regulated expression for that gene. The Δcys-3 data are not in agreement with the expected pattern of expression for a gene under control of CYS3, nor do they agree with a model of regulation where CYS3 acts as a negative regulator of scon-3 in expression. The unusual expression pattern suggests several possibilities, where scon-3 may (i) encode a more stable component of the SCF complex; (ii) be subject to posttranscriptional controls; or (iii) be involved in other, as-yet-undefined, cellular functions.

Skp1p is a major component in two evolutionarily conserved ubiquitin-conjugating E3 complexes: the anaphase-promoting complex and SCF complexes (14, 14, 28). SCF complexes were first identified in S. cerevisiae and have subsequently been identified in a number of eukaryotic organisms (14). SCF complexes are composed of at least three common subunits: Skp1p, Cdc53p, and Rbx-1p. SCF complexes also contain a modular ubiquitin-conjugating E3 complexes: the anaphase-promoting complex; (ii) be subject to posttranscriptional controls; or (iii) be involved in other, as-yet-undefined, cellular functions.


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