MOLECULAR AND CELLULAR BIOLOGY, Oct. 1990, p. 5207–5214
0270-7306/90/105207-08$02.00/0

Vol. 10, No. 10

Molecular Cloning and Analysis of the *scon-2* Negative Regulatory Gene of *Neurospora crassa*

JOHN V. PAIETTA

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

Received 21 May 1990/Accepted 12 July 1990

The sulfur regulatory system of *Neurospora crassa* is composed of a group of highly regulated structural genes (e.g., the gene encoding arylsulfatase) that are under coordinate control of *scon*$^+$ (sulfur controller) negative and *cys-3*$^+$ positive regulatory genes. In *scon-1* (previously designated *scon*^{C}) and *scon-2* mutants, there is constitutive expression of sulfur structural genes regardless of the sulfur level available to the cells. The *scon-2*$^+$ gene was cloned by sib selection screening of a cosmid-based gene library. The screening was based on the use of chromate, a toxic sulfate analog, which is transported into *scon-2* cells grown on high sulfur but is not transported into cells that have regained normal sulfur regulation. Restriction fragment length polymorphism analysis was used to confirm that the cloned segment mapped to the proper chromosomal location. In wild-type cells, Northern (RNA) blot analysis showed that a 2.6-kilobase *scon-2*$^+$ transcript was present at a substantial level only under sulfur-derepressing conditions. Kinetic analysis showed that *scon-2*$^+$ mRNA content increased as the cells became sulfur starved. Further, *scon-2*$^+$ RNA was detectable in a nuclear transcription assay only under derepressing conditions. In *scon-1*, the levels of *scon-2*$^+$ mRNA were found to be constitutive. In the *cys-3* regulatory mutant, there was a reduced level of *scon-2*$^+$ transcript. *cys-3*$^+$ and *ars-1*$^+$ mRNAs were present under both derepressing and repressing conditions in the *scon-2* mutant. Repeat-induced point mutation-generated *scon-2* mutants were identical in phenotype to the known mutant.

Sulfur uptake and assimilation in *Neurospora crassa* is carried out by a set of coordinately expressed structural genes. The structural genes are controlled by a set of genetically defined *trans*-acting regulatory genes and are expressed under conditions of sulfur limitation (i.e., derepressing conditions) (18). The unlinked structural genes encode arylsulfatase, choline sulfatase, sulfate permeases I and II, methionine permease, and an extracellular protease (12, 14, 19, 28, 31), which allow for the uptake and assimilation of a variety of sulfur compounds. The sulfur regulatory circuit of *N. crassa* provides a useful model system for studying regulatory interactions in a multigene network and how a cell regulates its sulfur status.

The *cys-3*$^+$ regulatory gene plays a central role in the sulfur regulatory circuit. *cys-3* mutants show a pleiotropic loss of the entire set of sulfur-controlled enzymes and, in contrast to the wild type, are unable to use a variety of compounds as sources of sulfur. The cloning of *cys-3*$^+$ by Paietta et al. (29) and subsequent work (10) has shown the encoded gene product to be a protein in the bZIP class (basic region-leucine zipper) (16, 37). *cys-3*$^+$ shows homology to the oncogene *fos* product and the yeast general control regulator GCN4 (10). By analogy to these proteins, *cys-3*$^+$ appears to encode for a DNA-binding transcriptional activator (10). Nuclear transcription assays using the cloned arylsulfatase structural gene support a model of transcriptional control of the sulfur structural genes (28).

Another class of regulatory mutant results in constitutively derepressed sulfur enzyme expression (e.g., arylsulfatase), wherein the repression by high sulfur seen in the wild type no longer occurs. *scon*^{C} (sulfur controller constitutive), isolated by Burton and Metzenberg (4), was the first reported of this mutant class. In *scon*^{C} (here referred to as *scon-1*), the message content was elevated and constitutive for the *cys-3*$^+$ regulatory and *ars-1*$^+$ structural genes (10, 28). On the basis of heterokaryon studies with an electrophoretic variant of arylsulfatase, it was shown that *scon-1*$^+$ exerted only intranuclear control of arylsulfatase gene expression (i.e., nuclear limitation) (4, 20). Furthermore, in double-mutant studies, *cys-3* was found to be epistatic to *scon-1* (8). On the basis of these results, *scon-1*$^+$ can be placed in a regulatory hierarchy in which it is a negative effector of *cys-3*$^+$ expression; *cys-3*$^+$ then acts as a positive regulator of sulfur structural gene expression.

A second gene which when mutant results in constitutive derepression of sulfur enzymes (e.g., arylsulfatase) was identified by P. S. Dietrich (M.S. thesis, University of Wisconsin, Madison, 1972). The mutation is designated here as *scon-2*. The *scon-2* mutation is recessive and unlinked to other known loci in the system. *scon-2* does not display the nuclear limitation effect seen in *scon-1*. The strain was isolated by a selection protocol which set up a condition in which growth was dependent on methionine permease, followed by repression of the methionine permease and then selection for a mutation that released the permease from control. The *scon-2* mutant is a prototroph. Arylsulfatase activity in *scon-2* is constitutive and is not repressible by methionine or sulfate (Dietrich, M.S. thesis, 1972).

Regulatory mutants showing constitutive expression of arylsulfatase have also been reported in *Aspergillus nidulans* and *Klebsiella aerogenes*. A number of *A. nidulans sul-reg* (sulfur regulatory) mutants representing six complementation groups have been isolated (1). These *A. nidulans* mutants show constitutive arylsulfatase synthesis when grown on sulfate, but Apte et al. (1) report that they are still subject to cysteine and methionine repression. In *K. aerogenes*, a regulatory gene, *atsR*, has been identified which when mutant results in constitutive arylsulfatase synthesis on sulfate and cysteine (25). An operon organization for the linked *atsR*, *atsC*, and *atsA* genes has been suggested (25).

I report here the cloning and characterization of the *scon-2*$^+$ gene of *N. crassa*. The *scon-2*$^+$ gene was cloned by sib selection, using a screening based on growth of transformants on chromate and high-sulfur medium. *scon-2*$^+$ mRNA

was detected when the system became derepressed, and the *scon-2*$^+$ gene appeared to be subject to transcriptional control. The appearance of *scon-2*$^+$ mRNA paralleled the appearance of *cys-3*$^+$ mRNA seen as the cells became sulfur derepressed. The previous cloning of the *cys-3*$^+$ and *ars-1*$^+$ genes allowed for examination of regulatory interactions at the mRNA level. *scon-2* was found to result in constitutive levels of *cys-3*$^+$ and *ars-1*$^+$ mRNAs. The effect of the other regulatory gene mutations on *scon-2*$^+$ message content was also examined.

MATERIALS AND METHODS

Strains and plasmids. The *N. crassa scon-2*(PSD272) and *scon-1* (36-18, *scon*^{C}) strains were generously provided by Robert Metzenberg (University of Wisconsin). The use of *scon-1* and *scon-2* locus designations originates in this report. The *scon* (sulfur controller) designation of Burton and Metzenberg (4) has been maintained. The *cys-3*(p22) strain was obtained from the Fungal Genetics Stock Center (Kansas City, Mo.). Wild-type 74-OR23-1a was used as the wild-type strain for these studies. *N. crassa* strains used for restriction fragment length polymorphism (RFLP) were FGSC 4411 to 4430, isolated by Metzenberg et al. (24) from a cross of Mauriceville-1cA with RLM 1-33a *al-2 arg-12 cot-1 inl nuc-2*. The cosmid clone bank in the pSV50 vector was as reported by Vollmer and Yanofsky (38). The benomyl resistance gene used for cotransformation experiments was obtained from M. Orbach (Stanford University) and carried on pBT3 (27). The pGEM3Z vector was from Promega Biotec. pJP11 carried the *am*$^+$ gene on a 2.4 kilobase (kb) *Bam*HI fragment in pBR322 (15, 30). pJP18 carried the *ars-1*$^+$ gene on a 5.6-kb *Eco*RV fragment in pUC8 (28). pJP14 carried the *cys-3*$^+$ gene on a 1.8-kb *Eco*RV fragment in pUC8 (29).

Media, crosses, and culture conditions. Minimal Vogel medium (7), with supplements as required, was used. Crosses were done according to standard techniques, using cornmeal agar or Westergaard-Mitchell medium (7). *N. crassa* cultures were grown at 30°C except for experiments involving temperature-sensitive *cys-3* mutants. Repression and derepression experiments were conducted by transfer of mycelia by filtration harvesting and transfer to Vogel minus-sulfur medium with high-sulfur (5.0 mM methionine) and low-sulfur (0.25 mM methionine) medium, respectively (28). In other cases, experiments involved inoculation of spores directly into Vogel minus-sulfur medium with high or low sulfur concentrations as with the mycelial transfer experiments.

DNA isolation. Plasmid DNA was isolated from *Escherichia coli* by the alkaline extraction technique (3). Cosmid DNA was isolated according to the modified alkaline extraction technique of Vollmer and Yanofsky (38) and included the use of LiCl precipitation. *N. crassa* chromosomal DNA was prepared by extraction of freeze-dried mycelial samples in pronase–lithium EDTA–Triton X-100 and precipitation in ethanolic perchlorate, followed by DNA spooling according to the technique of Metzenberg and Baisch (21).

Transformation. Transformation of *N. crassa* was carried out with the Novozyme 234 spheroplasting technique of Vollmer and Yanofsky (38). Cotransformation was done by mixing electroeluted DNA fragments to be tested with the *Sal*I fragment containing the benomyl resistance gene from pBT3 as described by Paietta (28). For rapid screening of complementing fragments in *N. crassa*, the DNA electroeluted from gel bands was used directly for transformation (28). *E. coli* transformation was carried out with competent cells produced by calcium chloride treatment (6).

RIP inactivation. *N. crassa* wild-type 74-OR23-1a was transformed with the *scon-2*$^+$ gene on a 5.5-kb *Sal*I fragment that was blunt ended with S1 nuclease and cloned into the pCSN44 vector (35). pCSN44 carries the *A. nidulans trpC* promoter coupled to the hygromycin B phosphotransferase-coding sequence (35). Hygromycin-resistant transformants were crossed to wild-type 74-OR23-1a, and the progeny were analyzed for sulfur metabolic defects. The specificity of the repeat-induced point mutation (RIP) events to the *scon-2*$^+$ locus was confirmed by allelism tests (i.e., crosses to *scon-2*) and complementation by transformation with the *scon-2*$^+$ gene.

Arylsulfatase assays. Arylsulfatase assays were performed by monitoring *p*-nitrophenol liberation at 405 nm from *p*-nitrophenyl sulfate according to standard methods (23, 28).

Selection for chromate resistance. Transformation mixes (i.e., spheroplasts and DNA) were overlaid on benomyl plates with high sulfur (5 mM methionine). After a 24-h incubation, 4 ml of sterile filtered 10 mM potassium chromate in 1.5% agar was added as an overlay. Transformants that had recovered wild-type regulation (i.e., became *scon-2*$^+$) survived under these conditions, whereas the *scon-2* strain did not.

DNA labeling and Southern blots. Oligolabeling (9) was used to prepare DNA probes with [^{32}P]dCTP (3,000 Ci/mmol). Southern blot (34) transfers of DNA from 0.85% agarose gels to nitrocellulose were subsequently hybridized at 65°C in 3× SSC (1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate)–10× Denhardt solution with 0.2% sodium dodecyl sulfate for 16 h. Washes ranged from 2× SSC to 0.1× SSC at 65°C.

mRNA isolation and analysis. The phenol extraction procedure of Reinert et al. (32) was used for the isolation of total RNA, with modifications as in reference 28. Basically, mycelial samples were frozen in liquid nitrogen after harvest and subsequently homogenized in the extraction buffer. After phenol-chloroform extractions and sodium acetate washes, the poly(A)$^+$ mRNA was isolated from total mRNA by oligo(dT)-cellulose chromatography (2). Poly(A)$^+$ mRNA levels were determined spectrophotometrically, and samples were compared in subsequent Northern (RNA) blot experiments by probing with the constitutively expressed *am*$^+$ (15) and β-tubulin (27) genes. [^{32}P]dCTP (3,000 Ci/mmol)-labeled probes were prepared by oligolabeling of DNA fragments (9). Hybridization was at 42°C for 24 h in 50% formamide–5× SSPE (SSPE is 0.18 M sodium chloride, 1 mM EDTA, and 10 mM sodium phosphate) (pH 7.7) and washes as described previously (28). Nuclear transcription was carried out with Percoll gradient-isolated nuclei, using [^{32}P]UTP for labeling, and analyzed as described previously (28).

Materials. Restriction enzymes were from New England BioLabs, Inc., and Promega Biotec. Klenow DNA polymerase was from Pharmacia. Oligo(dT)-cellulose and DNA ligase were obtained from Boehringer Mannheim Biochemicals. Novozyme 234 was supplied by Novo Industries. ^{32}P isotopes were from Amersham Corp. Other biochemicals were from Sigma Chemical Co.

RESULTS

Cloning strategy. The approach taken for cloning the *scon-2*$^+$ gene was that of sib selection, using the pSV50 cosmid-based gene library of Vollmer and Yanofsky (38). The screening technique developed relied on the altered

TABLE 1. Arylsulfatase activity and chromate resistance of wild-type and sulfur mutant *N. crassa* strains

Strain	Arylsulfatase sp act[a]		Chromate resistance	
	High S[b]	Low S[c]	High S	Low S
Wild type	<0.05	7.2	+	−
scon-1 (36-18)	7.5	7.9	−	−
scon-2(PSD272)	8.3	8.6	−	−
cys-3(p22)	<0.05	<0.05	+	+

[a] Expressed as nanomoles per minute per milligram of total protein (23).
[b] High-sulfur medium with 5.0 mM methionine.
[c] Low-sulfur medium with 0.25 mM methionine.

sulfur regulation of the *scon-2* mutant. Table 1 demonstrates the constitutively derepressed phenotype of *scon-2* with regard to arylsulfatase. Note, in particular, the high arylsulfatase activity detectable in *scon-2* cells grown on high sulfur that could not be detected in the wild type. Other sulfur-regulated enzymes, such as sulfate permease, are also derepressed in the *scon-2* mutant (data not shown). The selection for *scon-2*$^+$ transformants is based on the finding that chromate, a toxic sulfate analog, is transported by sulfate permease into the cell (17). A wild-type strain (i.e., *scon-2*$^+$) will be resistant to chromate when grown in high sulfur as a result of repression of sulfate permease. Because the *scon-2* mutant is not repressible for sulfate permease (or other sulfur enzymes), it is chromate sensitive under high sulfur levels (Table 1). Therefore, transformation of *scon-2* to *scon-2*$^+$ was selected for on the basis of chromate resistance on high-methionine medium. *scon-2*$^+$ transformants will have regained wild-type control of sulfate permease, with the permease now subject to sulfur repression. The pSV50 cosmid library of Vollmer and Yanofsky (38) was subdivided and screened. A single clone, designated pJP20, transforming *scon-2* to *scon-2*$^+$ at high frequency was subsequently isolated.

Characterization of the clone. The 40-kb *N. crassa* segment in the cosmid clone isolated, pJP20, was digested by a series of restriction endonucleases to find the minimal complementing fragment that would transform *scon-2* to *scon-2*$^+$. In successive steps, this was determined to be a *Sal*I-*Xho*I fragment of 3.7 kb (Fig. 1A). Further deletion of DNA at either end of the *Sal*I-*Xho*I fragment resulted in noncomplementing clones. The 5.5-kb *Sal*I fragment from pJP20 was subcloned into pGEM3Z and designated pSCON2.

The *Sal*I-*Xho*I 3.7-kb fragment will transform *scon-2* to *scon-2*$^+$, with restoration of normal sulfur regulation. The *scon-2*$^+$ transformants isolated were grouped into two classes on the basis of Southern blot analysis. One group resulted from homologous integration at the *scon-2* resident site, and the other had integration events at heterologous sites. Streak purification was used to isolate homokaryons from the initially heterokaryotic transformants (28, 30). When the heterologous and homologous transformants were examined, both types showed the presence of normal sulfur gene regulation (data not shown). The normal sulfur regulation of heterologous site *scon-2*$^+$ transformants suggested that the cloned segment contained all of the necessary regulatory and coding sequence. In addition, the pattern of complementation of the *scon-2* mutant with DNA fragments was consistent with the detection of hybridizing *scon-2*$^+$ mRNA, using the same fragments as DNA probes in Northern blots (Fig. 1B).

The *scon-2* mutation has been mapped to chromosome III near *tyr-1* (Dietrich, M.S. thesis, 1972). The RFLP analysis

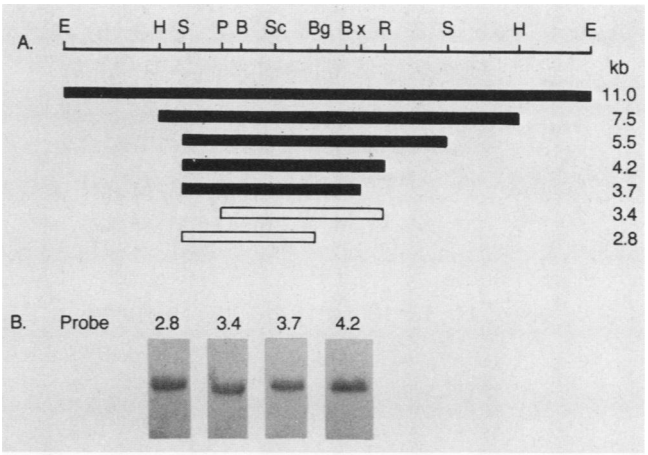

FIG. 1. Cloning and characterization of the *scon-2*$^+$ gene. (A) Localization of the *scon-2*$^+$ gene in pJP20. Cosmid pJP20 was initially cleaved with a number of restriction endonucleases, with screening of fragments for transformation of *scon-2* to *scon-2*$^+$ by selective plating on high-sulfur–chromate medium. An 11.0-kb *Eco*RI fragment in the original 40 kb of the cosmid clone was initially found to transform *scon-2* to *scon-2*$^+$. A restriction map of the complementing 11-kb *Eco*RI fragment is shown (E, *Eco*RI; Bg, *Bgl*II; H, *Hin*dIII; S, *Sal*I; Sc, *Sca*I; R, *Eco*RV; X, *Xho*I). The filled blocks beneath the restriction map indicate fragments that would transform *scon-2*. The smallest fragment capable of transforming *scon-2* to *scon-2*$^+$ was a 3.7-kb *Sal*I-*Xho*I segment. Open blocks indicate nontransforming segments. (B) Northern blot hybridization analysis of the 2.8- to 4.8-kb fragments. Northern blots of mRNA from sulfur-derepressed wild-type cultures were probed with labeled fragments as indicated. Probe number refers to fragments of corresponding sizes from panel A. Hybridization was observed with the 2.8-, 3.4-, 3.7-, and 4.2-kb fragments. Nonhybridizing fragments flanking the *Sal*I and *Eco*RV regions are not shown. These fragments include 2.5-kb *Eco*RI-*Sal*I, 2.8-kb *Eco*RI-*Pst*I (left) and 4.3-kb *Eco*RI-*Eco*RV, 4.8-kb *Eco*RI-*Xho*I (right). The constitutively expressed *am*$^+$ gene was used as a control to standardize comparisons (data not shown).

of Metzenberg et al. (22, 24) was performed to localize the map position of the cloned segment and to essentially rule out the cloning of a suppressor gene. An RFLP was identified between the parental tester strains (Mauriceville-1cA wild type and Oak Ridge *al-2 arg-12 cot-1 inl nuc-2*) by using the complementing 5.5-kb *Sal*I fragment from pJP20 to probe Southern blots of genomic DNA digested with a variety of restriction endonucleases. *Eco*RI provided a useful RFLP. Metzenberg et al. (24) have crossed the Mauriceville 1cA and Oak Ridge *al-2 arg-12 cot-1 inl nuc-2* strains and isolated a set of progeny characterized for the inheritance of genetic markers and molecular markers. The set of RFLP tester progeny was screened by probing with Southern blots of *Eco*RI digests of genomic DNA from these strains with the 5.5-kb *Sal*I *scon-2*$^+$ fragment (Fig. 2A). Upon comparison with known markers, it was found that the tightest linkage (16%) was to Fsr-45, a 5S gene (22) on the right arm of linkage group III. The map position indicated by the data were consistent with the mapping of *scon-2* to chromosome III near *tyr-1* by Dietrich (M.S. thesis, 1972). An additional RFLP test used a cross of the *scon-2* mutant and the Mauriceville-1cA (*scon-2*$^+$) strain. *scon-2* and *scon-2*$^+$ progeny from the cross were scored as to the presence of the *Eco*RI RFLP. If the cloned segment contained the *scon-2*$^+$ gene, then one would predict a perfect correlation between the *scon-2* or *scon-2*$^+$ phenotype and the particular RFLP associated in the parental strains. In 20 *scon-2*$^+$ and 18

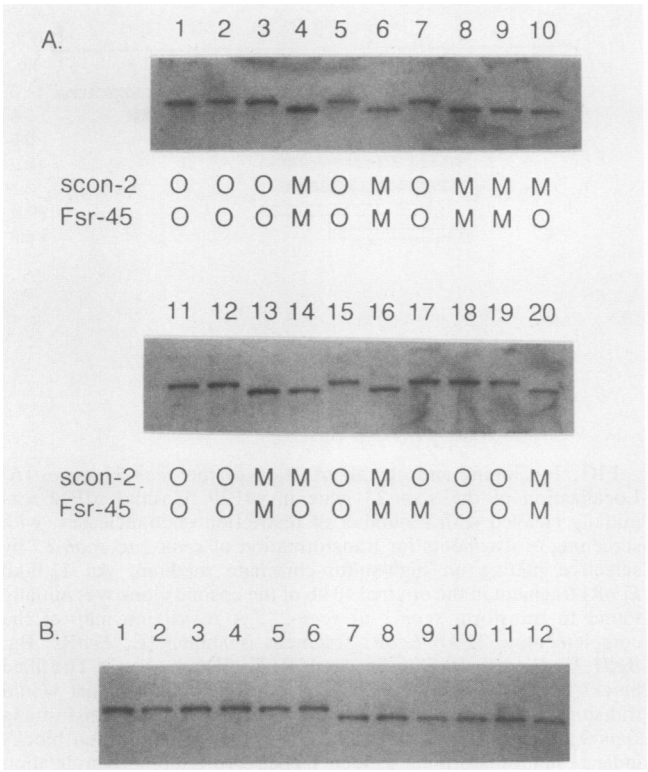

FIG. 2. (A) Chromosomal localization of the *scon-2*$^+$ cloned segment by RFLP analysis. Lanes 1 to 20 represent progeny of the cross Mauriceville-1cA × Oak Ridge *al-1 arg-12 inl nuc-2*. Lane 1 represents the Oak Ridge (O) and lane 6 represents the Mauriceville (M) parental RFLP pattern. Genomic digests with *Eco*RI provided a useful RFLP. The *Eco*RI fragments were probed with a smaller, internal 5.5-kb *Sal*I fragment. *scon-2* represents the RFLP segregation pattern seen in a Southern blot using the putative *scon-2*$^+$ segment as a probe. Fsr-45 represents a 5S ribosomal gene (22) on the right arm of chromosome III which shows the closest linkage of known markers to *scon-2*$^+$. (B) RFLP analysis using a *scon-2* × *scon-2*$^+$ cross. *scon-2* was crossed with Mauriceville-1cA (which is *scon-2*$^+$), and the progeny were scored for phenotype and an *Eco*RI RFLP. Representative progeny are shown. Lanes 1 to 6 are *scon-2* and show the parental (Oak Ridge) pattern; lanes 7 to 12 are *scon-2*$^+$ and show the Mauriceville pattern. The probe was as in panel A.

scon-2 progeny that were tested, an exact concordance of molecular marker and phenotype was found (Fig. 2B).

Analysis of *scon-2*$^+$ gene expression. *scon-2*$^+$ mRNA size and level were initially assayed in wild-type *N. crassa* grown on high and low levels of sulfur (i.e., repressing and derepressing conditions, respectively). Poly(A)$^+$ mRNA was isolated, and Northern blots were prepared and probed with the cloned *scon-2*$^+$ gene. In the blot shown in Fig. 3, mRNA was isolated from cultures grown for 12 h in low- and high-sulfur media inoculated with wild-type conidia. A 2.6-kb message showed hybridization to the *scon-2*$^+$ probe and was clearly detectable only under sulfur-derepressing conditions. A shift experiment in which mycelia grown on Vogel standard medium were transferred to low- or high-sulfur medium for 12 h produced the same result (data not shown). The experiments indicated that the steady-state level of *scon-2*$^+$ mRNA was substantially increased upon derepression. The constitutively expressed *am*$^+$ gene was used as a control probe for the Northern blots to ensure that the bulk RNA levels in the samples were comparable.

The kinetics of *scon-2*$^+$ message accumulation was also

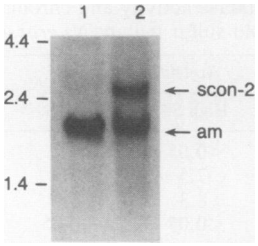

FIG. 3. Northern hybridization analysis of *scon-2*$^+$ mRNA. Wild-type cells were grown on low- or high-sulfur medium, poly(A)$^+$ mRNA was isolated, and Northern blots were prepared and probed with the *scon-2*$^+$ gene and *am*$^+$ gene. The probes were the ^{32}P-labeled 2.4-kb *Bam*HI fragment *am*$^+$ gene from pJP11 and the ^{32}P-labeled 4.2-kb *Eco*RV-*Sal*I fragment from pSCON2. The numbers 1.4, 2.4, and 4.4 represent size markers in kilobases. The blot was probed simultaneously with *scon-2*$^+$ and *am*$^+$. Lanes: 1, mRNA extracted from sulfur repressed cultures; 2, mRNA from sulfur-derepressed cultures. The experiment shows a transcript of approximately 2.6 kb that hybridizes to the *scon-2*$^+$ probe (lane 2). The *am*$^+$ probe, representing the constitutively expressed glutamate dehydrogenase gene, was present to provide a control for comparing message levels between the high- and low-sulfur samples.

examined. The *scon-2*$^+$ mRNA level was compared with the level of *cys-3*$^+$ mRNA, which has been shown to increase upon sulfur limitation (29). The kinetics of the appearance of *ars-1*$^+$ mRNA upon sulfur derepression has been previously documented (28). For a time course assay of *scon-2*$^+$ transcript levels, poly(A)$^+$ mRNA was isolated, and replicate Northern blots were prepared and probed with *am*$^+$, *cys-3*$^+$, and *scon-2*$^+$. The total sampling interval of 12 h was chosen because arylsulfatase enzyme activity and transcript level have been shown to rise and peak during this time period as the system becomes derepressed (28). The Northern blots demonstrate a substantial increase in *scon-2*$^+$ and *cys-3*$^+$ message content as the system became derepressed (Fig. 4).

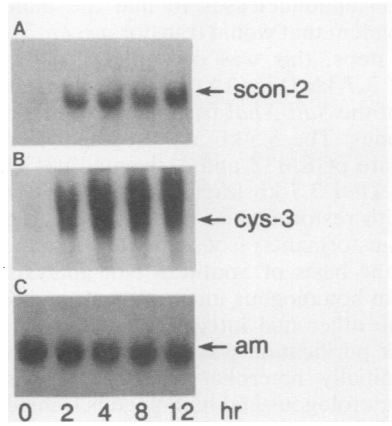

FIG. 4. Time course assay of *scon-2*$^+$ and *cys-3*$^+$ message levels. Mycelial wild-type cultures were shifted to derepressing (sulfur-limiting) conditions, and samples were taken at the indicated times. Poly(A)$^+$ mRNA was extracted, and Northern blots were prepared and hybridized with ^{32}P-labeled probe. (A) Probe of a Northern blot with a 4.2-kb *Eco*RV-*Sal*I *scon-2*$^+$ fragment. (B) Probe of a replicate blot with a 1.8-kb *Eco*RV *cys-3*$^+$ fragment. Arrow indicates the position of the 1.3-kb *cys-3*$^+$ transcript (see Results). (C) Probe of a replicate blot with a 2.4-kb *Bam*HI *am*$^+$ fragment. Use of the constitutively expressed *am*$^+$ gene confirmed that approximately equal levels of bulk mRNA were loaded.

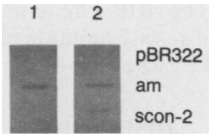

FIG. 5. Nuclear transcription assay of the *scon-2*$^+$ gene. Mycelial wild-type cultures were grown under derepressing and repressing conditions, and nuclei were isolated. In vitro transcription of the nuclei was performed, and the synthesized RNA was hybridized to slot blots of nitrocellulose-bound *am*$^+$, *scon-2*$^+$, and pBR322 DNAs. The bound DNAs were the *am*$^+$ 2.4-kb *Bam*HI and *scon-2*$^+$ 4.2-kb *Eco*RV-*Sal*I fragments. pBR322 was included as a control to assess nonspecific background hybridization. Lanes: 1, blot hybridized with labeled RNA from repressed nuclei; 2, blot hybridized with labeled RNA from derepressed nuclei.

Starting at 2 h, an initial 20-fold increase in *scon-2*$^+$ mRNA estimated by densitometry was observed. Parallel increases were seen in *cys-3*$^+$ mRNA levels during this time period. The presence of two major *cys-3*$^+$ transcripts was detectable, as has been reported previously (10, 28). To confirm that the system was indeed becoming derepressed, the arylsulfatase enzyme activity was assayed in the time course samples. The arylsulfatase assays confirmed that the cells became progressively sulfur derepressed with lengthening incubation time on low-sulfur medium (data not shown). The control experiment done with a shift to high-sulfur medium (i.e., repressing conditions) showed no increase in *cys-3*$^+$ or *scon-2*$^+$ mRNA level or any detectable arylsulfatase enzyme activity (data not shown). Again for these experiments, the constitutively expressed *am*$^+$ gene was used as a control to demonstrate the comparability of bulk RNA in the samples tested.

To test for the possibility of transcriptional regulation for the *scon-2*$^+$ gene, a nuclear transcription assay was performed. Such assays allow for assessment of RNA polymerase II loading levels for a gene (11, 36). Sulfur-derepressed and -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 *scon-2*$^+$ and *am*$^+$ DNA but not to pBR322 (control) DNA. Conversely, RNA that was synthesized by repressed nuclei hybridized only to *am*$^+$ DNA, not to *scon-2*$^+$ DNA or to the control pBR322 DNA (Fig. 5).

***scon-2*$^+$ expression in sulfur regulatory mutants.** The effect of the *cys-3*, *scon-1*, and *scon-2* regulatory mutations on *scon-2*$^+$ gene expression was examined by Northern blot analysis (Fig. 6). The *scon-1* mutation results in a phenotype in which the sulfur repression normally seen in the wild type does not occur (i.e., arylsulfatase activity is constitutively present) (4) (Table 1). Previous analyses of mRNA levels assayed in *scon-1* have shown that there are constitutive, high levels of *cys-3*$^+$, *ars-1*$^+$, and *cys-14*$^+$ mRNA present (10, 14, 28). The effect of *scon-1* on *scon-2*$^+$ mRNA level was therefore a question of considerable interest. *scon-2*$^+$ mRNA was constitutively present in a *scon-1* strain grown under either high- or low-sulfur conditions (Fig. 6A, lanes 1 and 2). The levels of *scon-2*$^+$ transcript observed were essentially equal for the two conditions.

The effect of the *scon-2* mutation on *scon-2*$^+$ mRNA level was also assayed. The Northern blots show that the *scon-2*$^+$ mRNA level seen under high- and low-sulfur conditions in the wild type was unaffected by the *scon-2* mutation (Fig. 6B, lanes 1 and 2). That is, *scon-2*$^+$ mRNA was detectable in sulfur-derepressed but not in sulfur-repressed cultures. Thus, the defect in the *scon-2* mutant appears not to involve regulation of the gene.

In *cys-3* mutants, there are low levels of *cys-3*$^+$ message (29) and essentially no *ars-1*$^+$ message (28) detectable even under sulfur-derepressing conditions. The level of *scon-2*$^+$ mRNA detected was substantially lower (Fig. 6C, lane 2) than in the wild type grown under derepressing conditions (Fig. 3). Under high-sulfur conditions in the *cys-3* mutant, *scon-2*$^+$ mRNA was essentially undetectable, as in the wild type (Fig. 6C, lane 1). The specific effect of the mutation in *cys-3* on *scon-2*$^+$ mRNA level was confirmed by using a temperature-sensitive mutant of *cys-3* (*ts47*). When *cys-3* (*ts47*) was grown under derepressing and permissive temperature conditions, *scon-2*$^+$ mRNA was easily detected; when the mutant was grown under derepressing but restrictive temperature conditions, *scon-2*$^+$ mRNA was present only at a low level (data not shown).

***ars-1*$^+$ and *cys-3*$^+$ expression in the *scon-2* mutant.** The previous cloning of the *cys-3*$^+$ regulatory and *ars-1*$^+$ (10, 28) structural genes provided probes to determine the effect of the *scon-2* mutation on mRNA levels for these genes. As has been noted, the *scon-2* mutation results in constitutive appearance of arylsulfatase enzyme activity (Table 1). mRNA levels were examined for *ars-1*$^+$ in a *scon-2* strain

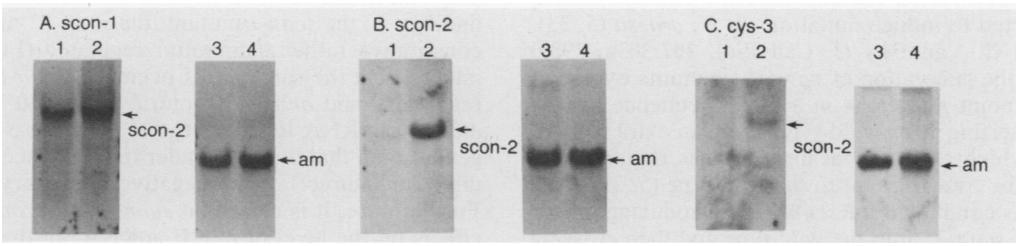

FIG. 6. Effect of sulfur regulatory mutants on *scon-2*$^+$ mRNA levels. The *scon-1*, *scon-2*, and *cys-3* regulatory mutants were grown under high- and low-sulfur conditions. Poly(A)$^+$ mRNA was isolated, and blots were prepared and probed with ^{32}P-labeled *scon-2*$^+$ DNA. Replicate samples were probed with the constitutively expressed *am*$^+$ gene. In each panel, lanes 1 and 2 were probed with a 4.2-kb *Eco*RV-*Sal*I *scon-2*$^+$ fragment and lanes 3 and 4 were probed with a 2.4-kb *Bam*HI *am*$^+$ fragment. Lanes 1 and 3 represent poly(A)$^+$ mRNA extracted from high-sulfur-grown cells; samples in lanes 2 and 4 were from low-sulfur-grown cells. (A) *scon-1* analysis. Northern blots of *scon-1* high- and low-sulfur mRNA preparations were probed with *scon-2*$^+$ and *am*$^+$. The *scon-2*$^+$ mRNA levels, under the two conditions were comparable. (B) *scon-2* analysis. Northern blots of *scon-2* high- and low-sulfur mRNA preparations were probed with *scon-2*$^+$ and *am*$^+$. Regulated expression of the *scon-2*$^+$ gene as in the wild type was observed. (C) *cys-3* analysis. Northern blots of high- and low-sulfur mRNA preparations from *cys-3* were probed with *scon-2*$^+$ and *am*$^+$. Lanes 1 and 2 represent an autoradiographic exposure twice as long as that of other blots. Note depressed levels of *scon-2*$^+$ mRNA under derepressing conditions (lane 2) as compared with Fig. 3, lane 2.

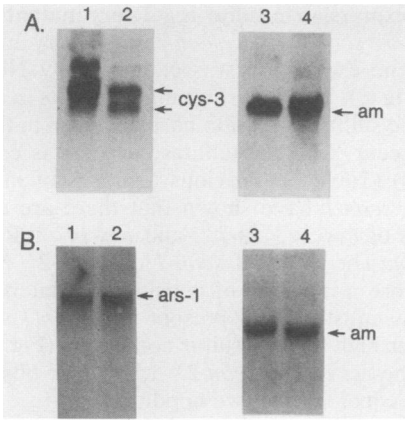

FIG. 7. *cys-3*$^+$ and *ars-1*$^+$ expression in the *scon-2* mutant. Poly(A)$^+$ mRNA was isolated from the *scon-2* mutant grown under high- and low-sulfur conditions and probed with either the *cys-3*$^+$ gene (A, lanes 1 and 2) or the *ars-1*$^+$ gene (B, lanes 1 and 2). (A) High-sulfur (lane 1) and low-sulfur (lane 2) mRNA isolated from *scon-2* and probed with the *cys-3*$^+$ gene. (B) High-sulfur (lane 1) and low-sulfur (lane 2) mRNA from *scon-2* probed with the *ars-1*$^+$ gene. In both panels, lanes 3 and 4 (high and low sulfur, respectively) represent replicate blots probed with the constitutively expressed *am*$^+$ gene.

grown under high- and low-sulfur conditions (Fig. 7B). Under both conditions, consistent with the arylsulfatase enzyme assays, *ars-1*$^+$ mRNA was detected. Next, the effect of *scon-2* on the *cys-3*$^+$ regulatory gene was assayed. Northern blots of mRNA preparations from *scon-2* showed the presence of *cys-3*$^+$ mRNA upon growth in both low-sulfur (derepressing conditions) and high-sulfur (repressing conditions) media. Under repressing conditions in *scon-2*, the same 1.3- and 1.6-kb transcripts were observed as in either *scon-2* or the wild type under derepressing conditions (Fig. 7A). The 1.3-kb transcript has been investigated most thoroughly and was shown previously to encode the *cys-3*$^+$ protein (10). A third transcript of about 2.1 kb in size that hybridized to the *cys-3*$^+$ probe was also detectable at higher levels in *scon-2* repressed versus derepressed cells in a number of replicate experiments. The significance and nature of the 1.6 kb- and 2.1-kb transcripts detected in previous studies (10, 29) are still under investigation. Previous work has shown that the other negative regulator, *scon-1*, also results in constitutive *cys-3*$^+$ mRNA levels (10).

Induction of new *scon-2* mutants by RIP. The use of RIP has been reported to induce mutations in *N. crassa* (5, 33). C. Staben and C. Yanofsky (J. Cell Biol. **107**:303a, 1989) have reported the generation of new *al-1* mutants by use of RIP. Multiple point mutations in a target sequence can be obtained by inserting into a wild-type strain an extra copy of the gene for which a functional disruption is required and then crossing the transformant to the wild type (5, 33). This experiment was conducted for *scon-2* by introducing one or more copies of *scon-2*$^+$ into the wild type and then crossing the transformant by the wild type. The progeny were then analyzed as to phenotype. To ensure that no sulfur-dependent mutants were overlooked (i.e., a different type of *scon-2* mutant than previously identified), methionine supplementation was used for germination and culturing of the cross progeny. Screening of the progeny revealed no sulfur auxotrophs but did reveal that about 10% of the 200 progeny tested had the *scon-2* phenotype (i.e., constitutive expression of arylsulfatase) and were indistinguishable from *scon-2* (data not shown). The RIP phenomenon, therefore, produced a new series of *scon-2* mutants phenotypically like the original one isolated by Dietrich (M.S. thesis, 1972).

DISCUSSION

I have reported the cloning and characterization of the *scon-2*$^+$ negative regulatory gene of *N. crassa*. The isolation of *scon-2*$^+$, the initial sulfur negative regulator to be cloned, is an important step in analyzing the regulatory interactions involved in controlling structural genes, such as the gene encoding arylsulfatase, associated with sulfur acquisition and processing. The restoration of normal sulfur regulation in *scon-2*$^+$ transformants as well as the RFLP analysis provide data confirming the identity of the clone.

Manipulation of the sulfur regulatory system with respect to chromate resistance or sensitivity on high-sulfur medium provided a powerful means of selecting transformants and of screening for the *scon-2*$^+$ clone. The selection technique should be a generally useful one for cloning genes of the sulfur controller class. It also provides a tool for the isolation of new sulfur mutants (J. Paietta, unpublished data).

Interestingly, expression of the *scon-2*$^+$ gene was responsive to the sulfur level on which the cells were grown (Fig. 3). In this regard, a similar response has been reported for all of the cloned genes in the sulfur control system studied to date (*ars-1*$^+$, *cys-3*$^+$, *cys-14*$^+$, and *scon-2*$^+$) in that the particular mRNAs are detectable at a substantial level only after cells are sulfur starved (i.e., the system becomes derepressed) (10, 14, 28). The mRNAs for these genes are either not present or are at extremely low levels when cells are cultured under high-sulfur (i.e., repressing) conditions. A time course analysis of RNA levels confirmed that the appearance of *scon-2*$^+$ mRNA was correlated with the derepression of the system as the cells became sulfur starved (Fig. 4). The *cys-3*$^+$ mRNA content during the time course was seen to parallel that of *scon-2*$^+$ mRNA. Thus, the two regulators show similar patterns of expression. Since the *scon-2*$^+$ mRNA level was found to be sulfur regulated, the level of control of *scon-2*$^+$ was examined. Nuclear transcription assays (Fig. 5) showed that hybridizable *scon-2*$^+$ mRNA was detectable, although weakly, only from assayed nuclei that had been isolated under sulfur starvation conditions. The involvement of controls at other levels of regulation cannot be excluded at present.

On the basis of Northern blot analyses and other data, a tentative regulatory hierarchy can be constructed (Fig. 8). The placement of *scon-1*$^+$ was indicated by the following findings. In the *scon-1* mutant, the *scon-2*$^+$ mRNA level was constitutive rather than sulfur regulated (Fig. 6A). It is of interest that the same effect occurs in *scon-1* for the *cys-3*$^+$ regulatory and *ars-1*$^+$ structural genes (10, 28). The alterations in mRNA levels of the *ars-1*$^+$, *cys-3*$^+$, and *scon-2*$^+$ genes argue that they are under the influence (which may be direct or indirect) of the negative regulatory gene *scon-1*$^+$. Furthermore, it is clear that *scon-1* and *scon-2* have similar effects on the level of *cys-3*$^+$ mRNA. In the *scon-2* mutant (Fig. 7A), the constitutive presence of *cys-3*$^+$ mRNA was detected as in *scon-1*. One therefore can place the *scon*$^+$ genes as effectors of the *cys-3*$^+$ positive regulatory gene, which in turn controls the expression of *ars-1*$^+$ and other structural genes. *cys-3*$^+$ is the most directly acting regulator of *ars-1*$^+$ gene expression, as determined from previous mutant studies, epistatic relationships (8, 18), and the RNA analyses presented here. The placement of *scon-2*$^+$ in the regulatory hierarchy (Fig. 8) was based on the finding that

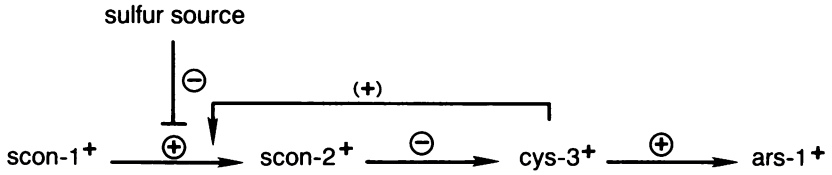

FIG. 8. Regulatory hierarchy for the sulfur regulatory system. Symbols: +, Positive effect; −, negative effect. The order is based on mRNA studies, epistatic relationships, and mutant phenotypes.

scon-2$^+$ expression becomes constitutive in *scon-1* just as *cys-3*$^+$ and *ars-1*$^+$ does (Fig. 6A). However, the *scon-2* mutation causes *cys-3*$^+$ to be expressed constitutively, so it is positioned at an intermediate point. The finding that the levels of *ars-1*$^+$ mRNA were constitutive in the *scon-2* mutant (Fig. 7B) is probably a result of an effect on *cys-3*$^+$ expression.

In addition, the *scon-2* mutant has normal sulfur regulation in terms of *scon-2* mRNA level (Fig. 6B). That the *scon-2* gene demonstrates a normal regulatory pattern suggests that *scon-2*$^+$ is not involved in the detection or sensing of the sulfur level. Instead, *scon-2*$^+$ would be involved in signal transmission from *scon-1*$^+$ (along with effector) to the *cys-3*$^+$ gene. For *scon-1*$^+$ (under low-sulfur conditions) or *scon-1* (regardless of the sulfur level), the effect on *scon-2*$^+$ or *scon-2* can be interpreted as positive (Fig. 8).

The question of whether the *scon-2* mutant phenotype results from loss of function of a gene product or whether the constitutive sulfur enzyme expression phenotype represents the only phenotypic possibility at this locus was examined. A RIP experiment was used to generate new mutant alleles that are probably functionally equivalent to a gene disruption (5, 33). Subsequent testing of the cross progeny revealed only the appearance of isolates showing the *scon-2*(PSD272) phenotype. Thus, the phenotypic properties of *scon-2* that suggest an important role for *scon-2*$^+$ in the sulfur regulatory system are present in the new mutants and include (i) derepression of arylsulfatase on methionine or sulfate and (ii) no alterations in various sulfur anabolic enzyme levels (e.g., sulfite reductase [26] or γ-cystathionase [4]; Paietta, unpublished data) that would indicate a secondary effect.

The effect of the *cys-3* mutation on *scon-2*$^+$ transcript level also needs to be considered. In the *cys-3* mutants tested, the level of *scon-2*$^+$ mRNA detected under derepressing conditions was considerably lower than in the wild type (Fig. 6C). Also clear is that the *scon-2*$^+$ product is a negative regulator of *cys-3*$^+$ transcription (Fig. 7A). One interpretation of these results suggest that the *cys-3*$^+$ gene product acts in a synergistic manner with the *scon-1*$^+$ gene product (if the sulfur level is low) to stimulate the transcription of *scon-2*$^+$. A control loop with the *cys-3*$^+$ positive regulator stimulating the synthesis of the *scon-2*$^+$ negative regulator would result (Fig. 8), with, as mentioned above, the *scon-2*$^+$ product being involved in signal transmission. An additional point in the overall model is that *cys-3* mutations also reduce the *cys-3*$^+$ mRNA level, and this may indicate that the *cys-3*$^+$ gene is subject to autoregulatory control (29). The roles and possible interaction of *scon-1*$^+$ and *scon-2*$^+$ in the regulation of *cys-3*$^+$ are now under study.

The signal for cellular sulfur status (i.e., sulfur sufficiency or starvation), which would set off the regulatory cascade leading to structural gene expression, is thought to be the intracellular level of cysteine, as indicated by the studies of Jacobson and Metzenberg (13) with a serine auxotroph. The molecular basis for the regulatory interactions involving *scon-2*$^+$, whether direct between gene products or indirect by involvement in, for example, corepressor formation, should now be amenable to experimental test. The control hierarchy (Fig. 8), based primarily on the mRNA data, serves as a starting point for testing possible regulatory interactions.

ACKNOWLEDGMENTS

I am grateful to Robert Metzenberg for his generous gift of the *scon-2* mutant. I thank Melinda Knudson-Stanley for assistance with mRNA isolation and crosses and Linda Barsalou for gene library screening and subcloning. I also thank the reviewers for comments on control models.

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

LITERATURE CITED

1. **Apte, B. N., P. N. Bhavsar, and O. Siddiqi.** 1974. The regulation of aryl sulphatase in *Aspergillus nidulans*. J. Mol. Biol. **86**:637–648.
2. **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.
3. **Birnboim, H. D., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
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. **Dagert, M., and S. D. Ehrlich.** 1979. Prolonged incubation in calcium chloride improves the competence of *Escherichia coli* cells. Gene **6**:23–28.
7. **Davis, R. H., and E. F. DeSerres.** 1970. Genetic and microbiological research techniques for *Neurospora crassa*. Methods Enzymol. **17A**:79–143.
8. **Dietrich, P. S., and R. L. Metzenberg.** 1973. Metabolic suppressors of a regulatory mutant in *Neurospora*. Biochem. Genet. **8**:73–84.
9. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132**:6–13.
10. **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.
11. **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.
12. **Hanson, M. A., and G. A. Marzluf.** 1973. Regulation of a sulfur-controlled protease in *Neurospora crassa*. J. Bacteriol. **116**:785–789.
13. **Jacobson, E. S., and R. L. Metzenberg.** 1977. Control of arylsulfatase in a serine auxotroph of *Neurospora*. J. Bacteriol. **130**:1397–1398.
14. **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.

15. **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.
16. **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.
17. **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.
18. **Metzenberg, R. L.** 1979. Implications of some genetic control mechanisms in *Neurospora*. Microbiol. Rev. **43**:361–383.
19. **Metzenberg, R. L., and S. K. Ahlgren.** 1970. Mutants of *Neurospora* deficient in arylsulfatase. Genetics **64**:409–422.
20. **Metzenberg, R. L., and S. K. Ahlgren.** 1971. Structural and regulatory control of arylsulfatase in *Neurospora*: the use of interspecific differences in structural genes. Genetics **68**:369–381.
21. **Metzenberg, R. L., and T. J. Baisch.** 1981. An easy method for preparing *Neurospora* DNA. Neurospora Newsl. **28**:20–21.
22. **Metzenberg, R. L., and J. Grotelueschen.** 1989. Restriction polymorphism maps of *Neurospora crassa*: update. Fungal Genet. Newsl. **36**:51–57.
23. **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.
24. **Metzenberg, R. J., J. N. Stevens, E. U. Selker, and E. Morzycka-Wroblewska.** 1984. A method for finding the genetic map position of cloned DNA fragments. Neurospora Newsl. **31**:35–39.
25. **Murooka, Y., T. Adachi, H. Okamura, and T. Harada.** 1977. Genetic control of arylsulfatase synthesis in *Klebsiella aerogenes*. J. Bacteriol. **130**:74–81.
26. **Nadolska-Lutyk, J., and A. Paszewski.** 1988. A new gene controlling sulphite reductase in *Aspergillus nidulans*. Genet. Res. **51**:1–3.
27. **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.
28. **Paietta, J. V.** 1989. Molecular cloning and regulatory analysis of the arylsulfatase structural gene of *Neurospora crassa*. Mol. Cell. Biol. **9**:3630–3637.
29. **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.
30. **Paietta, J. V., and G. A. Marzluf.** 1985. Gene disruption by transformation in *Neurospora crassa*. Mol. Cell. Biol. **5**:1554–1559.
31. **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.
32. **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.
33. **Selker, E., E. Cambareri, P. Garrett, B. Jensen, K. Haack, E. Foss, C. Turpen, M. Singer, and J. Kinsey.** 1989. Use of RIP to inactivate genes in *Neurospora crassa*. Fungal Genet. Newsl. **36**:76–77.
34. **Southern, E.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
35. **Staben, C., B. Jensen, M. Singer, J. Pollock, M. Schechtman, J. Kinsey, and E. Selker.** 1989. Use of a bacterial hygromycin B resistance gene as a dominant selectable marker in *Neurospora crassa* transformation. Fungal Genet. Newsl. **36**:79–81.
36. **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.
37. **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.
38. **Vollmer, S. J., and C. Yanofsky.** 1986. Efficient cloning of genes of *Neurospora crassa*. Proc. Natl. Acad. Sci. USA **83**:4869–4873.