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Novel Mutation Causing Derepression of Several Enzymes of Sulfur Metabolism in *Neurospora crassa*

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A group of enzymes of sulfur metabolism (arylsulfatase, cholinesulfatase, and a number of others) are normally repressed in *Neurospora crassa* by an abundant supply of a "favored" sulfur source such as methionine or inorganic sulfate. A mutant called *scon*^{c} was isolated in which the formation of each of these enzymes is largely or completely nonrepressible. The structural genes for three of these enzymes have been mapped; *scon*^{c} is not linked to any of them. It is also not linked to *cys-3*, another gene which is involved in control of the same group of enzymes. Two alleles of the structural gene for arylsulfatase [*ars*$^+$ and *ars(UFC-220)*] produce electrophoretically distinguishable forms of arylsulfatase. Heterokaryons with the constitution *scon*^{c} *ars*$^+$ + *scon*$^+$ *ars(UFC-220)* were prepared. These heterokaryons produce both forms of arylsulfatase under conditions of sulfur limitation, but produce only the wild-type (*ars*$^+$) form under conditions of sulfur abundance. When the alleles of *ars* and *scon* are in the opposite relationship, only the *ars(UFC-220)* form of arylsulfatase can be detected under conditions of sulfur abundance. Thus the effect of the *scon*^{c} mutation seems to be limited to its own nucleus. The implications of these findings are discussed.

In *Neurospora*, a number of sulfohydrolases and permeases appear to serve the organism by providing it with a sulfur source. The formation of these proteins is strongly repressed by "favored" sulfur sources, such as methionine or inorganic sulfate, and is derepressed during sulfur starvation or limitation by a less readily used sulfur source such as cysteic acid (23). Mutations at the *cys-3* (cysteine) locus eliminate the formation of most or all of these enzymes (15, 16, 19, 26, and G. A. Marzluf, *personal communication*). In our laboratory, we have concentrated our attention on arylsulfatase because it is extremely easy to assay and because its range of activity from full repression to full derepression is as much as 1,000-fold, which is unusual among eukaryote systems (19). The structural gene for arylsulfatase (*ars*) has been defined by mutations that eliminate only the activity of this enzyme (*ars*) and by an allele which gives rise to an electrophoretically distinguishable form of the enzyme, *ars(UFC-220)* (19, 21, 22). This gene is in linkage group VII and is therefore on a different chromosome from *cys-3*, which is on the left arm of linkage group II (19). Likewise, Marzluf (15) has mapped what are probably the structural genes for two distinct forms of sulfate permease, which function maximally at two different stages in the life cycle of the mold. These genes, *cys-13* and *cys-14*, map in linkage groups I and IV, respectively, and are therefore unlinked to *ars*, to *cys-3*, and to each other.

Heterokaryons can be constructed in which *cys-3* is present in one nuclear type and *cys-3*$^+$ in the other. Under conditions that allow derepression of wild type, such heterokaryons produce arylsulfatase, cholinesulfatase, and, presumably, the rest of the enzymes that are normally regulated together (16). If the heterokaryon is so arranged that the *cys-3* component carries an allele for one electrophoretic form of arylsulfatase and the *cys-3*$^+$ component carries an allele for the other form, both forms of arylsulfatase are made by the heterokaryon under conditions of sulfur limitation (21). We have interpreted this, along with

other evidence, to mean that the product of the *cys-3*$^+$ gene is needed to turn on the synthesis of the several enzymes. The *cys-3*$^+$ product might be made in the common cytoplasm and act in the cytoplasm; or it might be made in the common cytoplasm and transported into both types of nuclei, where it could exert its effect. Conversely it might be made in the *cys-3*$^+$ nuclei but be transported into the cytoplasm for use, or it could even be made and used in the *cys-3*$^+$ nuclei, with part of it reaching the *cys-3* nuclei via the common cytoplasm. The only mechanism that can be excluded is that the *cys-3*$^+$ product is made solely for local use in its own nucleus. It would be misleading to say that *cys-3*$^+$ is "*trans*-dominant" over *cys-3*, as the two alleles in the heterokaryon are separated from one another by two nuclear membranes.

In this paper, we report the isolation of a new kind of mutant called *scon*^{c} which is, in many respects, the obverse of *cys-3*. All of the enzymes that fail to be made in *cys-3* are, in the mutant, almost completely nonrepressible by methionine or inorganic sulfate, or by both together; hence the name *scon* which stands for "sulfur controller" and which we pronounce "ess-con" *scon*^{c} is not linked to *ars*, *cys-13*, *cys-14*, *cys-3*, or *eth-1*^{r} (ethionine-resistant) (13, 18, 23), nor closely linked to any other known gene governing sulfur metabolism.

Heterokaryons have been constructed containing *scon*^{c} *ars*$^+$ nuclei and *scon*$^+$ *ars*(*UFC-220*) nuclei, where *scon*$^+$ is the wild-type allele for repressible synthesis of arylsulfatase. Under conditions of sulfur limitation, both forms of arylsulfatase are, of course, made. When the heterokaryon is grown with excess methionine, sulfate, or both, only the *ars*$^+$ arylsulfatase is made, insofar as we can determine. Conversely, if the alleles are arranged so that *scon*^{c} and *ars*(*UFC-220*) are in coupling, only the *ars*(*UFC-220*) arylsulfatase is made under conditions of excess sulfur. It appears that, in addition to the diffusable product of the *cys-3*$^+$ gene, there is a nucleus-restricted control element produced by the *scon* gene. We postulate the existence of a normal allele (*scon*$^+$) that can mutate to a form (*scon*^{c}) that gives nonrepressible or constitutive synthesis of arylsulfatase and its allied enzymes.

MATERIALS AND METHODS

Strains. Wild-type strains used in this study were the Oak Ridge stocks, 74-OR8-1a and 74-OR23-1A (Fungal Genetics Stock Center no. 988 and 986, respectively). These strains of the two mating types are essentially isogenic.

All of the strains except *scon*^{c} have been described previously (21). Those that originated in this laboratory were isolated in the Oak Ridge genetic background. The rest of the strains were kindly furnished by the Fungal Genetics Stock Center, Humboldt State College, Arcata, Calif. These were outcrossed to the Oak Ridge wild types a number of times (usually five) to reduce the variability of genetic background, especially at the loci for heterokaryon compatibility. Except where otherwise specified, the *scon*^{c} strain used was always the same reisolate R136-a (*see below*). The nuclear ratio in heterokaryotic cultures was determined by plating conidia on selective media as described by Atwood and Mukai (1).

Media for growth and for crossing, temperatures, details of harvesting, etc., have all been described previously (21). All cultures for enzyme assays were grown on Fries' minimal medium lacking the usual sulfur source and supplemented with sulfur sources as indicated in the tables. Growth was at 25 C as described by Metzenberg (18) for the length of time indicated in the tables. The carbon source was 1.5% sucrose except where otherwise indicated.

Electrophoresis. Electrophoresis of crude extracts of *Neurospora* heterokaryons and homokaryons and the qualitative and quantitative assay of the two forms of arylsulfatase were performed as described by Metzenberg and Ahlgren (19, 21).

Preparation of substrates. *p*-Nitrophenyl ^{35}S-sulfate was prepared as described by Metzenberg and Ahlgren (20).

^{35}S - adenosine - 3 - phosphate - 5' - phosphosulfate (^{35}S-PAPS) was prepared enzymatically by use of an extract of *Chlorella*, essentially as described by Hodson and Schiff (11). The strain of *Chlorella pyrenoidosa* was a stock strain used in the Wisconsin Water Resources Laboratory and was kindly furnished by George P. Fitzgerald. The incubation mixture included, in a volume of 10 ml: 300 μmoles of adenosine triphosphate (ATP); 250 μmoles of $MgCl_2$; 800 μmoles of tris(hydroxymethyl)aminomethane-(Tris)-hydrochloride buffer, *p*H 9.0; 250 μmoles of β-mercaptoethanol; 150 μmoles of $Na_2{}^{35}SO_4$ (0.3 mCi); and 6 ml of dialyzed extract of *C. pyrenoidosa*. The mixture was incubated under N_2 for 10 hr at 37 C, and the reaction was then terminated by heating for 3 min at 100 C. Denatured protein was removed by centrifugation, and the volume was then adjusted to 70 ml with distilled water. The ^{35}S-PAPS which had been formed was purified by column chromatography by using a system similar to one which was used for other purposes by Smith and Khorana (28). Diethylaminoethyl (DEAE)-Sephadex A-25 was converted to the HCO_3^- form by washing with 1 M $KHCO_3$ and was then washed with distilled water. The washed material was packed in a chromatography column (1.6 cm diameter) to give a bed height of 26 cm and was then equilibrated with 2 mM triethylamine bicarbonate (TEA-HCO_3) buffer, *p*H 7.2. The diluted reaction mixture was applied to the column which was then eluted with a linear gradient formed with 1,000 ml of 2 mM TEA-HCO_3 and 1,000 ml of 500 mM TEA-HCO_3. Fractions of approxi-

mately 10 ml were collected at a flow rate of 100 ml/hr. Unreacted $^{35}SO_4^{2-}$ was eluted between 200 and 300 ml. Fractions containing ^{35}S-PAPS, which eluted between 850 and 950 ml, were pooled and taken to dryness on a rotary evaporator. The matieral was dissolved in distilled water, taken to dryness a second time, and finally dissolved in 7.0 ml of distilled water.

Assays. Cell-free extracts were prepared as described by Metzenberg (18) by using the appropriate extraction buffer described in the individual reference for each enzyme. The following enzymes were measured by methods used and described previously. The systematic name, Enzyme Commission number, and relevant references are given after the trivial name of each enzyme: arylsulfatase [aryl-sulfate sulfohydrolase EC 3.1.6.1 (18)]; cholinesulfatase [choline-*O*-sulfate sulfohydrolase EC 3.1.6 group (23)]; γ-cystathionase [L-homoserine hydro-lyase (deaminating) EC 4.2.1.15 (8)]; invertase [β-D-fructofuranoside fructohydrolase EC 3.2.1.26 (17)]; isocitrate lyase [L-isocitrate glyoxylate-lyase EC 4.1.3.1 (6)]; alkaline phosphatase [orthophosphoric monoester phosphohydrolase EC 3.1.3.1 (24)].

Sulfate uptake by the sulfate permeases of mycelial and conidial preparations was assayed as described by Marzluf and Metzenberg (16). The choline sulfate permease of intact mycelia was measured by a similar procedure except that 2 mM ^{35}S-choline-*O*-sulfate was used instead of inorganic sulfate (G. A. Marzluf, *personal communication*). The methionine-specific permease was measured by following the uptake of 0.03 mM ^{14}C-L-methionine in the presence of 100 mM glycine as described by Pall (26).

S-Adenosylmethionine (SAM) synthetase (ATP: L-methionine *S*-adenosyltransferase EC 2.4.2.13) was assayed by a modification (E. Jacobson, *personal communication*) of the incubation system described by Cantoni and Durrell (4). The complete reaction mixture contained, in a volume of 1.0 ml: 10 μmoles of $MgCl_2$; 200 μmoles of KCl; 25 μmoles of *N*-2-hydroxyethylpiperazine-*N'*-2-ethansulfonic acid-KOH buffer, *p*H 7.4; 1.25 μmoles of ATP-8-^{14}C (0.05 μCi); 20 μmoles of L-methionine; and 0.1 ml of cell extract. Tubes were incubated for 30 min at 25 C, and the reaction was then terminated by the addition of 1.0 ml of absolute ethanol. The *S*-adenosylmethionine formed was isolated by the Dowex-50 (Na^+) column procedure of Shapiro and Ehninger (27). Samples of the 6 N HCl fraction were evaporated to dryness and dissolved in 1.0 ml of water, and radioactivity was determined by liquid scintillation counting.

Choline sulfotransferase (3'-phosphoadenylylsulfate: choline sulfotransferase EC 2.8.2 group) was assayed by a procedure similar to that of Kaji and Gregory (12). The complete reaction mixture contained, in a volume of 0.6 ml: 10 μmoles of potassium phosphate buffer, *p*H 7.2; 2.5 μmoles of $MgCl_2$; 2.5 μmoles of cysteine hydrochloride; 10 μmoles of choline chloride; 0.25 μmole of ^{35}S-PAPS (0.42 μCi); and 0.1 ml of cell extract. Tubes were incubated for 20 min at 37 C, and the reaction was then terminated by heating the tubes for 2 min in a boiling water bath. The radioactive choline sulfate formed was separated from excess ^{35}S-PAPS on columns of Dowex-1(Cl^-) (12), and radioactivity in the product was determined by liquid scintillation counting.

The protein content of cell extracts was determined by the method of Lowry et al. (14), with bovine serum albumin as the standard.

Specific activities of all enzymes are expressed as nanomoles per minute per milligram of protein.

RESULTS

Isolation of scon^c mutants. Conidia of the colonial temperature-sensitive strain *cot-1-a* (essentially isogenic with the Oak Ridge wild-type strains) were suspended in water at a density of 1.8×10^6/ml and irradiated with an ultraviolet sterilamp to about 50% killing. The suspension was diluted 100-fold into a solution of methionine and K_2SO_4 (80 mM and 200 mM, respectively), and samples (0.5 ml) were plated onto modified medium P (19). The plates were incubated at 33 C for 2 days and were treated with indoxyl sulfate as described in the above reference. Four blue colonies were observed among about 250,000 total colonies. These were picked to BC salts without K_2HPO_4 (19) with 1.5% sucrose and 5 mM methionine. Three of the four cultures proved to be derepressed. We refer to these as *scon^c* mutants. Conidia from these cultures were replated on modified medium P, and after two days the colonies were treated with indoxyl sulfate as usual. Well-stained colonies from each of the original isolates were again picked, allowed to grow, and replated. This process was repeated twice more to insure that the strains were homokaryotic. The resulting cultures were crossed to 74-OR23-1A. All of the crosses gave many colorless spores (about 50%) which failed to germinate. Germinated sporelings (about 20 from each cross) were picked to BC salts plus sucrose, supplemented with methionine and K_2SO_4 (each 5 mM). To our initial surprise, all of the viable progeny from all of the *scon^c* mutants were found to be repressible. One of the *scon* strains was arbitrarily chosen as the "type" strain, and all of the work subsequently reported in this paper was done with this mutant (referred to simply as *scon^c cot-1-a*).

Isolation of scon^c from a cross: nonlinkage to cys-3, cys-13, and cys-14. The failure of the trait of nonrepression to be transmitted with normal frequency in a cross left open the possibility that oversynthesis of the enzymes of sulfur anabolism might cause lethality in some stage of meiosis or spore maturation. It seemed possible that a cross to *cys-3* might

favor recovery of viable spores bearing *scon*^{c}. Although further experience has not borne out this hope, the first successful recovery of *scon*^{c} as a meiotic product was from such a cross. Reciprocal crosses were made on Westergaard-Mitchell slants with 1 mM methionine, in which *scon*^{c} *cot-1-a* and *cys-3-A* were both used as female and as male parents. The recovery of *cys-3* progeny from any kind of cross is always low or very low, and, as we have noted, the recovery of *scon*^{c} is very low, so it was not surprising that there were many pale nonviable spores from both of these crosses. However, it is significant that many spores (about 25%) did germinate, and almost all of these were fully repressible prototrophs, suggesting that the two genes are not linked. It is clear that these prototrophs are not the double mutant, *cys-3 scon*^{c}; strains of this constitution are phenotypically indistinguishable from *cys-3* (Dietrich and Metzenberg, *manuscript in preparation*).

From the cross *cys-3-A* ♀ × *scon*^{c} *cot-1-a* ♂, 48 viable *cys-3*$^+$ sporelings were examined. None was *scon*^{c}. Exactly half of the cultures were *cot-1*, suggesting that *scon* is also unlinked to that gene. This is of some interest, since *cot-1* is linked to *cys-14* (15). In the reciprocal cross, in which *scon*^{c} *cot-1-a* was the female parent, 43 *cys-3*$^+$ sporelings were picked, and the cultures were examined. Forty-two of the cultures were *scon*$^+$. Twenty-seven were *cot-1*, and 22 were mating type A, again showing that *scon* is not perceptibly linked to either *cot-1* or to mating type; [*cys-13* is quite closely linked to mating type (15)]. The sole *scon*^{c} isolate was non-*cot* and mating type a. It will be referred to henceforth as *scon*^{c}*R136-a*. Plating out of conidia of this strain and staining in the usual fashion with indoxyl sulfate showed that it was homokaryotic for the derepression trait and stable in vegetative culture. To obtain *scon*^{c} in the opposite mating type, the R136-a isolate was crossed to wild type (74-OR23-1A). Of 100 sporelings that were picked and tested, one proved to be nonrepressible, and it was of the desired mating type. It will be called *scon*^{c}*R212-A*.

It should be remarked that the failure of the *scon*^{c} trait to be transmitted with high frequency even when *scon*^{c} is the female parent seems to exclude the possibility of maternal inheritance. The failure of the trait to be transmitted by heterokaryosis (*see below*) is also in harmony with this interpretation.

Nonlinkage of scon with ars. *scon*^{c}*R212-A* was crossed by simultaneous inoculation to a strain with two markers on linkage group VII, *nic-3*, *ars(101)*. Random ascospores were heat-shocked and plated on minimal medium plus nicotinamide (2 μg/ml). One hundred forty-one sporelings were picked to BC medium with nicotinamide and with 5 mM methionine as the sulfur source, and the resulting cultures were tested as usual with a drop of *p*-nitrophenyl sulfate. None gave any color, showing that every culture was either *scon*$^+$, or *ars*, or both. Transfers were made to a nonrepressing medium (BC plus nicotinamide plus 1 mM cysteic acid), and after the cultures had grown up they were spot-tested for arylsulfatase. There were 71 *ars*$^+$ cultures and 70 *ars* cultures. There were 17 *nic*, *ars*$^+$ recombinants and 27 *nic*$^+$, *ars* recombinants, values which were consistent with our previous mapping of the *ars* locus (19). *ars scon*^{c} recombinants, if they occurred, would not have been distinguished from *ars scon*$^+$ parentals in this experiment. However, the high frequency (50%) of *ars*$^+$ *scon*$^+$ cultures argues that the two genes could not be closely linked.

Isolation of other derivatives of scon^c. In the process of mapping *scon*, we have crossed it to a number of markers in all seven linkage groups of *Neurospora*. The data are rather complex because of the very low viability of *scon*^{c} spores, and they will be presented in detail elsewhere (Totten and Metzenberg, *unpublished data*). Suffice it here to say that the *scon* locus appears to be far out on the right arm of linkage group V, beyond *hist-6*, and is not closely linked to any previously recognized gene that affects the metabolism of sulfur compounds. As a by-product of our efforts to map *scon*, some useful auxotrophic and other derivatives of it were obtained. These allowed us to make heterokaryons containing nutritional forcing markers, such as *pdx-1* (pyridoxine), as well as *al-2* (albino) and *act-2* (actidione resistance).

To obtain *scon*^{c} carrying a different electrophoretic variant of arylsulfatase, we crossed *scon*^{c}*R136-a* to *ars(UFC-220)R1001-A* and plated heat-shocked ascospores as in the original isolation of *scon*^{c}. After staining the plates with indoxyl sulfate, we picked 15 blue colonies. Fourteen of them carried the *ars*$^+$ allele, and one was the desired strain, *scon*^{c} *ars(UFC-220)-A*. We felt that the lopsided segregation ratio reflected the extremely low viability of spores of the desired type—much lower even than *scon*^{c} *ars*$^+$—rather than some previously undetected linkage between the two genes. To test this, we outcrossed the double mutant to an *scon*$^+$ *ars*$^+$ strain. The cross was of un-

usually low fertility, even for crosses containing *scon*^{c}, but, among 29 *scon*$^+$ progeny that were examined, 12 were *ars*(*UFC-220*). Two *scon*^{c} colonies were obtained by visual screening of indoxyl sulfate-stained plates; these were picked and cultures of them were prepared. Both proved to carry the *ars*$^+$ allele. Thus the original 14:1 ratio of segregants is best explained by differential viability, not by linkage.

We desired an auxotrophic derivative of *scon*^{c} *ars*(*UFC-220*) for preparing forced heterokaryons and attempted to make one by crossing to a number of auxotrophs. Because of the very low recovery of the double mutant from ascospores, we failed repeatedly. As a last resort, we mutagenized and selected an auxotrophic derivative of *scon*^{c} *ars*(*UFC-220*)-*A*. Conidia of this strain suspended in water were irradiated with a germicidal lamp to about 70% killing. From the irradiated suspension, we selected a good, nonleaky arginine mutant by the filtration technique of Woodward et al. (31). This strain will be referred to as *scon*^{c} *ars*(*UFC-220*) *arg-X-A*, since we have not made any attempt to map the mutation to arginine auxotrophy.

Derepression of arylsulfatase in scon^c. The isolation procedure which was used to obtain *scon*^{c} mutants depends on the qualitative observation of hydrolysis of *p*-nitrophenylsulfate by intact cells grown in the presence of both high methionine and high sulfate concentrations. Table 1 presents quantitative data on the lack of repression of arylsulfatase by methionine plus sulfate in *scon*^{c}. In this particular experiment *scon*^{c} produced 40% as much arylsulfatase in the presence of sulfate plus methionine as it did when grown on cysteic acid. In numerous other similar experiments we have observed levels of arylsulfatase in *scon*^{c} grown on high methionine or high sulfate or both, ranging from 15 to 100% of the levels obtained when growth is on cysteic acid. By contrast, arylsulfatase in wild type is invariably repressed several 100- to 1,000-fold on either high sulfate or high methionine. We have been unable to observe any consistent pattern in this variability of arylsulfatase enzyme level in *scon*^{c} on high sulfate or methionine media, but the degree of repression control retained by the mutant, if any, is slight compared to that of wild type.

TABLE 1. *Levels of arylsulfatase in mycelia of wild type and scon^c*

Strain[a]	Sulfur source (mM)	Specific activity[b]
Wild type	Cysteic acid (1)	9.2
	L-Methionine (5), K_2SO_4 (2)	0.03
scon^c	Cysteic acid (1)	7.3
	L-Methionine (5), K_2SO_4 (2)	2.8

[a] Cultures were grown for 12 hr.

[b] Expressed as nanomoles per minute per milligram of protein.

Generalized derepression in scon^c of the same group of enzymes not produced by cys-3. The activities of enzymes known to be both regulated in wild type by the sulfur source available for growth and absent from *cys-3* (15, 16, 26) were determined in *scon*^{c} under conditions suitable for their repression and derepression in wild type. Wild-type and *cys-3* values obtained under the same growth conditions are included for comparison. Table 2 shows that *scon*^{c} produces essentially derepressed levels of each enzyme under all conditions tested. Arylsulfatase, cholinesulfatase, and choline sulfate permease are repressed in wild type by either high methionine or high sulfate, whereas in *scon*^{c} these enzymes are present at derepressed levels even when both high methionine and high sulfate are present during growth. Sulfate permease cannot be assayed reliably when sulfate is included in the growth medium, but high methionine, which represses wild type, does not strongly repress this activity in *scon*^{c} mycelia.

The predominant form of sulfate permease in conidia differs from the form that predominates in mycelia (15). Table 3 shows that this conidial sulfate permease is likewise not strongly repressible in *scon*^{c}. The methionine permease cannot be assayed when methionine is included in the growth medium. In addition we find that this activity is not repressible by sulfate in young, rapidly growing wild-type mycelia. However when 2- to 3-day-old mycelia are used as originally described (26), methionine permease in wild type is 25-fold repressed by high sulfate (Table 2). When *scon*^{c} is grown on high sulfate, it produces the methionine permease at about the same level as is found in wild type derepressed by growth on limiting sulfate. When *scon*^{c} is grown on limiting sulfate it produces even higher levels (about fourfold) of the permease.

Activity of three other sulfur-related enzymes in wild type, scon^c, and cys-3. Table 4 lists the specific activities of γ-cystathionase, SAM synthetase, and PAPS:choline sulfotransferase in wild type, *scon*^{c}, and *cys-3*. These enzymes had not previously been shown to be present in *cys-3*. γ-Cystathionase and

TABLE 2. *Levels of arylsulfatase, cholinesulfatase, sulfate permease, choline sulfate permease, and methionine permease in mycelia of wild type, scon[c] and cys-3 grown on several sulfur sources*

Strain[a]	Sulfur source (mM)	Specific activity[c]				
		Aryl-sulfatase	Choline-sulfatase	Sulfate permease	Choline sulfate permease	Methionine permease
Wild type	Cysteic acid (1)	9.8	0.614	3.02	7.69	2.6
	L-Methionine (0.25)	6.5	0.393	4.78		
	L-Methionine (5)	0.05	0.018	0.13	0.103	
	K_2SO_4 (2)	0.04	0.017		0.470	2.0
	L-Methionine (5) + K_2SO_4 (2)	0.03	0.014		0.095	
	K_2SO_4 (0.025)[b]	72.5				0.59
	K_2SO_4 (2)[b]	0.05				0.023
scon[c]	Cysteic acid (1)	8.9	0.292	2.84	6.81	2.4
	L-Methionine (0.25)	6.5	0.391	4.37		
	L-Methionine (5)	6.3	0.396	2.64	3.95	
	K_2SO_4 (2)	6.6	0.248		5.42	4.0
	L-Methionine (5) + K_2SO_4 (2)	7.1	0.176		2.38	
	K_2SO_4 (0.025)[b]	76.0				1.6
	K_2SO_4 (2)[b]	12.3				0.39
cys-3	L-Methionine (0.25)	<0.05	<0.01	<0.4	0.14	
	L-Methionine (5)	<0.05	<0.01	<0.05	0.08	
	L-Methionine (0.05)	<0.05				0.012
	L-Methionine (0.10)[b]	<0.05				0.0035

[a] Cultures were grown for 13 hr except where otherwise indicated.
[b] Cultures were grown for 60 hr.
[c] Expressed as nanomoles per minute per milligram of protein.

SAM synthetase were expected to be present in this strain, however, because both of these enzymes are presumably necessary for growth on methionine as sole sulfur source. The PAPS:choline sulfotransferase was of interest since choline sulfate is thought to be a major storage form of sulfate in *Neurospora* and certain other fungi (12, 25, 29). *cys-3* would presumably be unable to utilize any choline sulfate formed by this enzyme. The results (Table 4) show that PAPS: choline sulfotransferase levels are affected relatively little by the sulfur source and are present at similar levels in all three strains tested except that they may be slightly lower in *scon[c]*. The γ-cystathionase is derepressed in wild type by growth on limiting sulfur sources (9), and we observed a 16-fold derepression of this enzyme in wild type grown for 2 days on limiting sulfate. This agrees well with a previous report (9). The effect is substantial only under conditions of prolonged sulfur starvation. The two mutant strains differ from wild type in the control of this enzyme: *scon[c]* appears to have derepressed levels of γ-cystathionase under all conditions tested, whereas *cys-3* is unable to respond to sulfur starvation by increasing the level of this enzyme even though young cultures of *cys-3* appear to have levels of the enzyme similar to wild type.

TABLE 3. *Levels of sulfate permease in conidia of wild type, scon[c], and cys-3*

Strain[a]	L-Methionine (mM)	Specific activity of sulfate permease[b]
Wild type	0.25	1.28
	5.0	0.024
scon[c]	0.25	0.93
	5.0	1.14
cys-3	0.25	<0.01
	5.0	<0.005

[a] Conidia were harvested from plates of Fries' minimal medium, supplemented with L-methionine as indicated, after 7 days of growth at 25 C.
[b] Expressed as nanomoles per minute per milligram of protein.

Regulation in scon[c] of enzymes unrelated to sulfur metabolism. The results presented in Table 5 show that three enzymes which are

TABLE 4. *Levels of γ-cystathionase, S-adenosylmethionine synthetase and PAPS: choline sulfotransferase in mycelia of wild type, scon^c, and cys-3 grown on several sulfur sources*

Strain	Sulfur source[a] (mM)	Specific activity[c]		
		γ-Cystathionase	SAM synthetase	PAPS: choline sulfotransferase
Wild type	Cysteic acid (1)	2.78	0.484	0.116
	L-Methionine (0.25)	1.74	0.767	
	L-Methionine (5)	0.89	1.30	0.0704
	K_2SO_4 (2)	0.62	0.746	0.0794
	L-Methionine (0.1)[b]	7.2		
	L-Methionine (5)[b]	0.44		
scon^c	Cysteic acid (1)	4.9	0.252	0.0672
	L-Methionine (0.25)	6.0	0.953	
	L-Methionine (5)	6.6	1.48	0.0336
	K_2SO_4 (2)	5.6	0.849	0.0582
	L-Methionine (0.1)[b]	8.5		
	L-Methionine (5)[b]	7.0		
cys-3	L-Methionine (0.25)	0.94	0.483	0.116
	L-Methionine (5)	1.23	1.55	0.0823
	L-Methionine (0.1)[b]	1.24		
	L-Methionine (5)[b]	0.58		

[a] Cultures were grown for 13 hr except where otherwise indicated.
[b] Cultures were grown for 48 hr.
[c] Expressed as nanomoles per minute per milligram of protein.

TABLE 5. *Control of invertase, isocitrate lyase, and alkaline phosphatase levels in wild type and scon^c*

Strain	Sulfur source (mM)	Specific activity					
		Invertase[a]		Isocitrate lyase[b]		Alkaline phosphatase[c]	
		75 mM Galactose	75 mM Mannose	Acetate	Sucrose	0.05 mM Phosphate	7.3 mM Phosphate
Wild type	Cysteic acid (1)	51	2.9	173	9.4		
	L-Methionine (5)	52	3.2	222	16.3	173	3.2
scon^c	Cysteic acid (1)	28	2.5	151	4.5		
	L-Methionine (5)	50	4.3	162	8.4	84	2.5

[a] Cultures were grown as described by Metzenberg (17) on the carbon sources indicated.
[b] Cultures were grown and induced as described by Flavell (6).
[c] Cultures were grown on Fries medium minus both sulfate and phosphate and supplemented as indicated. Growth was for 14 hr.

subject to repression and derepression, but are unrelated to sulfur metabolism (invertase, isocitrate lyase, and alkaline phosphatase) are under normal regulatory control in *scon^c*. Thus *scon^c* does not result in a general loss of all repression systems.

Preparation and properties of the heterokaryons. The homokaryons used for preparing heterokaryons were: 1, *scon$^+$ ars(UFC-220) tryp-3-a;* 2, *scon$^+$ ars(UFC-220) thi-3, thr-1-a;* 3, *scon$^+$ ars$^+$ tryp-3-A;* IV, *scon^c* ars$^+$ *pdx-1 act-2^r-a;* V, *scon^c ars(UFC-220) arg-X-A.* From these, heterokaryons 1 + IV, 2 + IV, and 3 + V were prepared by inoculating the pairs of strains into a minimal medium (BC with 5 mM K_2SO_4 as the sulfur source). The resulting nutritionally forced heterokaryons were transferred to flasks of Fries' minimal agar and allowed to grow for about 5 days at 25 C. The conidia from these flasks were used as inocula for flasks of liquid media as previously described (18). In each case the flasks were sparged with sterile air at 25 C for 16 hr. "Derepression medium" is Fries' medium without sulfur (that is, the $MgSO_4$ has been replaced with an equimolar amount of $MgCl_2$), to which

is added 1 mM cysteic acid. "Repression medium" is Fries' medium without sulfur, plus 5 mM K_2SO_4 and 5 mM L-methionine. (In the interest of brevity, we omit the results obtained with *only* 5 mM sulfate and *only* 5 mM methionine as sulfur sources. They were essentially indistinguishable from the results obtained when both were present.) Just before the mycelia were harvested, a sample was removed aseptically and pipetted onto Fries' minimal agar and allowed to conidiate. The conidia from these cultures were used in an attempt to estimate the ratio of the two nuclear types in each heterokaryon. Extracts of the mycelia were subjected to electrophoresis. Strips were stained with indoxyl sulfate to make the position of the bands of arylsulfatase activity visible. Duplicate strips were exposed to *p*-nitrophenyl ^{35}S-sulfate in the presence of barium ions, and the precipitated $Ba^{35}SO_4$ was detected by autoradiography. The bands were excised and counted as described previously (21). Some quantitative measurements on the heterokaryons are described in Table 6.

The interpretation of the results of indoxyl sulfate staining of strips appears very clear. In extracts from cultures grown on cysteic acid, both bands of arylsulfatase are obvious and usually of roughly equal intensity. In extracts from cultures grown on sulfate plus methionine, only the arylsulfatase form coded by the *scon*^{c} moiety was visible (Fig. 1). (Again, identical results were seen with strains grown on sulfate only, or methionine only, as sulfur sources.) The picture seen on the autoradiograms was qualitatively the same, but, perhaps because of a tendency of the enzyme to spread during the incubation with radioactive substrate, the bands appeared less sharp. In addition, the method is so much more sensitive than staining with indoxyl sulfate that one readily detects "trailing" of enzyme behind the main bands of both the fast (*ars*$^+$) and slow (*ars*(*UFC-220*)) components (Fig. 2). This is equally true of enzymes prepared from either homokaryon. Thus, the point at which one draws the line between "fast component" and "slow component" is somewhat arbitrary; and where it is drawn makes a big difference in

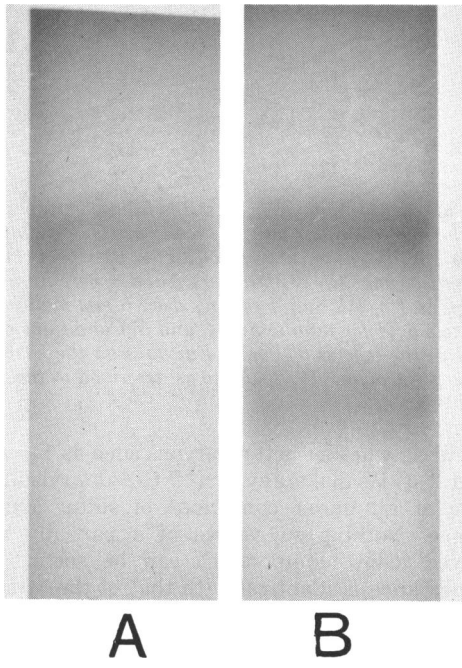

FIG. 1. *Electrophoretogram of arylsulfatase from heterokaryon 2 + IV (described in the text) which contains an scon*^{c} *ars*$^+$ *nucleus and an scon*$^+$ *ars(UFC-220) nucleus. Cultures were grown for 16 hr at 25 C in Fries' medium sans sulfur plus (A) 5 mM methionine and 5 mM* K_2SO_4*, and (B) 1 mM cysteic acid. Extracts were subjected to electrophoresis, and the strips were stained with indoxyl sulfate.*

TABLE 6. *Synthesis of "slow" and "fast" components of arylsulfatase in heterokaryons*

Strain[a]	Sulfur source	Arylsulfatase (total specific activity)	Heterokaryotic conidia (per cent of total viable conidia)	Nuclear ratio
Het 1 + IV	Cysteic acid	11.9	44	
	SO_4^{2-}, methionine	2.15	31	
Het 2 + IV	Cysteic acid	12.5	47	
	SO_4^{2-}, methionine	1.69	48	
Het 3 + V	Cysteic acid	6.65	37	34% component V(*scon*^{c})
	SO_4^{2-}, methionine	0.62	38	45% component V(*scon*^{c})
ars$^+$ (homokaryon)	Cysteic acid	28.2	0	100% *ars*$^+$
ars(*UFC-220*) (homokaryon)	Cysteic acid	15.0	0	100% *ars*(*UFC-220*)

[a] The identity of the heterokaryons (Het), each of which contains an *scon*$^+$ nucleus (Arabic numeral) and an *scon*^{c} nucleus (Roman numeral) are described in the text.

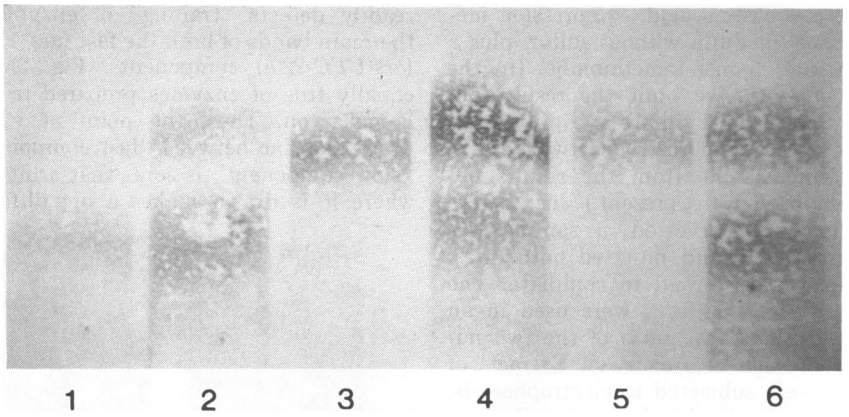

FIG. 2. *Autoradiogram of strips from electrophoresis of arylsulfatase from heterokaryons. The components of the heterokaryons were: 1, scon⁺ ars(UFC-220) tryp-3-a; 2, scon⁺ ars(UFC-220) thi-3, thr-1-a; 3, scon⁺ ars⁺ tryp-3-A; IV, sconᶜ ars⁺ pdx-1 act-2ʳ-a; V, sconᶜ ars(UFC-220) arg-X-A. Strips numbers 1 and 2 were from heterokaryons 3 + V; numbers 3 and 4 were from heterokaryons 2 + IV; numbers 5 and 6 were from heterokaryons 1 + IV. Sulfur sources were 5 mM methionine plus 5 mM K_2SO_4 for numbers 1, 3, and 5, and 1 mM cysteic acid for numbers 2, 4, and 6. Conditions of growth and electrophoresis were as described in the text and in the legend of Fig. 1. Enzymes on the strips were detected, and the relative amounts were estimated with p-nitrophenyl ^{35}S-sulfate as described in the text. The data corresponding to this figure are presented in Table 6.*

deciding whether heterokaryons such as 1 + IV and 2 + IV make any *ars(UFC-220)* arylsulfatase at all under conditions of sulfur abundance. Nothing suggestive of a partially resolved "slow component" can be seen; the appearance is identical with that of the "trailing" of activity that is seen in an *ars⁺* homokaryon. Because of the "trailing" and the arbitrariness about where to draw the line, it is rather difficult even to set a quantitative upper limit for the proportion of this "slow component."

The case of the third heterokaryon, 3 + V, in which the *sconᶜ* and *ars(UFC-220)* alleles are in the same nucleus, affords a cleaner test. Under conditions of sulfur abundance, it is the "fast component," coded by *ars⁺*, which is repressed. For the "slow component" to interfere in this test, it would have to trail ahead, rather than behind. Apparently this does not happen to any appreciable extent. In the electrophoretogram in question, no net counts at all can be detected in the area in which the "fast component" should be found. Indeed, there are slightly fewer counts than the background obtained with an equivalent area of electrophoretogram that had never been exposed to enzyme. In the culture of heterokaryon 3 + V grown on derepression medium, a well resolved "fast component" was obvious, and 22% of the total activity was in that component.

A puzzling phenomenon interfered with our attempts to determine the proportions of the two types of nuclei in heterokaryons 1 + IV and 2 + IV, as well as in a number of other *sconᶜ*-containing heterokaryons that we have prepared but have not discussed in this paper. After the heterokaryon had become established and been transferred a few times, it became impossible to recover *sconᶜ* homokaryons from it. Platings to supplemented media gave only a mixture of heterokaryotic colonies, which were prototrophic and only partly repressible, like the parent heterokaryon, and homokaryons that were identical with the original *scon⁺* moiety. It is as though the *sconᶜ* component rapidly picks up lethal mutations or, perhaps, chromosomal rearrangements or imbalances, and these lethal mutations are of selective value to this component when it is in a heterokaryon. Whatever the explanation, our inability to assess the frequency of *both* types of homokaryotic conidia as well as heterokaryotic conidia makes it impossible to apply the method of Atwood and Mukai (1). We have simply listed the proportion of the total viable conidia that gave rise to heterokaryotic, prototrophic colonies in each case. For some reason, this problem did not occur with heterokaryon 3 + V, from which both homokaryons could be readily resolved. Accordingly, we have presented nuclear ratios in this case, though with some trepidation, since we are not sure why this assessment failed in the other cases.

DISCUSSION

The *sconᶜ* mutation described here results in loss of repression control over several enzymes

involved in sulfur assimilation. All of the enzymes previously known to be controlled by the *cys-3* locus are shown here (Table 2) to be nonrepressible, or, at most, weakly repressible in *scon*^{c}. The *scon* locus is unlinked to *cys-3*, however, and is also unlinked to the putative structural genes, *ars*, *cys-13*, and *cys-14*, which specify three of the enzymes whose synthesis it affects. (Mutants lacking cholinesulfatase, choline sulfate permease, or methionine permease have not yet been observed, and consequently the location of the structural genes for these enzymes is not yet known.)

At first glance it might appear that *scon*^{c}, which acts in its own nucleus but seems to have no effect on its neighboring nuclei, is somehow analogous to the *cis*-dominance, *trans*-recessiveness of operator or promoter mutations in prokaryotes. The facile use of "cis" and "trans" in referring to processes in a heterokaryon is more deceptive than enlightening. "*Cis*-dominance-*trans*-recessiveness" in a diploid generally indicates that the gene that exhibits this property, such as an operator or a promotor, does not yield a diffusable product. It should be noted that *scon*^{c} cannot be similar to an operator-constitutive mutation in the *lac* operon of *Escherichia coli*, because it affects genes that are unlinked to it and to one another. Hence, the *scon* gene probably forms a product which is diffusible, albeit not through the cytoplasm. Since we have not yet obtained diploids or disomics, we have no evidence as to whether the *scon*$^+$ gene product functions by turning on the structural genes when sulfur compounds are limiting or by turning them off when sulfur compounds are abundant.

The apparent loss of control over γ-cystathionase in *scon*^{c} and the noninducibility of this enzyme in *cys-3* (Table 4) require special comment. It was reasonable to expect that *cys-3* would possess γ-cystathionase since growth on methionine as sole sulfur source is thought to require the action of this enzyme in formation of cysteine. As seen in Table 4, *cys-3* does seem to produce a basal level of γ-cystathionase but is unable to make significantly higher levels of the enzyme under conditions that are sufficient to derepress wild type 15-fold. Since the other enzymes found to be nonrepressible in *scon*^{c} (Table 2) are either undetectable or present at the lower limits of detectability in *cys-3*, it is worth considering the possibility that the inducible "γ-cystathionase" activity is actually due to an enzyme different from the basal γ-cystathionase activity. This postulated new enzyme might differ in substrate specificity or kinetic properties and might normally serve for utilization of some sulfur compound other than cystathionine (e.g. *S*-methylcysteine). It is perhaps significant that *S*-methylcysteine does not support growth of *cys-3*. It is also interesting to note that *S*-methylcysteine was not thought to be a substrate of γ-cystathionase (7) prior to studies on the enzyme from derepressed cultures (9). Alternatively the altered levels of γ-cystathionase in *scon*^{c}, or *cys-3*, or both, might be an indirect result of the altered control of other enzymes of sulfur metabolism.

Could the nonrepressibility of *scon*^{c} be the result of failure to produce effective levels of the true corepressor? It is clear that neither sulfate nor methionine is the true corepressor of arylsulfatase synthesis in *N. crassa*. Mutants blocked in early steps of sulfate assimilation such as *cys-2*, *cys-4*, *cys-5*, *cys-10*, and *cys-11* (all blocked before sulfide formation) are not repressible by sulfate although they are repressed by high levels of methionine (23). Similarly, the *eth-1*^{r} mutant, which is now thought to be defective in SAM formation (13, 22), is not repressible by methionine but is repressed by high levels of sulfate (23). The double mutant *cys-11*, *eth-1*^{r} resembles *scon*^{c} in being derepressed in the presence of both high sulfate and high methionine. (Unlike *scon*^{c}, this double mutant does not grow on sulfate alone.) The results of the heterokaryon studies (Table 6) argue against any model in which the *scon* locus is assumed to affect the level of some product of intermediary metabolism which functions as corepressor. Arylsulfatase production coded by nuclei containing the *scon*^{c} gene is not strongly repressed by the presence of *scon*$^+$ nuclei in the common cytoplasm, even though arylsulfatase production coded by the *scon*$^+$ nuclei is repressed by the sulfate and methionine present. Therefore, although the *scon* locus must produce some diffusible product in order to exert its influence on several unlinked genes, this product is not freely accessible to other nuclei. The data do not favor models in which the *scon* product is made in the common cytoplasm or made in the nucleus and transported into the common cytoplasm for use. Instead they are consistent with a model in which the *scon* product is made and used in the vicinity of its own nucleus, perhaps in its own nucleus.

It is impossible, at present, to rule out the possibility that, for some reason, *scon*^{c} nuclei tend to segregate themselves in one part of the mycelium and *scon*$^+$ nuclei in other parts. The only limitations we can place on this possibility are (i) that nutritionally forced heterokaryons between these two kinds of homokaryons do grow, and (ii) that platings of conidia

from such heterokaryons reveal that many of the conidia are heterokaryotic (see Table 6). We cannot say anything about how the gene would behave in a diploid in which *scon*^{c} and *scon*$^{+}$ were present in the same nucleus. Unfortunately, full diploids in *Neurospora* occur only as a single transient cell after karyogamy in mating, and disomics, though they can be prepared, are very unstable.

Valone, Case, and Giles (30) have recently described a regulatory gene in *N. crassa*, *qa-1*, which can exist in alternative mutant forms resembling either *cys-3* or *scon*^{c}. Mutation at the *qa-1* locus has a pleiotropic effect on the expression of three putative structural genes for three inducible enzymes involved in the catabolism of quinic acid. Mutants of the *qa-1* type are noninducible for all three enzymes, and most of them are recessive in heterokaryons. Thus they resemble *cys-3*. Mutants of the *qa-1*^{c} type are constitutive for all three enzymes and show various degrees of partial dominance in heterokaryons, thus resembling *scon*^{c}. In the case of quinic acid catabolism, however, no electrophoretic variants of the enzymes have been described. Therefore, it is not known whether partial dominance of the constitutive mutants in heterokaryons represents partially derepressed synthesis by both types of nuclei or fully derepressed synthesis by one type and no synthesis by the other. The *cys-3* and *scon* loci differ from the *qa-1* locus in at least one respect. The latter is closely linked to the genes it regulates, whereas *cys-3* and *scon* are unlinked to at least some of the genes they control and are not linked to one another. Furthermore, all revertants of *cys-3* which have been obtained so far have had normal repression control over synthesis of arylsulfatase. Despite these differences, the overall pattern of control may really be the same.

If the evidence is taken at face value that the *scon* product is limited to its own nucleus, it becomes of interest to inquire what the nature of that product might be. It is hard to imagine that it is not a macromolecule. A regulatory protein is an attractive possibility. It would not necessarily have to be synthesized in the nucleus proper; it could be made on the ribosomes of the outer surface of the nuclear envelope and transported directly into the nucleus. On the other hand, the final product of the *scon* gene could be a regulatory ribonucleic acid (RNA) molecule. Recently, it has become clear that much of the RNA that is made in the eukaryotic nucleus is degraded without ever leaving the nucleus (10). The suggestion has been made that much of this RNA may not be messenger at all, but may have a regulatory role (3). It will be interesting to see if other control mutants with the formal properties of *scon*^{c} are found in eukaryotes.

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