STRUCTURAL AND REGULATORY CONTROL OF ARYL SULFATASE IN NEUROSPORA: THE USE OF INTERSPECIFIC DIFFERENCES IN STRUCTURAL GENES

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SOME time ago, we reported that *cys-3* mutants of *Neurospora crassa* are unable to make aryl sulfatase and some other enzymes of sulfur anabolism (MARZLUF and METZENBERG 1968). We suggested that the pleiotropic-negative character of these mutants is best explained on the assumption that the *cys-3*$^+$ locus normally produces a regulatory macromolecule that is necessary for the expression of the various structural genes. The main basis for concluding that *cys-3* is a regulatory gene, and does not code for a polypeptide common to the several enzymes was that revertants of *cys-3*$^-$, including temperature-conditional strains, synthesize aryl sulfatase with normal physicochemical properties, including normal thermostability *in vitro* and *in vivo*. The grounds for believing that *cys-3*$^-$ mutants lack a positive regulator rather than being burdened with a super repressor of the structural genes were:

1. Revertants of *cys-3*$^-$ are all normally repressible by methionine and by inorganic sulfate. If *cys-3*$^-$ were a super repressor, most of the revertants would be expected to be nonrepressible (cf. WILLSON *et al* 1964).

2. Heterocaryons between *cys-3*$^-$ and *cys-3*$^+$ can synthesize aryl sulfatase, even when *cys-3*$^-$ is the majority nuclear type. Thus *cys-3*$^-$ cannot be a super repressor.

Each of these arguments can be faulted. One could imagine that a *totally* derepressed revertant might be lethal, leaving the first line of evidence in doubt. As to the second argument, one could suppose that *cys-3*$^-$ does make a super repressor, but that the super repressor (and by implication, the normal repressor coded by *cys-3*$^+$) is confined to the nucleus in which it was synthesized, and therefore fails to affect its neighbors. In principle, this could be tested in heterozygous diploids or disomics. In practice, diploids are unknown in Neurospora, and while disomics can be prepared, they are very unstable. What was needed for testing the possibility of a nucleus-limited super repressor was a heterocaryon between *cys-3*$^-$ and *cys-3*$^+$ in which the two nuclear types carried two distinguishable forms of aryl sulfatase. Then one could ask the simple question: can *cys-3*$^-$ nuclei in such a heterocaryon make aryl sulfatase?

We have described the isolation of mutants (called *ars* mutants) that lack only this enzyme (METZENBERG and AHLGREN 1970a). If it were certain that those mutants define the structural gene for aryl sulfatase, one could answer the

question simply by preparing a heterocaryon between *cys-3*$^-$ and *ars*$^-$ strains and noting whether it could synthesize any aryl sulfatase. However, if *ars* mutants are regulatory mutants that have intact structural information for the enzyme, the results could be highly misleading, since there would be no way to tell which nucleus made the enzyme. In the accompanying paper (METZENBERG, CHEN and AHLGREN 1971) we record our isolation of revertants of *ars* mutants, and, unfortunately, the absence among the revertants of structural variants that would either establish *ars* as the structural locus for aryl sulfatase, and/or provide material for the most definitive test of the super-repressor model of *cys-3*$^-$.

Sometimes naturally occurring electrophoretic variants within a species, especially in "exotic" races or in related species, can be used to advantage. Having failed to find such variants in the laboratory, we "cast our loaves upon the waters" in hopes of finding some rewarding molds. We examined the aryl sulfatase of *N. crassa* isolates from 11 different countries of North America, Central America, Africa, Asia, and Oceania without finding any significant differences in electrophoretic mobility. However, when we looked at the related, but pseudohomothallic species *N. tetrasperma*, we found that some isolates were natural aryl sulfataseless "mutants," one had an aryl sulfatase that was indistinguishable from the enzyme of *N. crassa*, while some others had an enzyme of lower electrophoretic mobility under our standard conditions. By a rather lengthy program of introgression, we were able to move the genes for absence of the enzyme and for altered mobility into the genetic background of the rest of the strains so that all the strains were fully heterocaryon-compatible.

The genes for absence of aryl sulfatase and for the "slow" electrophoretic trait in *N. tetrasperma* are closely linked to the *ars* locus in *N. crassa*, and are probably allelic with it. We have prepared heterocaryons of *cys-3*$^-$ and *cys-3*$^+$ in which one nuclear type carries the *ars* locus of normal *N. crassa* and the other carries that of the variant enzyme from *N. tetrasperma*. Such heterocaryons make both forms of aryl sulfatase. Therefore the super-repressor notion may be discarded.

MATERIALS AND METHODS

The assay procedures and sources of chemicals used in this study have been listed elsewhere (METZENBERG and AHLGREN 1970a). Electrophoresis was conducted somewhat differently from the manner described in the companion paper (METZENBERG, CHEN and AHLGREN 1971) for the following reason. On the advice of a colleague, we modified the Gelman electrophoresis tray, replacing the short platinum electrodes with longer ones that ran the length of the trays so as to achieve faster separations, and more uniform migration at the ends of the tray. After doing so, we found that the pH 9.07 buffer we had used no longer produced optimal separation of the variant forms of aryl sulfatase reported in this paper. Except where otherwise noted, all electrophoreses reported herein were performed in Tris-acetate buffer, pH 8.2 (50 mM Tris), with sulfate (added as the Tris salt) at a concentration of 0.5 mM. The latter did not affect the mobility of the enzymes, but acted as a carrier for the precipitation of $Ba^{35}SO_4$ in the radiochemical detection method described below. Extracts of Neurospora were prepared as described by MARZLUF and METZENBERG (1968). Hemoglobin A was added and electrophoresis was performed at 300 volts for 75 min, and the strips were stained with indoxyl sulfate (METZENBERG, CHEN and AHLGREN 1971).

When very low levels of aryl sulfatase were to be detected, and when the proportion of two

forms of aryl sulfatase in a heterocaryon was to be quantitatively estimated, we used a method based on the hydrolysis of ^{35}S-*p*-nitrophenyl sulfate and precipitation of the $^{35}SO_4^=$ as the barium salt. The details were as follows. The Sepraphore strips, after electrophoresis, were placed on a sheet of Whatman 3 MM paper that had been dampened with a mixture of equal volumes of the radioactive assay cocktail described by METZENBERG and AHLGREN (1970b) and 0.05 M $BaCl_2$. The paper and strips were sandwiched tightly between sheets of plastic to insure good contact and were incubated at 40°C for 2 hr. Then the Sepraphore strips, which contained very little of the $BaSO_4$, were discarded, and the paper was soaked twice for an hour, and once overnight in a solution of $BaCl_2$, NH_4OH, and nonradioactive *p*-nitrophenyl sulfate (10 mM, 100 mM, and 0.25 mM, respectively) to remove all unreacted radioactive substrate. The paper was then rinsed for a few minutes in 1 M acetic acid, dried, and autoradiographed on Kodak Royal Blue X-ray film. When quantitative results were desired, the band or bands on the paper corresponding to aryl sulfatase on the Sepraphore strips were excised and wet-ashed in glass vials by the method of JEFFAY, OLUBAJO and JEWELL (1960). The colorless residues were dissolved in 1 ml of water and 10 ml of the scintillator solution of BRAY (1960), and were counted in a scintillation spectrometer.

Except where otherwise noted, all strains were either obtained from the Fungal Genetics Stock Center or isolated in our laboratory.

Our wild-type *N. crassa* strains are 74-OR8-1*a* and the essentially isogenic strain of opposite mating type, 74-OR23-1*A* (FGSC# 988 and 986, respectively). *N. crassa* mutants used in this study were outcrossed a number of times (commonly four to six times) to eliminate polymorphism at loci for heterocaryon compatibility. The type homocaryotic strains for *N. tetrasperma* are 85 (FGSC# 1270 and 1271, resp.). The mating-type heterocaryon is designated 85*A/a*. These, and the *N. tetrasperma* stocks 343.6*ae*, 394.4*Ae*, 340.6*aE*, 394.5*Ae/ae* and *pan* 124*A* (FGSC# 606, 608, 605, 609, and 1266, resp.) are from the collection of B. O. DODGE, and apparently originated from Surinam. The *N. tetrasperma* stocks Honduras UFC-220 *A/a* and Liberia *A/a* are FGSC # 850 and 965, resp. *N. tetrasperma* T-220-7*A/a* originated from Borneo, and was kindly provided by Drs. A. M. SRB and D. NOVAK. The pseudohomothallic strain called *N. toroi*, CBS# 259.35 *A/a* (FGSC# 688) is from Puerto Rico. It gives 4-spored asci containing heterocaryotic ascospores, like those of *N. tetrasperma.* We have separated the two components by plating conidia and isolating homocaryotic cultures (DODGE 1928) and find that they are extremely fertile with their opposite mating types of 85*A* and 85*a*, but completely infertile with *N. crassa.* We regard *N. toroi* as being very closely related to *N. tetrasperma*, if not identical with it. P202 Gianjor-1*A/a*, kindly furnished by Dr. DAVID PERKINS (now assigned FGSC# 1794) has not been designated as a particular species, but appears to us to be *N. tetrasperma* by these same criteria. The 4-spored (pseudo?) homothallic strains isolated by PERKINS in Florida and called LaBelle-1, Groveland-1, Homestead-1f, and Lake Alfred-1b (FGSC# 1941–1944, resp.) are also listed as "species uncertain." We have not tested them for fertility with type stocks of *N. tetrasperma.* Strain Argentina 2521.12*ae* (FGSC# 604) is reputed to be *N. tetrasperma.* We find this strain to be completely infertile with the type strains 85*A* and 85*a*, as well as with wild-type *N. crassa.* It crosses quite well with hybrids[1] between *N. tetrasperma* and *N. crassa*, especially *C1,T1-A* and *C1T3-A*, giving many black perithecia and ultimately a mixture of black spores and white, inviable spores. This Argentina strain was also fertile with *N. intermedia* NIT-*A* (kindly provided by Drs. SRB and NOVAK); this species has long been thought to be intermediate in character between *N. sitophila* and *N. crassa.* The Argentina strain mated well with a hybrid between the above *N. intermedia* strain and *N. crassa.* It formed perithecia with *N. sitophila*, but all of the spores were white and failed to mature. On these grounds, we feel that this strain should not be thought of as *N. tetrasperma*, but might be a natural hybrid be-

[1] The following examples illustrate the notations we use for interspecific hybrids. A strain with one pure *N. crassa* parent and one pure *N. tetrasperma* parent would be called *C1,T1*; a strain with 1 *N. crassa* great-grandparent and 7 *N. tetrasperma* great-grandparents would be called *C1,T7*, etc.

tween the latter and *N. crassa*. The point is of interest because this is the only "*N. tetrasperma*" that makes *crassa*-type aryl sulfatase.

Crosses were generally performed by simultaneous inoculation onto the medium of WESTERGAARD and MITCHELL (1947) with appropriate supplements for auxotrophic strains. All amino acid supplements were provided at 1 mM; all vitamins were used at a concentration of 2 μg/ml. For some crosses (especially interspecific ones) sterility was a problem, and better results were obtained with cornmeal medium (4×) of HOWE and PRAKASH (1969). Random ascospores were heat-shocked and the germinated sporelings were picked to 1 ml of liquid BC medium with suitable supplements (METZENBERG and AHLGREN 1970a).

RESULTS

Aryl sulfatase in N. tetrasperma *and putative* N. tetrasperma *strains:* Many isolates of *N. tetrasperma* grow very poorly if not at all with cysteic acid as the sole sulfur source. Therefore we used a limiting level of K_2SO_4 to produce conditions of derepression. Table 1 shows that strains from Surinam, from Liberia, and two of the strains from Florida do not produce detectable amounts of aryl sulfatase. They were also grown on media containing 1 mM taurine and 1 m*M* S-methyl cysteine as sole sulfur sources. Both of these allow complete derepression of *N. crassa* strains, and gave limited but satisfactory growth of the *N. tetrasperma* strains. In no case did the latter strains produce aryl sulfatase on these sulfur sources when they ha dnot done so on limiting inorganic sulfate. Subsequent studies showed that the failure to produce aryl sulfatase and the failure to grow on cysteic acid are not controlled by the same gene, and can be regarded as coincidental. P202 Gianjor *A/a* produces low levels of aryl sulfatase on limiting sulfate (and also on 1 mM cysteic acid, on which it grows satisfactorily). Strain Argentina 2521.12 has a level of the enzyme roughly comparable with that of *N. crassa* and electrophoretically indistinguishable from it. The *N. tetrasperma* strains from Honduras and from Borneo, and the putative ones from Puerto Rico and from Groveland and Homestead, Florida, have an aryl sulfatase of strikingly different electrophoretic mobility from that of *N. crassa*. The mobility of the enzyme from P202 Gianjor (Bali) was intermediate between these two electrophoretic forms.

We wished to conduct the gene for the electrophoretic variant in Honduras UFC-220 into an *N. crassa* background so that it could be studied in combination with our standard stocks. Though crosses between the species are usually completely sterile, a few viable ascopores are sometimes produced. By backcrossing such first-generation hybrids, we have developed a "transfer kit" of strains containing various proportions of *N. crassa* and *N. tetrasperma* ancestry (METZENBERG and AHLGREN 1969). Using a cline of interspecific hybrids[1], each of which is reasonably fertile with its neighbors, one can easily perform introgression of genes from *N. tetrasperma* to *N. crassa* (or vice versa). Such a procedure provides little assurance, however, that the final isolate will be isogenic with *N. crassa* stocks, since there may be persistent co-selection of certain *N. tetrasperma* chromosomes in the progeny. To circumvent this, we incorporated into the schedule of introgression some markers that allowed us to play "genetic ping-pong." (1) The partly introgressed strain was crossed to *alcoy*. This *N. crassa* strain carries

TABLE 1

Activity and electrophoretic mobility of aryl sulfatase of various species and races of Neurospora

Strain	FGSC #	Species of Neurospora	Geographic origin	Aryl s'ase sp. act.	Aryl s'ase, mobility (R_{HbA})
85a	1271	tetrasperma	Surinam	<0.1	-
85A	1270	"	"	0.1	-
340.6aE	605	"	"	<0.1	-
343.6ae	606	"	"	<0.1	-
394.4Ae	608	"	"	<0.1	-
394.5Ae/ae	609	"	"	<0.1	-
Liberia A/a	965	"	Liberia	<0.1	-
Honduras UFC-220	850	"	Honduras	23.6	1.1
T-220-7A/a	-	"	Borneo	25.2	1.1
CBS#259.35A/a	688	toroi (tetrasperma?)	Puerto Rico	51.7	1.1
P202 Gianjor-1A/a	1794	species (tetrasperma?)	Bali, Indo.	2.7	1.5
LaBelle-1	1941	"	Florida	0.1	-
Groveland-1	1942	"	Florida	4.1	1.1
Homestead-1f	1943	"	Florida	11.3	1.05
Lake Alfred-1b	1944	"	Florida	0.1	-
Argentina 2521.12ae	604	debatable	Argentina	47.3	2.0
74-OR8-1a	988	crassa	Louisiana	33.4	2.0

All strains were grown on FRIES' salts containing 0.1 mM sulfate and 1.5% sucrose in standing culture for 3 days at 25°C. Specific activity of aryl sulfatase and electrophoretic mobility of this enzyme relative to hemoglobin A were determined as described in the text. The activities of P202 Gianjor-1*A/a* and of Groveland-1 were just at the limit of detection by indoxyl sulfate staining, and the mobilities of these enzymes were confirmed by the radiochemical detection method.

three reciprocal translocations—between linkage groups I and II, with the breakpoint marked by *albino-1*, between IV and V, with the breakpoint marked by the temperature colonial, *cot-1*, and between III and VI, with the breakpoint marked by *yellow-1* (PERKINS 1966). Progeny containing the desired electrophoretic trait, plus the traits *al*, *cot*, and *ylo* were selected. A minor complication is introduced by the fact that *al* is epistatic to *ylo*, so that strains carrying the latter can only be identified retrospectively in the next cross. (2) The next cross was to wild-type *N. crassa*, and a strain carrying the desired electrophoretic trait, and free of *al*, *cot*, and *ylo* was chosen. (3) To remove undesired *N. tetrasperma* genes from linkage group VII, which carries the *ars* locus, we crossed the partly introgressed strain to *thi-3*, *ars (101)*, *thr-1*. The two nutritional markers lie on

opposite sides of *ars*, and are each very roughly 1 centimorgan from it (METZENBERG and AHLGREN 1970a). (It turned out that the desired electrophoretic trait is probably allelic with *ars*, see below). By choosing the rare recombinants from two such crosses, the triple mutant, *thi-3*, *"slow electrophoretic trait"*, *thr-1* was obtained. (4) The auxotrophic markers were then removed by two successive crosses to *ars (101)*, again selecting the desired recombinants. The details of the introgression schedule are presented in Table 2.

The aryl sulfatase negative trait of *N. tetrasperma* 343.6*ae* was moved into the genetic background of *N. crassa* by a similar schedule of introgression, but without use of *ars (101)*, since it could not be distinguished from the trait in question. We did not start with the type strains 85*A* or 85*a* because, in our hands, they were completely infertile with *N. crassa*. 343.6*ae* is highly fertile with 85*A*. During the introgression procedure, it became evident that the gene for the aryl sulfatase negative trait, like the electrophoretic trait of UFC-220, maps between *thi-3* and *thr-1*.

Mapping of the gene for the electrophoretic trait: Spores from Cross #9, Table 2 were used so that, if recombinants with electrophoretically normal, wild-type *N. crassa* aryl sulfatase were detected, the crossover events could be mapped with respect to *thi-3* and *thr-1*. The spores were germinated on plates supplemented with thiamin, but not threonine. A total of 142 threonine-independent sporelings were picked to BC with 1 mM cysteic acid as sulfur source, and 2 μg/ml of thiamin-HCl. Of these, 140 were ars^+. The two ars^-, thr^+ recombinants were thi^-, as expected. Of the 140 ars^+ strains, 2 were thi^- recombinants. One hundred of the thi^+, ars^+ strains were grown under the conditions described in the legend of Table 1. The two thi^-, ars^+ strains were grown on the same medium supplemented with thiamin-HCl (2 μg/ml). Extracts of the strains were subjected to electrophoresis, and strips were stained as described by METZENBERG, CHEN and AHLGREN (1971). The mobility of aryl sulfatase from 102 cultures was identical with that of the original Honduras UFC-220.

From Cross #10, 185 thr^- sporelings were picked to BC medium with thiamin and cysteic acid as before, and 1 mM threonine. One of these was ars^+. It showed the UFC-220 electrophoretic trait, and was thi^-, as expected. In Cross #11, 148 thr^+ sporelings were tested, one of which was ars^+; it was thi^- and carried the UFC-220 trait as expected. In Cross #12, 100 random spores were picked. There were 48 thi^+, ars^- and 43 thi^-, ars^+ cultures (parental), and 4 thi^-, ars^- and 5 thi^+, ars^+ cultures (recombinant), giving a map distance of 9 centimorgans between *thi-3* and the *ars* locus. All of the ars^+ strains carried the UFC-220 trait. We do not know why the recombination rate found in this final cross is substantially higher than we have seen in previous crosses in Table 2, or in the original linkage tests of *ars* mutants to *thi-3* (METZENBERG and AHLGREN 1970a). In any event, it is clear that the electrophoretic trait of UFC-220 maps between *thi-3* and *thr-1*, and it is probably allelic with the *ars* mutations. We will refer to the gene in question as *ars(UFC-220)*.

*Mapping of the gene for the absence of aryl sulfatase in 343.6*ae: In the introgression cross analogous to Cross #10, Table 2, 191 thr^- sporelings were tested

TABLE 2

Introgression of the gene for low electrophoretic mobility of aryl sulfatase in Honduras UFC-220 (N. tetrasperma) *into* N. crassa

Cross # 1: C1,T3-a (FGSC# 1772) ♀ x UFC-220 (FGSC# 850) ♂[1]; check elect. trait[2].

Cross # 2: UFC-220R.01a x C1,T1-A (FGSC# 1770). Check elect. trait[2].

Cross # 3: UFC-220R19a x 74-0R23-1A (FGSC# 986). Select elect. trait.

Cross # 4: UFC-220R108A x ars(101)a (FGSC# 1864). Check elect. trait[2].

Cross # 5: UFC-220R253a x 74-0R23-1A. Select elect. trait.

Cross # 6: UFC-220R306A x alcoy-a (FGSC# 998). Select elect. trait, cot, and al-2 (albino) [several isolates, since one carrying ylo-1 (hypostatic) was needed].

Cross # 7: UFC-220R406a (carrying cot, al-1, and ylo-1) x 74-0R23-1A. Select elect. trait, non-cot, non-al-1, non-ylo-1 cultures.

Cross # 8: UFC-220R507A x 74-0R8-1a (FGSC# 988). Select elect. trait.

Cross # 9: UFC-220R609a x thi-3, ars(101), thr-1A. Select thi^-, ars^+ crossover; thr^+. Check elect. trait[2].

Cross #10: thi-3, UFC-220R782A x thi-3, ars(101), thr-1a. Select thi^-, ars^+, thr^- crossover. Check elect. trait[2].

Cross #11: thi-3, UFC-220, thr-1R801a x ars(101)A. Select thi^-, ars^+, thr^+ crossover. Check elect. trait[2].

Cross #12: thi-3, UFC-220R951A x ars(101)a. Select thi^+, ars^+ crossover. Check elect. trait[2].

Final strain: UFC-220R1001-A (FGSC# 1909)

[1] In the first cross, it seemed desirable to remove as much *N. tetrasperma* cytoplasm as possible by using it as the male parent. The *C1,T3-a* parent was inoculated onto WESTERGAARD & MITCHELL minimal slants, and, after protoperithecia had formed (5 days) a suspension of conidia of UFC-220*A*/*a* was added. This also avoided the problem of self-fertility of the latter strain, which was pseudohomothallic. All subsequent crosses were made by simultaneous inoculation.

[2] In crosses #1, 2, 4, 9, 10, 11, and 12, the non-UFC-220 parent was aryl sulfatase negative. All of the ars+ progeny showed the slow-moving electrophoretic trait.

for aryl sulfatase production, of which one was found to be negative. This shows that the gene for absence of aryl sulfatase is closely linked to *thr-1*. In the analog of Cross #11, Table 2, 138 *thr*$^+$ sporelings were picked, of which two proved to be aryl sulfatase-negative recombinants. These were *thi*$^-$, as expected. In the analog of Cross #12, Table 2, 177 random sporelings were picked. Of these 83 were *thi*$^+$, *ars*$^+$, 77 were *thi*$^-$, *ars*$^-$ (parentals); 4 were *thi*$^+$, *ars*$^-$, and 13 were *thi*$^-$, *ars*$^+$ (recombinants), giving a map distance of 9.6 centimorgans. Again, this genetic distance is significantly greater than we had observed in intraspecific crosses, and suggests that there might be some stimulation of crossing over by heterozygosity in this region. In a cross of the introgressed strain, *ars(343.6)-a* to *ars(101)-A*, no aryl sulfatase positive cultures were found among 100 isolates.

Is the ars *locus in some strains of* N. tetrasperma *solely responsible for the aryl sulfatase negative character of such strains?:* An aryl sulfatase-positive segregant from the original interspecific cross (343.6*ae* × 74-OR23–1*A*) was isolated and called *C1,T1-A(ars*$^+$*)*. This strain was backcrossed to 343.6*ae*. An aryl sulfatase positive segregant from this cross, called *C1,T3-a(ars*$^+$*)* was crossed to 85*A*, and 97 *C1,T7* progeny were picked to WESTERGAARD and MITCHELL (1947) slants. Fifty were self-sterile homocaryons, and the rest were self-fertile. Thirty-five of the self-sterile isolates and 28 of the self-fertile ones were aryl sulfatase negative. The ratios of *ars*$^+$/*ars*$^-$ suggest the possibility that two genes for the absence of the enzyme exist in *N. tetrasperma*. However, the likelihood of distortion of the ratio by selection for viability could not be ignored. Accordingly, we introgressed the aryl sulfatase positive trait into 85*A* for three more generations, by which time almost all of the sporelings gave rise to self-fertile cultures. These were resolved into unisexual isolates by plating conidia (DODGE 1928). The final cultures were called *C1,T63(ars*$^+$*)*, mating types *A* and *a*. Using this cline of aryl sulfatase positive *N. tetrasperma* derivatives, *ars (101)* from *N. crassa* was then introgressed into *N. tetrasperma* by a series of five crosses. The final strains, containing *ars(101)* as the sole determinant of the aryl sulfatase negative trait, were obtained as homocaryons of both mating types. They were crossed to the 35 self-sterile aryl sulfatase negative *C1,T7* homocaryons described above, using the opposite mating types. Of the 35 crosses, 31 produced ascospores. A small scraping from the spore-print of each tube (a few thousand spores) was transferred en masse to a tube of BC medium + 1 mM cysteic acid + 0.05 mM K_2SO_4 and germinated to give a mass culture. After 3 days' incubation at 33°C the cultures were tested for aryl sulfatase by adding a drop of *p*-nitrophenyl sulfate. All of the cultures were negative, even after 3 days' additional incubation. Control crosses to the *C1,T7* homocaryons, in which the parent was *ars*$^+$, were similarly sampled; spores were germinated en masse, and the resulting cultures were found to be aryl sulfatase positive. It seems likely that the aryl sulfatase negative character of these strains of *N. tetrasperma* is due to a single gene difference from *N. crassa*. The possibility of a second, closely linked gene cannot be completely excluded. However, if a second gene for the absence of aryl sulfatase were closely linked to *ars* it would not explain the aberrant ratio among the *C1,T7*

progeny—the only basis for suspecting the presence of two aryl sulfatase negative genes in *N. tetrasperma* in the first place.

Heterocaryons between ars (101), ars (UFC-220), *and* cys-3: The component strains for producing heterocaryons were isolated by standard crossing methods. Strain C is *cys-3A*. Strain T is *tryp-3; ars(UFC-220)A*. Strain C′ is *cys-3; thi-3, ars(UFC-220)a*. Strain T′ is *tryp-3a*. Strain M is *al-2; arg-12; ars(101)A* of our previous paper (METZENBERG and AHLGREN 1970a). Heterocaryons were prepared, and nuclear ratios of the conidial inocula and of harvested mycelia from sparge flasks were determined as described in that paper. The medium used in every case was FRIES' salts (cited by BEADLE and TATUM 1945) lacking inorganic sulfate, with 1 mM cysteic acid as the sulfur source and 1.5% sucrose as carbon source. The mycelia were assayed for protein and aryl sulfatase and triplicate samples were subjected to electrophoresis to determine the proportion of the two species of aryl sulfatase (see METHODS AND MATERIALS).

Mixtures of extracts from wild-type *N. crassa* and *ars(UFC-220)R1001A* were subjected to electrophoresis and the strips were stained with indoxyl sulfate. The two forms of the enzyme were readily detected, with no evidence of formation of any hybrid enzyme. Similarly, no hybrid enzyme could be detected in any of several nutritionally balanced heterocaryons containing the two alleles of the aryl sulfatase gene. As expected, Het (M + T) contained only the *ars(UFC-220)* form of the enzyme.

To check the validity of the quantitative method for determining the ratio of the two forms of the enzyme, artificial mixtures containing known amounts of activity from wild-type *N. crassa* and *ars(UFC-220)R1001A* were prepared, and following electrophoresis, the strips were assayed with ^{35}S-nitrophenyl sulfate as described in METHODS AND MATERIALS. The results showed that the ratio could be determined with very good accuracy when the slower-moving form of the enzyme [from *ars(UFC-220)R1001A*] was the preponderant form, but that this form of the enzyme was greatly overestimated in the analysis when the fast-moving form of the enzyme was preponderant. This was to be expected, because there is always a small amount of trailing of the fast form during electrophoresis, so that a small percent of it overlaps the slow form of the enzyme. In mixtures in which the slow form predominates, the error from this source is small but the converse situation produces large errors. The heterocaryons discussed in this paper produce the two aryl sulfatases in proportions that are within the range of good accuracy for this method.

In order to estimate the extent to which *cys-3*$^+$ nuclei bearing one allele of *ars* can stimulate the production of aryl sulfatase by *cys-3*$^-$ nuclei bearing a different allele of the *ars* gene, it was necessary to know the relative intrinsic activities of the two enzyme forms in homocaryons. The *cys-3*$^-$ homocaryon produces no detectable aryl sulfatase; in addition, the *cys-3*$^+$ homocaryons used to construct balanced heterocaryons are necessarily auxotrophs that grow less rapidly than heterocaryons, even when suitably supplemented. Therefore, we felt that the most valid comparison of intrinsic activities of the two alleles of *ars*

TABLE 3

Activity of aryl sulfatase in prototrophic homocaryons

Strain	Aryl sulfatase specific activity	
74-OR8-1*a*	27.0	28.2
	29.4	
ars(UFC-220)R1001A	15.0	15.0
	15.0	

The medium was FRIES' salts with $MgCl_2$ substituted for $MgSO_4$ and with L-cysteic acid (1 mM) as the sole sulfur source. The carbon source was sucrose (1.5%). The strains were grown in duplicate by sparging with sterile air for 16 hours for 25°C. Assays for aryl sulfatase and for protein were performed in triplicate from mycelia from each of the four flasks.

would be between prototrophic, essentially isogenic strains. Table 3 shows such a comparison. It is seen that *ars(UFC-220)R1001A* produces 53% as much aryl sulfatase as does 74-OR8-1*a*.

Conidia of the heterocaryons, Het (C + T) and Het (C′ + T′) were inoculated into sparge flasks and grown exactly as described in the legend of Table 3. Samples of the inocula were plated as described by METZENBERG and AHLGREN (1970a) for determination of the nuclear ratios. This method is only a slight modification of that of ATWOOD and MUKAI (1955). At the time of harvest (16 hr) a sample of mycelium from each flask was allowed to conidiate on FRIES' minimal medium as previously described, and the nuclear ratio was determined as before. In both the inoculum and the harvested mycelium of Het (C + T), the proportion of *cys-3*$^-$ nuclei was found to be 46%. The agreement is fortuitously close, but it does show that the nuclear ratio could not have been changing drastically during the 16-hr incubation. The corresponding figures for Het (C′ + T′) were 59% and 63% *cys-3*$^-$ nuclei; we will take the mean value, 61%, as the best estimate.

The harvested mycelia were homogenized as usual and the extracts were assayed for gross specific activity of aryl sulfatase. Quadruplicate samples were subjected to electrophoresis, along with control strips charged with enzymes from 74-OR8-1*a* and *ars(UFC-220)R1001A*. The control strips, and one of the quadruplicate strips from each heterocaryon were stained with indoxyl sulfate. Both heterocaryons very clearly contained both forms of the enzyme. The remaining triplicate strips were treated with ^{35}S-*p*-nitrophenyl sulfate as described in METHODS AND MATERIALS, and after locating the bands of radioactive barium sulfate by autoradiography, the bands were excised and their radioactivity was determined. The results are presented in Table 4.

It will be seen that, in Het (C + T), 25.1% of the enzyme activity is of the *ars*$^+$ type, and must have been made by the *cys-3*$^-$ nuclei. This figure must be compared with 46%, which is the proportion of nuclei that code for this form of the enzyme. When it is recognized that the other nuclear type bears an *ars* locus that is only 53% as intrinsically active as that which is borne by the cys-3$^-$

TABLE 4

The formation of aryl sulfatase by cys-3⁻ *nuclei*

Heterocaryon	Percent *cys-3*⁻ nuclei	Total aryl sulfatase specific activity	Percent aryl sulfatase activity made by *cys-3*⁻ nuclei
Het (C + T)	46%	10.2	25.1%[1] (*ars*+type)
Het (C′ + T′)	61%	19.3	43.4%[2] (*ars(UFC-220)* type)

The preparation of the heterocaryons, determination of the nuclear ratios, and assay of the percent aryl sulfatase of each type is described in the text.

[1] The value 25.1% is the average of three determinations, with values of 23.1%, 26.3%, and 25.8%.

[2] The value 43.4% is the average of three determinations, with values of 44.4%, 43.4%, and 42.5%.

nucleus, the production of aryl sulfatase by the latter is much less than the amount to be expected (61.6%) on the basis of nuclear frequency. On the other hand, Het (C′ + T′) produces very nearly the expected proportions of the two types of aryl sulfatase, when the nuclear frequency and the difference in intrinsic activities of the homocaryons are taken into account (expected: 46.5%; found: 43.4%). It is not clear why one of these heterocaryons behaves quantitatively as expected, while the other does not.

Other heterocaryons between *cys-3*⁺ and *cys-3*⁻ containing *ars*⁺ and *ars(UFC-200)* have been prepared and shown qualitatively to contain both forms of aryl sulfatase. Unfortunately, they did not have sufficiently stable nuclear ratios to allow a quantitative comparison of the sort described for Het (C + T) and Het (C′ + T′) and the nuclear ratio rapidly became very lopsided in one direction or the other.

DISCUSSION

Some signal from the *cys-3*⁺ nuclei can apparently turn on the aryl sulfatase synthesizing machinery of *cys-3*⁻ nuclei. Therefore we can rule out the possibility that *cys-3*⁻ makes a super repressor that is restricted to its own nucleus. We must now inquire whether the element responsive to the *cys-3*⁺ signal is intranuclear or extranuclear.

If the *cys-3*⁺ product acts outside the nucleus, presumably at the translational level, there is no problem in understanding how the complementation occurs. On the other hand, if the site of action of the *cys-3*⁺ product is intranuclear, it must somehow find its way into the *cys-3*⁻ nuclei. If it is normally made *in* a particular nucleus for use by the same nucleus, it must be considered accidental that there is any mechanism at all for transfer of this product across the nuclear membrane. One last possibility must be considered. The *cys-3*⁺ product might be made in the nucleus for local use; at the time of mitosis, the nuclear membrane presumably disappears, and when it re-forms, a sample of the common cytoplasm, including some heterologous *cys-3* product, is included in the daughter nuclei. In

this way, *cys-3*$^-$ nuclei in a heterocaryon might regularly contain *cys-3*$^+$ product without its ever having been "transported" across a nuclear membrane. This is obviously highly speculative, all the more so because there is a continuing controversy in the literature about whether mitosis is "classical" in fungi, and whether the nuclear membrane ever disappears during mitosis (see AIST and WILSON 1967; WILSON, BRUSHABER and AIST 1966; also reviews by OLIVE 1953; ROBINOW and BAKERSPIGEL 1965).

It seems that *cys-3* is formally quite similar to a number of other pleiotropic control mutants in fungi that have been described by various authors (for review, see GROSS 1969). In each of these systems, a single mutation at a site far from the structural genes for the affected enzymes can decrease or abolish the expression of those structural genes. In no case do we know how this control is exercised, nor even whether it occurs at the level of transcription or of translation. It would be interesting not only to know the molecular mechanism involved in this sort of control, but also to know whether it is widespread in eucaryotes other than fungi.

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SUMMARY

Some natural isolates of *Neurospora tetrasperma* do not make aryl sulfatase, whereas others contain an aryl sulfatase that differs electrophoretically from that of *N. crassa*. These traits from *N. tetrasperma* have been introgressed into the genetic background of *N. crassa*, and appear to be controlled by alleles of the *ars* locus in Linkage Group VII.—It became possible to test the hypothesis that *cys-3*$^-$ mutants are unable to make aryl sulfatase and its congeners because it produces a super repressor; this super repressor would have to be confined to the nucleus in which it is produced. Heterocaryons were synthesized with two alleles of the *ars* locus that code for different forms of aryl sulfatase. One of the alleles was introduced along with *cys-3*$^-$, the other with *cys-3*$^+$. The heterocaryons produced both forms of the enzyme. The super-repressor hypothesis can therefore be discarded, and the interpretation that the *cys-3* locus specifies a positive control factor is correspondingly strengthened. Some possible sites of action of this control factor are discussed.

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