MOLECULAR AND CELLULAR BIOLOGY, Apr. 1988, p. 1504–1508
0270-7306/88/041504-05$02.00/0

Vol. 8, No. 4

Molecular Cloning and Analysis of the Regulation of *cys-14*$^+$, a Structural Gene of the Sulfur Regulatory Circuit of *Neurospora crassa*

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Received 24 August 1987/Accepted 21 December 1987

The *cys-14*$^+$ gene encodes sulfate permease II, which is primarily expressed in mycelia. *cys-14*$^+$ is one of a set of sulfur-related structural genes under the control of *cys-3*$^+$ and *scon*$^+$, the regulatory genes of the sulfur control circuit. We have cloned *cys-14*$^+$ from a cosmid library of *Neurospora crassa* DNA. A restriction fragment length polymorphism analysis showed that this clone maps to the region of chromosome IV corresponding to the *cys-14*$^+$ locus. Northern blot analyses were used to examine the regulated expression of the *cys-14*$^+$ gene. In the wild type, a 3-kilobase *cys-14*$^+$ transcript was highly expressed under sulfur-derepressing conditions but completely absent during sulfur repression. A *cys-3* mutant, which cannot synthesize any of the sulfur-controlled enzymes, including sulfate permease II, did not possess any *cys-14*$^+$ transcript under either condition. A *cys-3* temperature-sensitive revertant completely lacked any *cys-14*$^+$ mRNA at the conditional temperature but expressed the *cys-14*$^+$ transcript upon derepression at the permissive temperature. Mutation of a second sulfur regulatory gene, *scon*^{c}, causes the expression of sulfur-related enzymes in a constitutive fashion; the *scon*^{c} mutant showed a corresponding constitutive expression of *cys-14*$^+$ mRNA, such that it was present even in cells subjected to sulfur repression conditions. These results show that the *cys-14*$^+$ gene is regulated through the modulation of message content by the *cys-3*$^+$ and *scon*^{c} control genes in response to the sulfur levels of the cells.

The ability of an organism to regulate its metabolic processes in response to environmental change allows it to conserve scarce resources efficiently. The sulfur control circuit of *Neurospora crassa* provides an excellent example of one such system of metabolic controls, offering well-defined genetics and a high degree of regulation of its component loci (13). The synthesis of a number of sulfur-related enzymes is repressed in a concerted fashion by high intracellular levels of sulfur. In general, these enzymes are necessary in the uptake and early stages of assimilation of a variety of sulfur compounds from the environment. They include aryl sulfatase (18), two distinct sulfate permeases (10), choline-O-sulfate permease (12), choline sulfatase, glucose-6-sulfate permease (23), a methionine permease (22), and an extracellular protease (7). The *cys-3*$^+$ gene serves to positively regulate expression of these structural genes, as is evidenced primarily by the complete loss of all of the respective enzymes in *cys-3* mutants (14).

A second regulatory gene, *scon*$^+$, acts to repress expression of genes in the sulfur control circuit. The mutant allele, *scon*^{c}, releases the circuit from sulfur repression, resulting in pleiotropic, constitutive expression of the various enzymes (4). *scon*$^+$, *cys-3*$^+$, and all three known structural gene loci have been shown to be genetically unlinked to each other (4, 10, 14, 15). The *cys-3*$^+$ locus encodes a regulatory gene product capable of acting within nuclei other than the one which encoded it, whereas the *scon*$^+$ locus displays nucleus-limited behavior (4, 14, 16). *cys-3* has also been shown to be epistatic to *scon*^{c} (5).

One recent study to examine the operation of the sulfur control circuit involved the cloning and subsequent analysis of the expression of the *cys-3*$^+$ control gene (21). To understand the sulfur regulatory circuit, it is obviously very important to clone and study the expression of one or more of the structural genes of the circuit. *cys-13*$^+$ and *cys-14*$^+$, which encode sulfate permeases I and II, respectively (10), represent two well-characterized genes in the sulfur circuit. In addition to sulfur circuit control, *cys-13*$^+$ and *cys-14*$^+$ show developmental regulation. *cys-13*$^+$ is expressed primarily in germinating conidia, whereas *cys-14*$^+$ expression is limited almost entirely to mycelia (9). The availability of tight mutant alleles at both loci and the inability of the double mutant to utilize sulfate as the only sulfur source make these loci candidates for cloning by complementation with the sibling selection procedure (1). We report here the cloning and initial characterization of *cys-14*$^+$, the structural gene which encodes sulfate permease II of *N. crassa*. Results are also presented which demonstrate that *cys-14*$^+$ gene expression is highly regulated through modulation of message content.

MATERIALS AND METHODS

Cosmid library of *N. crassa* DNA. A cosmid library of *N. crassa* genomic DNA constructed by Vollmer and Yanofsky (27) was obtained from the Fungal Genetics Stock Center. This library is comprised of large (approximately 40-kilobase [kb]) *N. crassa Sau*3a partial DNA fragments cloned into vector pSV50, which contains pBR322 sequences for propagation in *Escherichia coli*, a benomyl resistance gene as a dominant selectable marker in *N. crassa* and a COS site for packaging into λ-like particles and subsequent infection of *E. coli*. The library is distributed in 32 microtiter plates, each containing 96 individual clones.

Transformation of *N. crassa*. Competent spheroplasts were prepared from conidia of a *cys-13 cys-14 al-1* strain of *N. crassa* by the method of Schweizer et al. (26), incorporating modifications developed by Orbach et al. (20) and Akins and Lambowitz (1). A *cys-13 cys-14* double mutant cannot im-

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port sulfate and thus will not grow on unsupplemented minimal medium, which contains only sulfate as a sulfur source. Selection for either *cys-13*$^+$ or *cys-14*$^+$, the two distinct sulfate permease genes, was accomplished by plating transformed cells in osmotically adjusted minimal top agar onto minimal bottom agar containing benomyl at 0.5 μg/ml.

DNA isolation. Cosmid and plasmid DNA were prepared by the alkaline lysis method (3). Cosmid preparations for screening the library were further purified by cesium chloride-ethidium bromide gradient ultracentrifugation of the cleared lysate (8). *N. crassa* DNA was isolated by using a slight modification of the protocol described by Metzenberg and Baisch (17).

Restriction digests and Southern blots. Restriction enzymes were obtained from Bethesda Research Laboratories, Inc., and from International Biotechnologies and were used according to the instructions provided by the manufacturers. In Southern transfers, the DNAs were blotted onto BA85 nitrocellulose (Schleicher & Schuell Co.) as described by Maniatis et al. (8). Nitrocellulose filters were baked for 30 to 60 min under vacuum and then prehybridized for 1 to 2 h in 0.9 M NaCl–0.09 M sodium citrate–0.25% dried milk–1 mM EDTA at 65°C. The DNA probe was nick translated by using [α-^{32}P]dCTP to incorporate radioactive label (25), heat denatured, and then added to the hybridization solution. Hybridization and washing conditions were otherwise as described by Maniatis et al. (8).

Poly(A)$^+$ mRNA isolation and Northern blots. *N. crassa* RNA was extracted by the method of Reinert et al. (24) from various strains grown with either high sulfur (5 mM methionine) or low sulfur (0.25 mM methionine). The poly(A)$^+$ fraction was isolated by using oligo(dT)-cellulose (2). Formaldehyde-agarose gels and Northern (RNA) blots were performed as described by Maniatis et al. (8), except that the alkaline denaturation and high salt renaturation steps were omitted. We have found that these steps are unnecessary and in some circumstances may compromise hybridization signal strength. Prehybridization and hybridization protocols were essentially as described by Fu and Marzluf (6).

RESULTS

Cloning of the *cys-14*$^+$ gene. Cosmid DNA was isolated and pooled from each of 32 subdivisions of the cosmid library of *N. crassa* wild-type DNA (27). Each sublibrary represented 96 individual clones and was purified by CsCl-ethidium bromide gradient ultracentrifugation (8). Competent spheroplasts were prepared (1, 20, 26) from conidia of a *cys-13 cys-14 al-1* strain of *N. crassa*. *cys-13 cys-14* double mutant strains lack both sulfate permease species and cannot transport and thereby utilize inorganic sulfate for growth. The albino mutation, *al-1*, allowed genuine transformants to be readily distinguished from any false-positive contaminant colonies. These spheroplasts were transformed with each of the 32 pools of cosmid DNA and double selected on minimal medium containing the fungicide benomyl at 0.5 μg/ml. Minimal medium contains sulfate as the only source of sulfur. Of the 32 sublibraries, 10 appeared to transform the *cys-13 cys-14 al-1* mutant strain, giving rise to colonies on minimal medium. Several of the most vigorous colonies from a number of the positive sublibraries were inoculated into liquid minimal medium and proved to be unambiguously capable of utilizing sulfate as the only source of sulfur. One sublibrary which showed a relatively high efficiency of transformation was selected for further screening and was divided into eight pools of 12 clones each. Each pool was tested for its ability to transform the *cys-13 cys-14 al-1* strain as described above. One pool of 12 clones gave rise to benomyl-resistant, sulfate-utilizing transformants. This pool was in turn divided into the 12 individual clones, of which a single clone efficiently complemented the *cys-13 cys-14 al-1* strain. Several complete restriction digests of this cosmid yielded fragments on an agarose gel which totaled approximately 45 kb in each case, which is in agreement with the expected size of one cosmid from this library (data not shown) (27).

Restriction fragment length polymorphism mapping of chromosomal location of cloned DNA. A restriction fragment length polymorphism analysis was employed to determine whether the cloned DNA fragment did indeed correspond to either the *cys-13*$^+$ or the *cys-14*$^+$ gene. The method described by Metzenberg et al. (19) was used. Several polymorphisms were identified between the parental strains, Mauriceville 1c-A wild type and RLM 33-1a (*al-2 arg-12 cot-1 inl nuc-2*), when their DNAs were treated with *Pst*I and Southern blots were probed with the cosmid. DNAs from the 18 progeny strains were then digested with *Pst*I, blotted, and probed with the cosmid (Fig. 1). The resultant pattern of inheritance of the chromosomal region corresponding to the cloned DNA was compared with that of a series of known markers. The cloned segment showed tight linkage to 5S genes 62 and 63 as well as a high degree of linkage to *cot-1*, all located on linkage group IV right, the location of *cys-14* (10). No linkage was evident to chromosome I, which contains *cys-13* (10), or to any other chromosome. We conclude that this clone represents the *cys-14*$^+$ gene of *N. crassa*.

Subcloning and localization of *cys-14*. Neither the *cys-13 cys-14 al-1* strain nor a closely related *cys-13 cys-14* strain (FGSC 1839) could be transformed efficiently with linear fragments of DNA. Thus, it was necessary to subclone restriction fragments of the cosmid DNA to determine whether the respective restriction enzyme inactivated *cys-14*, presumably by cutting within it. Small libraries for each restriction enzyme were constructed in a pIBI31-based vector which contained a benomyl resistance marker (20). Pools of plasmids from these libraries were screened for the ability to transform *cys-13 cys-14*. In this way a plasmid designated pJSK1 containing a 7.2-kb *Neurospora Hin*dIII fragment,

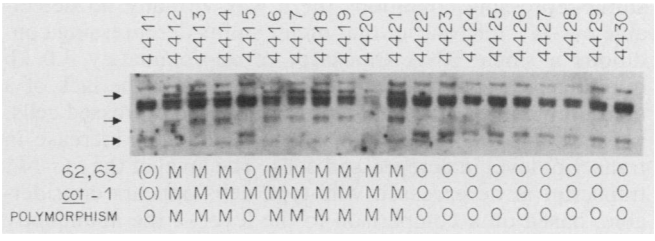

FIG. 1. Mapping the chromosomal location of the cloned cosmid DNA by restriction fragment length polymorphism analysis. A Southern blot of a 0.75% agarose gel loaded with *Pst*I-digested genomic DNAs from *N. crassa* FGSC strains 4411 through 4430 was probed with ^{32}P-labeled cosmid DNA. Strains 4411 and 4416 correspond to RLM 33-1a and Mauriceville-1c-A, respectively, the parental strains. The remaining 18 strains represent progeny of that cross (19). The arrows indicate the polymorphic bands. The pattern of inheritance of the cloned DNA for each progeny strain, is shown below each lane (M, Mauriceville; O, Oak Ridge). The inheritance of several closely linked markers is also shown; 62 and 63 indicate restriction fragment length polymorphism markers in the flanking regions of 5S genes 62 and 63.

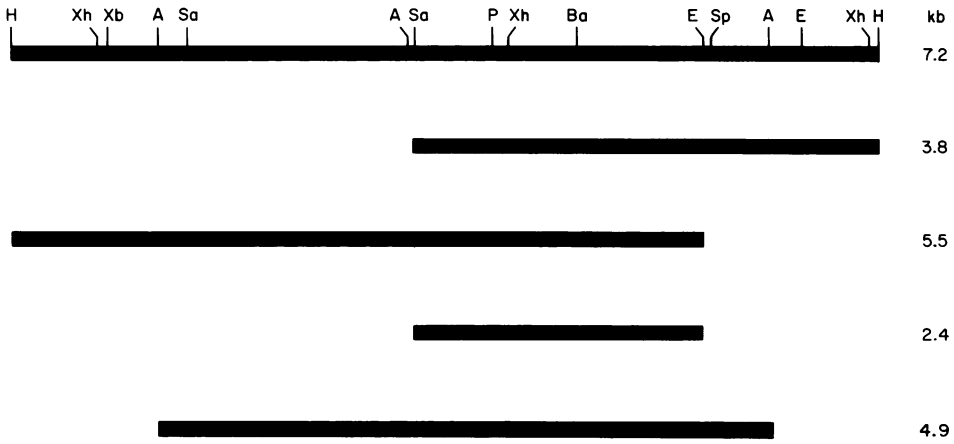

FIG. 2. Physical map of 7.2-kb *cys-14*$^+$ *Hin*dIII fragment and subfragments. The 7.2-kb fragment contains the entire functional *cys-14*$^+$ gene as determined by its ability to complement the *cys-13 cys-14 al-1* strain of *N. crassa*. Restriction enzyme sites are represented as follows: H, *Hin*dIII; Xh, *Xho*I; Xb, *Xba*I; Sa, *Sal*I; A, *Apa*I; P, *Pst*I; Ba, *Bam*HI; E, *Eco*RI; and Sp, *Sph*I. The 3.8-, 4.9-, and 5.5-kb fragments were subcloned and tested for transformation of the *cys-13 cys-14 al-1* strain with selection on minimal medium. None of these fragments complemented the double mutant strain. The 2.4-kb fragment was cloned for use as a probe for *cys-14*$^+$ mRNA in Northern blot experiments.

which contains *cys-14*$^+$ and efficiently complements the *cys-13 cys-14* double mutant, was isolated (Fig. 2). Several restriction fragments of pJSK1 were subcloned and tested for the ability to transform the *cys-13 cys-14* strain. The 3.8-kb *Sal*I-*Hin*dIII fragment (Fig. 2) was incapable of complementing the permease mutations, indicating that the *cys-14*$^+$ gene spans the *Sal*I site. The 5.5-kb *Eco*RI-*Hin*dIII fragment (Fig. 2) did not complement, thus showing that the gene must also extend through this *Eco*RI site. The 2.4-kb *Sal*I-*Eco*RI fragment was subcloned for use as a probe in subsequent Northern blot experiments. With such an internal fragment as a probe we expected that only *cys-14*$^+$ transcripts would be detected.

Northern blot analysis of *cys-14* expression. To examine the roles of *cys-3*$^+$ and *scon*$^+$ in the regulation of *cys-14*$^+$ expression, a series of Northern blot experiments was performed with mRNA isolated from cells of the wild type and several mutant strains. In each case, mRNA isolated from cells grown under sulfur-repressing (high-methionine) conditions was compared with mRNA from cells grown under sulfur-derepressing (low-methionine) conditions. A Northern blot of wild-type mRNA (Fig. 3A) shows that under sulfur-repressing conditions there was virtually no detectable *cys-14*$^+$ mRNA but that under sulfur-derepressing conditions a single RNA transcript of approximately 3.0 kb hybridized strongly to the probe. Because of the lack of a measurable level of *cys-14*$^+$ transcript in the repressed cells, it was not possible to estimate the amount of increase in transcript level in derepressed cells. The level of the *cys-14*$^+$ transcript in derepressed wild-type cells appears considerable, based on a comparison to the level of the abundantly expressed β-tubulin gene transcript. A similar experiment was performed with mRNA isolated from a *cys-3* mutant (Fig. 3B). In this case no *cys-14*$^+$ transcript was detected in mRNA from cells grown with either high or low methionine. To further examine the role of the *cys-3*$^+$ gene product in regulating the expression of *cys-14*$^+$, a temperature-sensitive revertant of *cys-3*, *cys-3*(REV65t), was employed. At 25°C this strain exhibits a nearly wild type degree of regulation and expression of sulfur-related enzyme activities normally under control of *cys-3*$^+$. At 37°C, however, this strain has the *cys-3* mutant phenotype and completely lacks these enzymes (14). The Northern blot results for *cys-3*(REV65t) (Fig. 3C) at 25°C are essentially indistinguishable from those for the wild-type strain (Fig. 3A), whereas at 37°C *cys-3*(REV65t) did not possess any *cys-14*$^+$ transcript under either condition, exactly as seen with the *cys-3* strain (Fig. 3B).

Unlike the wild type, the *scon*^{c} strain is not sensitive to

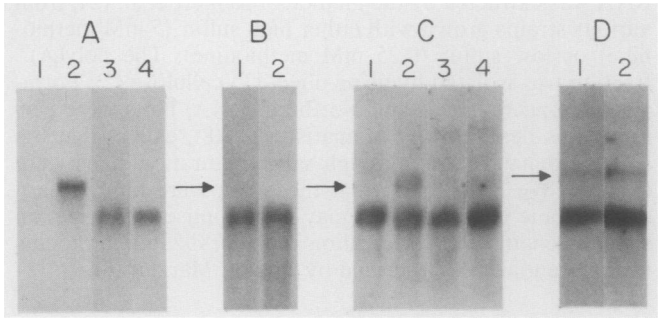

FIG. 3. Northern blot experiments. (A) The poly(A)$^+$ fraction of RNA isolated from wild-type (Oak Ridge 74A) *N. crassa* cells grown under either high or low sulfur was run on a 1% agarose–formaldehyde gel and blotted to nitrocellulose. Lanes: 1 and 3, RNA isolated from cells grown under high sulfur; 2 and 4, RNA isolated from low-sulfur conditions. Lanes 1 and 2 were probed with ^{32}P-labeled pJSK2, which consists of the 2.4-kb *Eco*RI-*Sal*I internal fragment of *cys-14*$^+$ (see text and Fig. 2) ligated into pIBI25 (International Biotechnologies). Lanes 3 and 4 were probed with pSV50, which contains the *N. crassa* β-tubulin gene. Lanes 1 and 2 were loaded with 25 μg of mRNA each, whereas lanes 3 and 4 were loaded with 10 μg each. (B) Poly(A)$^+$ mRNA (10 μg) from a *cys-3* strain grown under high sulfur (lane 1) or low sulfur (lane 2) was run and blotted as for panel A. The blot was probed with ^{32}P-labeled pJSK2 and pSV50. The arrow indicates the position where the *cys-14*$^+$ transcript would run. (C) Poly(A)$^+$ mRNA (10 μg) from *cys-3*(REV65t) grown at 25°C with high or low sulfur (lanes 1 and 2) or at 37°C with high or low sulfur (lanes 3 and 4) was electrophoresed and blotted as for panel A. *cys-3*(REV65t) is a heat-sensitive revertant of *cys-3*. All four lanes were probed with ^{32}P-labeled pJSK2 and pSV50. The arrow indicates the *cys-14*$^+$ transcript. (D) mRNA (10 μg) from *scon*^{c} cells grown under high sulfur (lane 1) or low sulfur (lane 2) were run and blotted as for panel A. Both lanes were probed with ^{32}P-labeled pJSK2 and pSV50. The arrow indicates the position of the *cys-14*$^+$ transcripts. Differential exposures were used for lanes in panels A, B, and D to normalize the β-tubulin message signal strengths.

sulfur catabolite repression and expresses sulfate permeases and other sulfur-related enzymes, even in the presence of excess sulfur (4). When the *scon*^{c} strain was examined, relatively high and approximately equal *cys-14*$^+$ transcript levels were detected in mRNA isolated from cells grown under sulfur-derepressed and sulfur-repressed conditions (Fig. 3D). In all of the Northern blot experiments β-tubulin transcript was used as an internal control to normalize hybridization signal strength in each lane. Expression of β-tubulin mRNA appears to be constitutive and clearly does not respond to the sulfur status of the cells.

DISCUSSION

We report here the cloning of the *N. crassa cys-14*$^+$ gene, which encodes sulfate permease II (10). Sulfate permease II is limited to the mycelial stage of growth, where it is more highly expressed than is sulfate permease I (9). The identity of this clone was established by demonstrating that a restriction fragment length polymorphism, revealed by using this DNA as a probe, mapped to the *cys-14* region of chromosome IV.

The cloning of *cys-14*$^+$ is significant in that it represents the first opportunity to examine directly the interaction of regulatory and structural genes in the sulfur regulatory circuit of *N. crassa*. It is known that the sulfur circuit contains two regulatory genes, *cys-3*$^+$ and *scon*$^+$, which act as positive and negative regulators, respectively, of a set of at least eight structural genes, including *cys-14*$^+$ (4, 14). However, the mechanism by which the regulatory genes control the expression of the structural genes has remained obscure, although it did not appear to take place by altering the activation or turnover of previously synthesized enzymes (11).

We have used the cloned *cys-14*$^+$ gene as a probe for Northern blot experiments to examine regulation of structural gene expression in the sulfur circuit of *N. crassa*. In the wild-type strain, no *cys-14*$^+$ transcript could be detected when the cells were grown under high-sulfur conditions. These growth conditions previously have been demonstrated to strongly repress sulfate uptake activity (9). A considerable content of the 3-kb *cys-14*$^+$ transcript was detectable, however, when sulfur was limited, the condition shown to derepress sulfate uptake (9). These results argue that the level of *cys-14*$^+$ transcript is sulfur controlled and that it in turn determines the production of sulfate permease II. In the *cys-3* mutant strain, which presumably lacks a functional *cys-3*$^+$ protein, there was no detectable *cys-14*$^+$ transcript, regardless of sulfur availability. This observation strongly suggests that the *cys-3*$^+$ gene positively regulates the level of *cys-14*$^+$ transcript. This conclusion was further strengthened by Northern blot experiments with mRNA from the temperature-sensitive revertant of *cys-3*, which showed that *cys-14*$^+$ transcripts were readily detected at the permissive temperature during sulfur limitation but were completely lacking at the conditional temperature. The constitutive presence of *cys-14*$^+$ mRNA in the *scon*^{c} strain, regardless of sulfur levels, demonstrates that the *scon*$^+$ gene also exerts its control function by directly or indirectly modulating *cys-14*$^+$ message content. In fact, recent experiments indicate that *scon*$^+$ regulates *cys-3*$^+$ expression, which in turn is responsible for expression of the unlinked structural genes of the sulfur circuit (D. G. Mannix and G. A. Marzluf, manuscript in preparation).

Recent work suggests that the *cys-3*$^+$ gene positively regulates its own expression, most likely at the level of transcription (21; D. G. Mannix and G. Marzluf, unpublished data), and the DNA sequence of *cys-3*$^+$ indicates that it encodes a protein of approximately 23,000 daltons (J. V. Paietta, D. G. Mannix, and G. A. Marzluf, manuscript in preparation). These results, plus the findings reported here, suggest a mechanism for the operation of the sulfur control circuit. During conditions of sulfur limitation, negative control of *cys-3*$^+$ by the *scon*$^+$ gene is lifted, leading to transcription of *cys-3*$^+$ and synthesis of a *cys-3*$^+$ regulatory protein, which, via autoregulation, even further enhances *cys-3*$^+$ expression. When present at a sufficient level in the nucleus, the *cys-3*$^+$ protein is visualized to interact with *cys-14*$^+$ and the other structural genes to turn on their transcription.

Support for the transcriptional control model presented above will require a direct demonstration that the *cys-3*$^+$ protein binds at the *cys-14*$^+$ gene and that this interaction is required for *cys-14*$^+$ expression. Mechanisms other than transcriptional control can regulate the content of mRNA in eucaryotic cells, namely, (i) the regulation of processing or export from the nucleus of mRNA species and (ii) regulation of mRNA stability or translation. Although we favor the transcriptional model for control of the sulfur circuit, the results presented here do not exclude the possibility that regulation occurs at one of these other steps.

It is noteworthy that *N. crassa* possesses two distinct sulfate permease species, encoded by unlinked genes, and that both are subjected to regulation by the *cys-3*$^+$ and *scon*$^+$ control genes (10). However, these two permeases are differentially expressed during development. It will be interesting to determine whether the developmental regulation of the two sulfate permeases is similar or even related to the mechanisms of sulfur regulation.

ACKNOWLEDGMENTS

We thank Steve Vollmer and Charles Yanofsky, for making their pSV50 cosmid library available to us, and Robert Metzenberg, who provided the *scon*^{c} strain of *N. crassa*.

This research was supported by Public Health Service grant GM-23367 from the National Institutes of Health.

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