CYTOGENETIC BEHAVIOR OF SPORE KILLER GENES IN NEUROSPORA[1]

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ABSTRACT

Crosses heterozygous and homozygous for *Sk-1*, *Sk-2* and *Sk-3* were examined by light microscopy. All three Spore killers behave similarly. In heterozygous killer × sensitive crosses, meiosis and ascospore development are normal until after the second postmeiotic mitosis when four of the eight ascospores in each ascus stop developing and degenerate. The four surviving ascospores carry the killer. Death of sensitives thus occurs only after killer and sensitive alleles, Sk^K and Sk^S, have segregated into separate ascospores. Homozygous killer × killer crosses do not show such a pattern of degeneration. Either all ascospores are normal or, if some fail to mature, they do not resemble the degenerating sensitive ascospores in heterozygous asci.——With *Sk-2*, it was shown that Sk^S nuclei do not abort when both Sk^K and Sk^S are present in the same ascospore. Mutants affecting ascus development were used to obtain large ascospores enclosing both Sk^K and Sk^S meiotic products in a common cytoplasm. Sk^S nuclei do not then undergo the degeneration that would be seen if they were sequestered into separate ascospores, and viable Sk^S progeny are recovered in undiminished numbers when the mixed multinucleate large ascospores are germinated. In a four-spored mutant, where each ascospore encloses a single nucleus following meiosis, degeneration of Sk^S ascospores nevertheless occurs, even though the third nuclear division is omitted. Cycloheximide and temperature treatments do not affect the expression of Sk^K.

THE genetic behavior of three Spore killer genes in Neurospora has been described in a companion paper by TURNER and PERKINS (1979). Crosses heterozygous for a given *Sk* gene typically produce four black viable and four dead ascospores in each ascus. The surviving ascospores carry the Spore killer allele, Sk^K, while ascospores having the normal *Sk*-sensitive allele, Sk^S, degenerate. Thus, *Sk* differs in a fundamental way from ascospore-autonomous lethal or semilethal mutants such as *asco*, *cys-3*, *le-1*, *gul-3*, and *ws-1*.

Similar instances of directed spore or gamete elimination are known in Drosophila, wheat, Nicotiana, tomato and Podospora (see DISCUSSION). They are of interest both for their consequences in populations as examples of meiotic drive and for the cellular and molecular mechanisms that underlie gamete elimination.

In Neurospora, independently segregating markers from the Sk^S parent can be recovered in the surviving Sk^K ascospores (TURNER and PERKINS 1979). This re-

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sult shows that the Sk^S nucleus is not grossly affected prior to the first meiotic division. Observations have been made by light microscopy to determine more directly the time and nature of the *Sk* effect on the developing asci and ascospores. These observations, reported here, show that *Sk* is expressed at a time following ascospore delimitation. The cytogenetic behavior of *Sk-2* was further examined in special situations where both Sk^K and Sk^S alleles were enclosed in the same ascospore. The results clearly show that death of ascospores and nuclei carrying Sk^S occurs only when Sk^K and Sk^S alleles have been sequestered into separate ascospores, but that death does not occur when Sk^S and Sk^K nuclei are together in the same ascospore. This paper reports cytological observations on all three *Sk* genes, and additional cytogenetic evidence bearing on the mode of action of *Sk-2*.

MATERIALS AND METHODS

The following strains were primarily employed: *N. sitophila* containing $Sk\text{-}1^K$ (FGSC 2664, 2665) and $Sk\text{-}1^S$ (FGSC 1134, 3191); *N. crassa* containing $Sk\text{-}2^K$ introgressed from *N. intermedia* (strains ancestral to FGSC 3114, 3115) and $Sk\text{-}2^S$ (FGSC 1690, 1838, 2218, 2220, 2230); and *N. intermedia* containing $Sk\text{-}3^K$ (FGSC 3193, 3194) and $Sk\text{-}3^S$ (FGSC 1766, 1767). (For origins, see TURNER and PERKINS 1979.) Crosses were made having each of the *Sk* genes in homozygous and in heterozygous condition.

N. crassa strains carrying $Sk\text{-}2^K$ were also crossed to strains that contained Banana (*Ban*) (RAJU and NEWMEYER 1977) and Four spored (*Fsp*) (RAJU 1979) to study the interaction between killer and sensitive nuclei when both were enclosed in the same ascospore. The mixed cultures obtained from these individual, large-sized ascospores were then crossed to both standard testers (fluffy, *fl a* and *fl A*) to determine their nuclear types.

Standard procedures were followed to obtain perithecia for cytological and cytogenetic studies (RAJU and NEWMEYER 1977). Samples of developing perithecia were fixed at intervals between four and ten days after fertilization, at 25°. The fixing fluid and the iron-hematoxylin procedure for nuclear staining were as described by RAJU and NEWMEYER (1977) and RAJU (1978). However, rosettes of maturing asci are best shown when eight- to ten-day-old perithecia are dissected without fixation or hydrolysis and are stained with very dilute solutions of the mordant and hematoxylin.

RESULTS

Since the cytological behavior of all three *Sk* genes is nearly identical, a unified general description is presented here; observations are most extensive for *Sk-2* in *N. crassa*, however. For descriptions and photographs of normal ascus development, nuclear divisions and ascospore formation in wild-type asci, see SINGLETON (1953) and RAJU (1978).

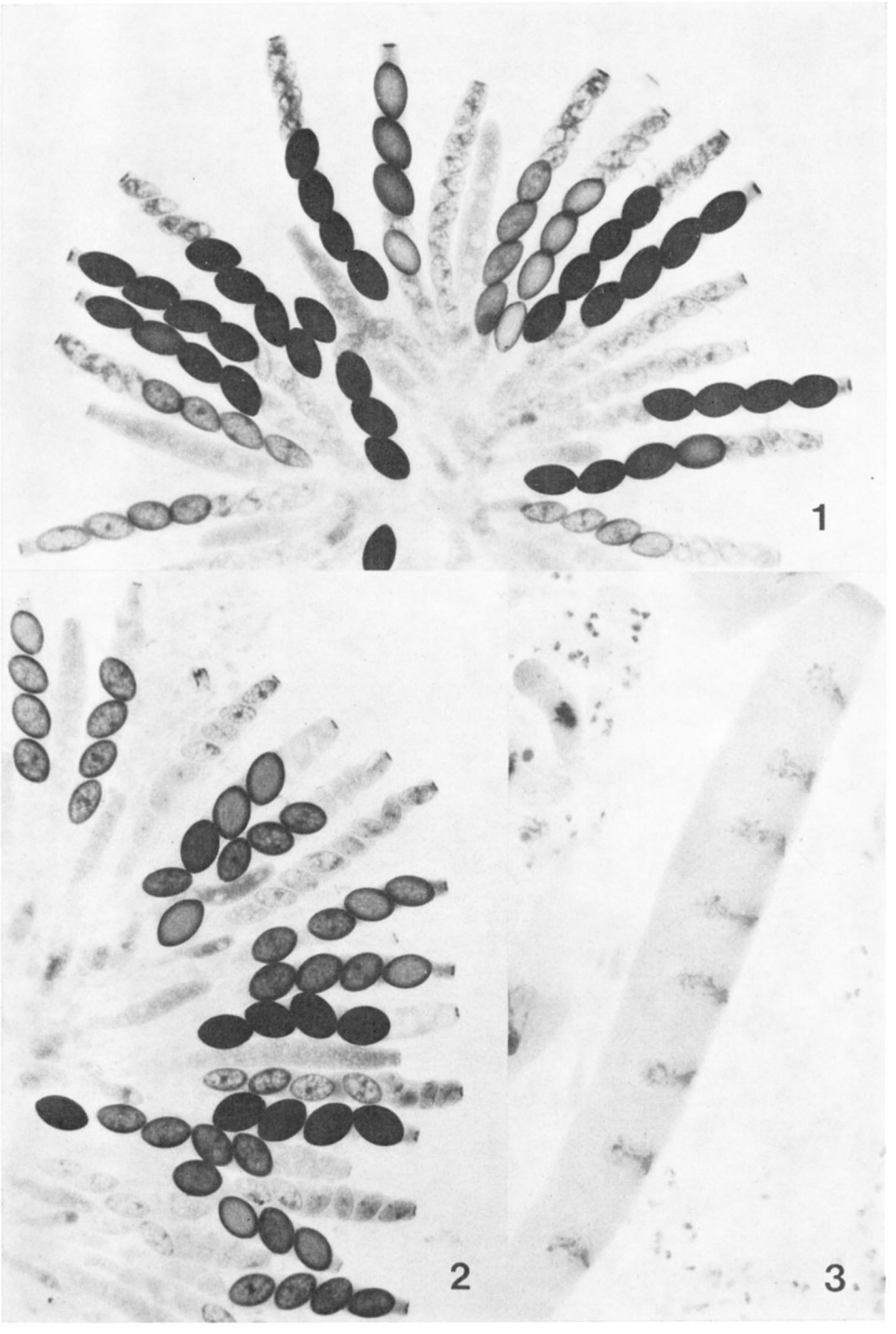
The manifestation of Sk *in heterozygous* Sk^K/Sk^S *asci*: Spore killer genes in Neurospora are clearly expressed in heterozygous crosses, regardless of which strain is used as female and which as fertilizing parent. The abnormality is not visible until after the second postmeiotic mitosis, when four of the eight ascospores begin to degenerate in each ascus. Figures 1 and 2 show rosettes of asci at various stages of ascospore development. The oldest asci clearly show four large black viable ascospores and four small hyaline dead ascospores. Only the asci that are immature fail to show such a 4:4 pattern. All three nuclear divisions in the ascus are completely normal, as is the delimitation of ascospores. Cursory observations

at pachytene showed no evidence of chromosomal abnormalities, although the existence of small rearrangements cannot be ruled out. As far as can be determined by light microscopy, the nucleolus and the spindle pole bodies (SPBs) behave normally through all nuclear divisions in the ascus. Soon after the first postmeiotic mitosis, all eight nuclei realign in single file with all eight SPBs on the same side of the ascus, just as in wild-type crosses (Figure 3). Then eight seemingly normal ascospores are delimited, each containing a single nucleus. All eight ascospores begin to develop normally as their nuclei undergo a normal mitosis, and they become binucleate. The nuclei then go into an interphase stage. The cytoplasm in all eight ascospores initially shows the normal "vacuolate" structure that is characteristic for normally maturing ascospores at that stage. (The original vacuolate nature of the cytoplasm was eliminated in Figures 3, 4 and 8 by soaking the hydrolyzed perithecia in a Carnoy solution containing chloroform; see RAJU 1978.) The nucleolus is conspicuous, and is surrounded by interphase-like chromatin. Up to this time, it is not possible to tell which four of the eight ascospores are destined to degenerate.

Abnormalities first became apparent some time after the ascospores have become binucleate. Four of the eight ascospores in each ascus stop further maturation at this time (Figure 4). The cytoplasm in arrested ascospores gradually loses the characteristic vacuolate nature of normal cytoplasm and becomes amorphous (Figure 5). The nuclei in the arrested ascospores slowly degenerate, while the other four spores enlarge and mature normally.

Sk usually segregates at the first division, with only a small percentage (0 to 5%) of asci showing second-division segregation patterns. *Sk-2* in *N. crassa* shows the least second-division segregation (<<1%) and *Sk-1* in *N. sitophila* shows up to 5%. The position of the surviving ascospores was scored in 1060 asci to determine if there is any preferential orientation of the Sk^K allele in the top half or bottom half of the ascus. Both types of asci are equally frequent (523:537), respectively). The degenerating ascospores are often pushed to the end of the ascus by the four normally maturing ascospores. This effect is most pronounced for *Sk-3* (Figure 2). The four degenerating ascospores are difficult to see in unstained rosettes of asci, so that asci with four mature ascospores sometimes resemble superficially the four-spored asci of *N. tetrasperma.*

The manifestation of Sk *in homozygous* Sk^K/Sk^K *asci: N. sitophila* crosses homozygous for $Sk\text{-}1^K$ are completely normal, with most of the asci producing eight black ascospores (Figure 6). Almost all of the black ascospores germinate and give rise to $Sk\text{-}1^K$ progeny. These crosses are indistinguishable from $Sk\text{-}1^S \times Sk\text{-}1^S$. When $Sk\text{-}2^K$ or $Sk\text{-}3^K$ is homozygous, all ascus events up to ascospore delimitation and the subsequent ascospore growth are apparently normal, but in most asci a variable number of ascospores (one to eight) fail to blacken (Figure 7). The proportion of these normal-sized nonblack, inviable ascospores varies widely depending upon the genotype of the strains involved. Some strains of $Sk\text{-}2^K$ or $Sk\text{-}3^K$ make a high proportion of normal asci in homozygous crosses (TURNER and PERKINS 1979).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Use of developmental mutants to determine the nature of Sk *action*: Sk^S ascospores are perfectly viable in crosses where Sk^K is not present. In crosses of killer and sensitive, the death of Sk^S ascospores occurs shortly after Sk^K and Sk^S are sequestered into separate ascospores. Therefore, Sk^K probably exerts its primary effect on Sk^S before ascospore delimitation and consequent severance of communication. We may now ask whether the death of ascospores occurs because of the presence of Sk^S or the absence of Sk^K. The two alternatives can be distinguished if ascospores containing both Sk^K and Sk^S can be generated and analyzed for ascospore and nuclear viability.

Two different genetic systems were used to ensure the regular inclusion of both Sk^K and Sk^S nuclei in the same ascospores: Banana (*Ban*) and Four spored (*Fsp*). The results from the two systems agree. The heterokaryotic ascospores containing both Sk^K and Sk^S are viable, and both types of nuclei are recoverable therefrom. Therefore the presence of Sk^K nuclei is necessary and sufficient for viability of the heterokaryotic ascospores and all the nuclei therein.

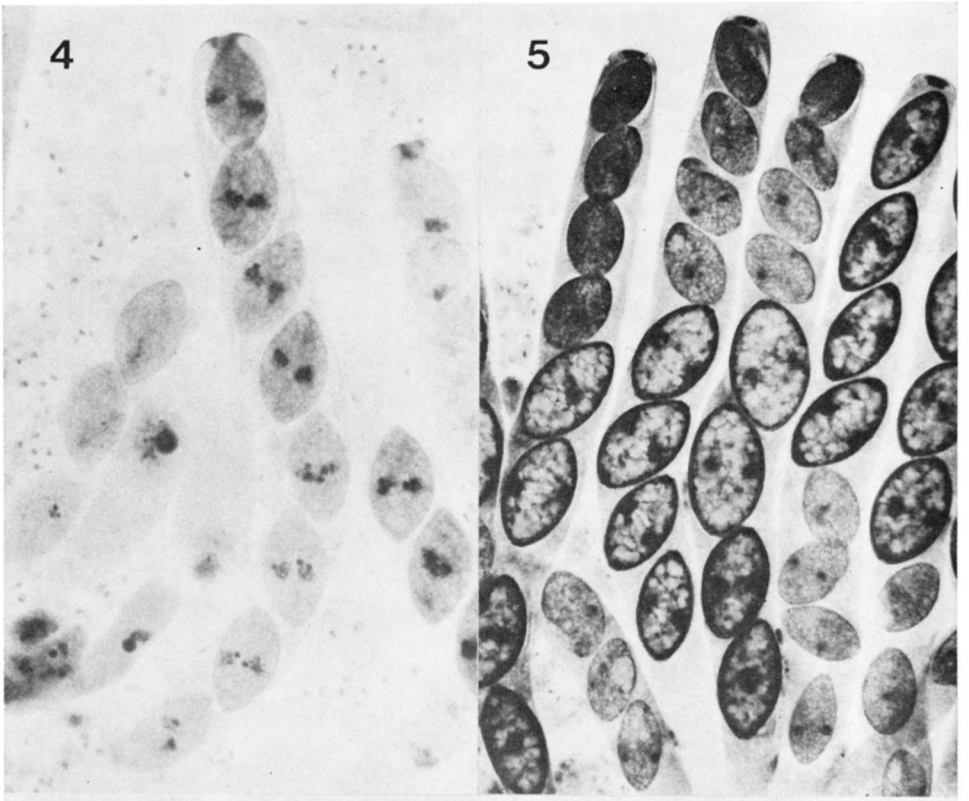
Experiments with Ban: The dominant *N. crassa* mutant, *Ban* produces asci consisting of a single giant ascospore enclosing all four products of meiosis and their mitotic derivatives (RAJU and NEWMEYER 1977). Crosses were made of *Sk-2*^{K}; *Ban*$^+$ × *Sk-2*^{S}; *Ban*, and these were monitored both cytologically and genetically in order to determine if the Sk^S nuclei degenerate in the Banana ascospores. Nuclear divisions in the Sk^K/*Ban* asci are normal and all eight nuclei are enclosed in each Banana ascospore. The nuclei in the Banana ascospores usually divide once (or, rarely, twice), so that they contain 16 (or 32) nuclei. The first of these two divisions is a normal event that occurs in both the eight-spored and *Ban* asci. The second division, giving 32 nuclei per ascus, is unique to some of the Banana ascospores.

If Sk^S nuclei degenerated following wall delimitation (and one subsequent mitosis) in Banana ascospores, just as they do in normal ascospores at a comparable stage, then eight healthy and eight "sick" nuclei should be seen (or 16 healthy and eight or 16 "sick" nuclei in the rare 32-nucleate giant ascospore). What was observed, in fact, was that all 16 (or 32) nuclei are morphologically alike and healthy-appearing in Banana ascospores of crosses heterozygous for Sk^S/Sk^K, as though no nuclei were being killed (Figure 8).

FIGURE 1.—*N. crassa*. *Sk-2*^{K} × *Sk-2*^{S}. A rosette of asynchronously developing asci at various stages of maturation. Each ascus shows four normal (larger) ascospores and four small aborted ascospores. The asci with darker ascospores are more mature than those with lighter ascospores. The few asci not showing the 4:4 pattern are still immature. All the maturing ascospores are Sk^K. (× 280).

FIGURE 2.—*N. intermedia*. *Sk-3*^{S} × *Sk-3*^{K}. Asci at various stages of maturation as in Figure 1. The cytoplasm in the degenerating ascospores is more amorphous than that in *Sk-1* or *Sk-2*. (× 240.)

FIGURE 3.—*N. crassa*. *Sk-2*^{S} × *Sk-2*^{K}. Ascus after the first postmeiotic mitosis with all eight nuclei lined up in single file. This event is normal and occurs in all asci prior to ascospore delimitation; each ascospore will enclose a single nucleus. (× 700.)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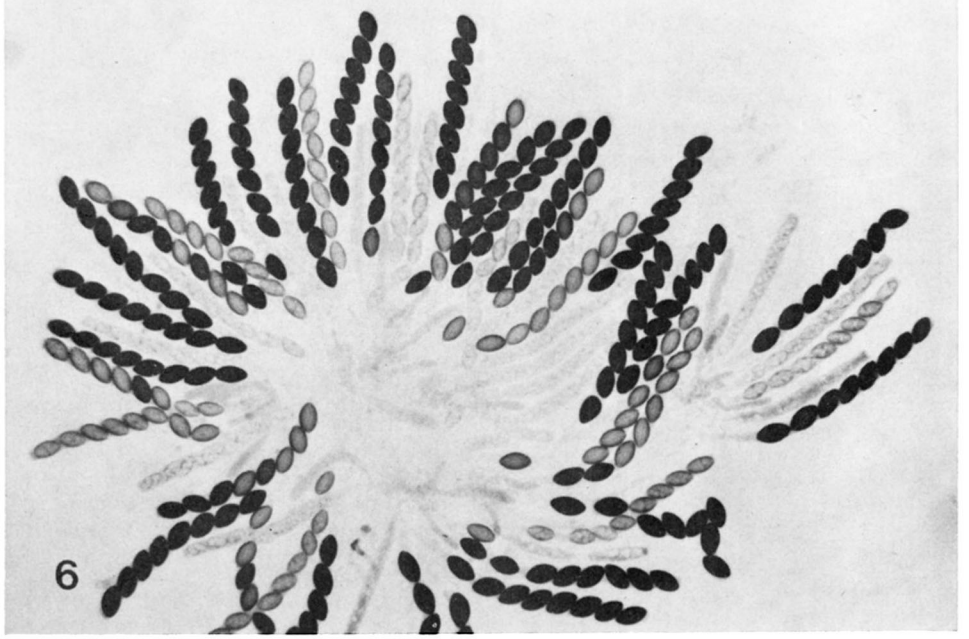
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It might be argued, however, that the normal-appearing Sk^S nuclei in the Sk^K/*Ban* giant ascospores are not viable. This was disproved by using progeny tests to show that viable Sk^S nuclei are present in such ascospores. To accomplish this, shot Banana ascospores from three- to four-week-old crosses were rehydrated overnight in sterile water and were then poured onto solidified 4% agar medium in petri dishes. When the Banana ascospores settled on the agar surface, excess water was drained and the agar was allowed to air-dry for about an hour to facilitate isolation of ascospores onto slants of complete medium in 10 × 75 mm glass tubes. (This was done to avoid any mechanical damage to the giant ascospores during spreading on the agar surface.) The Banana ascospores were given a 20- to 30-minute heat-shock at 60° and were then incubated at 25°.

The Banana ascospores usually germinated within four to eight hours. Germination was usually from both ends, but sometimes from one end only and occasionally from other places on the giant ascospore. The cultures from individual Banana ascospores were allowed to grow for five to ten days before they were crossed to standard testers for progeny testing. Both *A* and *a* standard strains were used to test each culture. Since each Banana ascospore encloses nuclei representing the mitotic derivatives of all four products of meiosis, genetic analysis of the resulting cultures may at first seem complicated, but the genes used in the cross have greatly facilitated the testing procedure.

Sk-2 in *N. crassa* is in linkage group III, tightly linked to its centromere (TURNER and PERKINS 1979). The dominant marker *Ban* is in linkage group I, distal to the mating-type locus (RAJU and NEWMEYER 1977). In a cross between Sk^K; *Ban*$^+$ *A* and Sk^S; *Ban a* (Table 1), each noncrossover Banana ascospore is expected to contain only two nuclear types: (1) Sk^K; *Ban*$^+$*A* and Sk^S; *Ban a*, or (2) Sk^K; *Ban a* and Sk^S; *Ban*$^+$ *A*, depending upon centromere segregation.

When the cultures from individual Banana ascospores are crossed to standard testers (whose genotypes are Sk^S; *Ban*$^+$ *A* and *a*), the two nuclear types that contain *Ban* will produce perithecia that again make only Banana ascospores. In such tests, it cannot be determined whether the tested nuclei are Sk^K or Sk^S without further tests for one or more additional generations. In contrast, the two nuclear types that contain *Ban*$^+$ would be expected to produce eight-spored asci when they are crossed with the Sk^S; *Ban*$^+$ *a* tester. These could be of two types: asci with four black and four white ascospores (4:4 asci) would be expected if a tested *Ban*$^+$ nucleus were Sk^K, while asci with eight black and zero white ascospores

FIGURE 4.—*N. crassa*. $Sk\text{-}2^S \times Sk\text{-}2^K$. Ascus showing eight binucleate spores following a normal second postmeiotic mitosis. The nuclei in all eight spores are normal and morphologically alike. The lower four ascospores appear smaller and are probably showing the first signs of arrest. (× 700.)

FIGURE 5.—*N. intermedia*. $Sk\text{-}3^S \times Sk\text{-}3^K$. In each ascus, the four Sk^S ascospores have degenerated and the cytoplasm in them has become amorphous. (× 450.)

FIGURE 6.—*N. sitophila*. $Sk\text{-}1^K \times Sk\text{-}1^K$. A normal rosette of maturing asci. Spore killer is not expressed in homozygous asci. (× 175.)

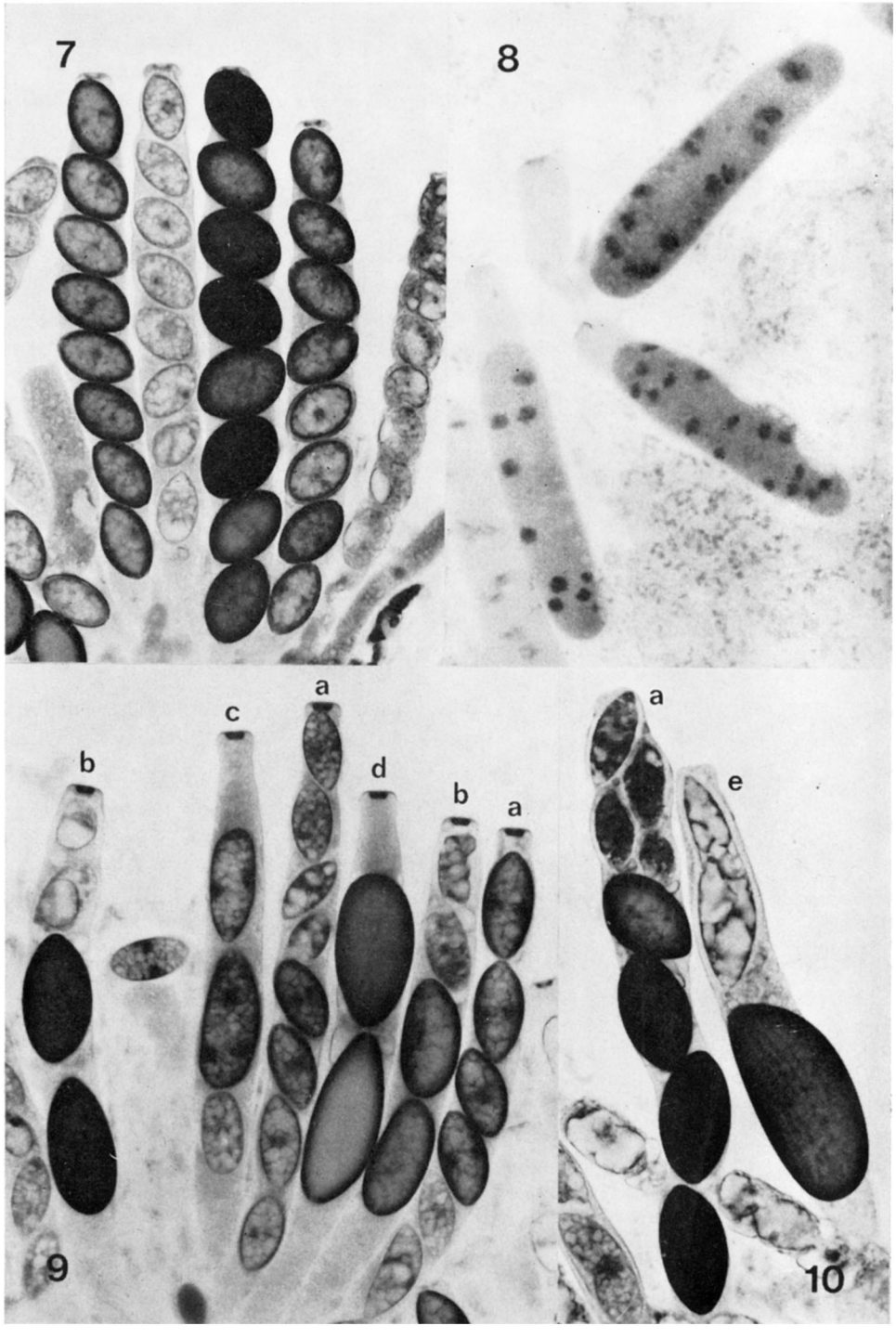
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TABLE 1

Expected results of testcrossing the mixed F_1 cultures from germinated one-spored Banana asci of the cross Sk^K; Ban+A × Sk^S; Ban a

Genotypes of nuclei in tested giant ascospores*	Testcross results expected for each nuclear type × tester Sk^S; *Ban+A*	× tester Sk^S; *Ban+a*
1. Ascus with parental-ditype segregation for *Sk* and *Ban*		
Sk^K; *Ban+A*	No perithecia	8-spored asci with
+		4 black†: 4 white
Sk^S; *Ban a*	Banana asci	No perithecia
2. Ascus with nonparental-ditype segregation for *Sk* and *Ban*		
Sk^K; *Ban a*	Banana asci	No perithecia
+		
Sk^S; *Ban+A*	No perithecia	8-spored asci with 8 black‡: 0 white

* For simplicity, only noncrossover asci are shown, consisting of parental and nonparental ditypes, which are expected to occur in equal numbers. Crossing over does in fact occur in the gene-gene and centromere-gene intervals, but is relatively infrequent (Table 2).
† All Sk^K.
‡ All Sk^S.

(8:0 asci) would be produced if the tested *Ban+* nucleus were Sk^S. Observation of 8:0 asci in these progeny tests can thus provide the needed evidence that Sk^S nuclei survive in Banana ascospores from Sk^S/Sk^K asci. No 8:0 asci should be found if the Sk^S nuclei have all degenerated in the giant ascospores; if tests are observed to produce 8:0 asci, then Sk^S nuclei must have survived.

Results of the progeny tests on 101 Banana ascospores are summarized in Table 2. Perithecia were opened in all tests for scoring the three ascus types: Banana, 8:0, or 4:4. Cultures from individual Banana ascospores normally produced a single ascus type on one tester and a different type on the tester of the opposite mating type. When a test showed two ascus types (on the same tester),

FIGURE 7.—*N. intermedia. Sk-3K* × *Sk-3K*. Ascospore delimitation and growth are normal, but some ascospores fail to become fully pigmented. This behavior is distinct from ascospore death in heterozygous Sk^K/Sk^S asci. (See Figure 2.) (× 360.)

FIGURE 8.—*N. crassa. Sk-2K*; *Ban+* × *Sk-2S*; *Ban.* The dominant mutant, Banana, causes each ascus to make a single giant ascospore. Both Sk^S and Sk^K nuclei are enclosed in the same giant ascospore. All nuclei appear morphologically normal. The ascospore left of center has not entered second postmeiotic mitosis, but one of the eight nuclei has divided asynchronously. (× 480.)

FIGURES 9 and 10.—*N. crassa. Sk-2S*; *Fsp* × *Sk-2K*; *Fsp.* When homozygous, *Fsp* makes: (a) 8-spored; (b) 4-spored; (c) 3-spored, and (d) and (e) 2-spored asci. In the 8- and 4-spored asci, one half of the ascospores abort. In the 3-spored ascus, the larger middle ascospore encloses both Sk^K and Sk^S nuclei. This large ascospore, as well as the top Sk^K ascospore, will mature. Among the rare 2-spored asci, roughly half are like *d* with both ascospores surviving, and half are like *e* with one ascospore degenerating. (× 480 and × 600, respectively.)

TABLE 2

Observed results of testcrossing the mixed F_1 cultures from 101 germinated one-spored Banana asci of the cross Sk^K; Ban+A × Sk^S; Ban a

	Ascus type observed* × tester *Sk^S; Ban+A*	× tester *Sk^S; Ban+a*	Number of asci tested†	Type of segregation‡ *Sk*, *Ban*	*Sk*, *mt*	*Ban*, *mt*
1.	*Ban*	4:4 (K)	17 (+3)			
	—	4:4 (K)	11 (+1)			
Total:	**Ban**	4:4 (K)	28 (+4)	**PD**	**PD**	**PD**
2.	*Ban*	8:0 (S)	17 (+2)			
	—	8:0 (S)	12 (+1)			
Total:	**Ban**	8:0 (S)	29 (+3)	**NPD**	**NPD**	**PD**
3.	**Ban**	—	16 (+1)	§	§	**PD**
4.	*Ban*, 8:0 (S)	—, —	2			
	—, —	*Ban*, 4:4 (K)	2			
	—, 8:0 (S)	*Ban*, —	1 (+1)			
	—, 8:0 (S)	—, —	1			
Total:	**Ban, 8:0 (S)**	**Ban, 4:4 (K)**	6 (+1)	**T**	**PD**	**T**
5.	*Ban*, 4:4 (K)	—, —	1			
	—, —	*Ban*, 8:0 (S)	1			
	—, 4:4 (K)	*Ban*, —	2			
	—, 4:4 (K)	—, 8:0 (S)	1			
	—, 4:4 (K)	—, —	2 (+1)			
Total:	**Ban, 4:4 (K)**	**Ban, 8:0 (S)**	7 (+1)	**T**	**NPD**	**T**
6.	**Ban, —**	**Ban, —**	1	§	§	**T**
7.	*Ban*, —	4:4, 8:0 (K,S)	1			
	—, —	4:4, 8:0 (K,S)	1			
Total:	**Ban, Ban**	4:4, 8:0 (S)	2	**T**	**T**	**PD**
8.	—, 8:0 (S)	—, 8:0 (S)	2			
Total:	**Ban, 8:0 (S)**	**Ban, 8:0 (S)**	2	**NPD**	**T**	**T**

The data are interpreted to fall into eight different classes based on the type of segregation. The total numbers of Banana asci observed in each class are shown in bold face. The major classes 1 and 2 correspond to classes 1 and 2 in Table 1.

* 4:4 signifies 8-spored asci with four ascospores aborted and four ascospores large, usually black. 8:0 signifies 8-spored asci with all eight ascospores large, usually black. Inferred *Sk* genotypes of the viable ascospores are given in parentheses: (K) = *Sk-2*^{K}, (S) = *Sk-2*^{S}. *Ban* signifies one-spored Banana asci; it was not feasible to determine *Sk* constitution in these asci. — (dash) indicates that these nuclear types have not been recovered. This is attributed to loss of nuclei representing one or more meiotic products, from the initially mixed Banana ascus; such loss is also commonly found in cultures from Banana asci not involving *Sk*. The missing products are inferred from their surviving siblings to have been of the type shown beneath them in the boldface "total" line for each class.

the two types were present in separate perithecia; individual perithecia always contained a single ascus type. From experience with Banana crosses not containing Sk^K, it is not expected that all meiotic products will be recovered from many of the one-spored Banana asci.

Classes 1 to 3 in Table 2 are most simply interpreted as noncrossover asci. Other classes most likely represent asci with a single crossover between *Ban* and mating type (4 to 6), or between mating type and the centromere (7), or a two-strand double exchange in both intervals (8). A few tests may have been misclassified owing to the failure to recover one or more of the meiotic products from some Banana ascospores, but this would not materially affect conclusions regarding survival of Sk^S nuclei. When all 83 tests that produced eight-spored asci were scored for *Sk*, a total of 43 tests showed the parent nucleus to have contained the Sk^S allele, and 43 the Sk^K allele. (Three tests showed both alleles, see classes 5 and 7.) The occurrence of 8:0 tests and 4:4 tests in equal numbers proves beyond doubt that the Sk^S nuclei are not killed when both Sk^K and Sk^S are enclosed in the same giant ascospore.

Among a total of 81 noncrossover Banana ascospores (Classes 1 to 3 in Table 2), the *Ban* component was not recovered from 25 Banana ascospores, and the Ban^+ component was not recovered from 17 other Banana ascospores. This suggests that proliferation of one nuclear component was suppressed at germination or relatively early in the growth. This behavior was also found in previous *Ban* crosses not involving Sk^K (RAJU and NEWMEYER, unpublished) and, therefore, it does not indicate death of Sk^S nuclei.

In *N. crassa*, *A* and *a* mating types are incompatible as vegetative heterokaryons. It is therefore expected that, when a Banana ascospore germinates, one or more component homokaryons grow together as mixed cultures but not as heterokaryons. To determine this more directly, observations were made on cut hyphae from 26 just germinated Banana ascospores from $Sk^K \times Ban$ crosses. The cut hyphae were grown separately and subsequently crossed to standard mating-type testers. The individual cultures almost always contained one or the other mating-type allele, but not both. With ten of the Banana ascospores, hyphae from both ends of each ascospore were recovered. The hyphae from one end contained either $Ban^+;Sk^S$ or $Ban^+;Sk^K$, while the hyphae from the other end contained *Ban* whose *Sk* constitution has not been determined. These results show that the Sk^S nuclei can exist in pure vegetative homokaryons, and that their survival does

† The numbers in parentheses are from a cross where the coupling phase of mating type was reversed with respect to the *Ban* and *Sk* parents; but the results are tabulated here according to appropriate segregation class.

‡ PD = parental ditype segregation; NPD = nonparental ditype; T = tetratype. The simplest interpretation is shown. *Sk-2* is assumed to segregate at first division, in accord with visual observations on thousands of intact asci.

§ = Segregation type could not be determined.

Linkage and recombination: Total numbers are 32 PD, 34 NPD, 17 T for *Sk*, *Ban*; 39 PD, 40 NPD, 4 T for *Sk*, *mt*; and 83 PD, 0 NPD, 18 T for *Ban*, *mt*. *Sk* is known to be at centromere of *III*, and *Ban* and *mt* in *IL*. The sequence (and percent recombination) in *IL* is therefore *Ban* (9) *mt* (2) centromere, based on these data.

not depend upon the presence of Sk^K. This further confirms that the Sk^S nuclei are completely undamaged in the developing Sk^K/Sk^S asci or giant ascospores.

Several alternative explanations have been ruled out. It might be argued that the *Ban* strains used in this study were in fact not genotypically *Sk* sensitive. However, in crosses of $Ban^+ \times Ban$, a few asci contain eight normal-sized ascospores. When such asci were found in $Sk^K \times Ban$ crosses, four of the eight ascospores aborted, confirming that the *Ban* parents used in this study were Sk^S. It is also conceivable that either (1) the Sk^S nuclei have become immune to killing while in the giant ascospores or, (2) that the Sk^K nuclei are rendered impotent or inoperative in the giant ascospores, so that they have no effect on Sk^S nuclei. If such changes occur at all, they are not permanent.

The possibility of immunity was examined by isolating four tetrads from one of the 8:0 tests (in Table 2). Four ascospores in each 8:0 ascus must contain derivatives of the Sk^S that had survived the Sk^K effect in the Sk^K/Ban giant ascospores, and the other four ascospores contain Sk^S nuclei from the tester strain. The cultures obtained from all eight ascospores were crossed to standard $Sk\text{-}2^K$ *N. crassa* strains. All tests produced 4:4 asci, meaning that the Sk^S nuclei that survived the Sk^K effect in the Banana ascospores had not been made permanently immune to killing. If Sk^S nuclei had been genetically altered, this should have been expressed as 8:0 asci in half of the tests for each of the four tetrads examined.

The second possibility, that the Sk^K nuclei are rendered impotent or inoperative, is also untenable. The $Sk\text{-}2^K$ alleles were fully potent in cultures derived from the Banana ascospores. No additional testing was necessary because the expected number (one-half) of the original Ban^+ tests (Table 2) contained 4:4 asci, proving that no heritable alteration had occurred in the Sk^K nuclei.

Although the possibility cannot be excluded that some killing of Sk^S nuclei still occurs in the giant ascospores, Table 2 results show that such killing certainly cannot be extensive.

Experiments with Fsp: Spore-killer behavior was also examined by combining $Sk\text{-}2^K$ with *Fsp* (Four spored), a semidominant mutant causing production predominantly of four ascospores per ascus rather than eight (RAJU 1979). In the four-spored asci of *Fsp*, one of the two postmeiotic mitoses is omitted, and four ascospores are delimited, each containing a single nucleus. The four ascospores later become binucleate. Meiosis, segregation of markers and ascospore viability are all normal. In the four-spored asci (from *Fsp*; $Sk^K \times Fsp;Sk^S$), two ascospores mature normally and the other two degenerate (Figure 9b). This proves that one postmeiotic mitosis is not essential in order for ascospore death to occur.

Fsp crosses occasionally produce asci in which only two very large ascospores are delimited. Initially, each ascospore contains a single nucleus that divides only once in the immature ascospores. Since diploid mitoses are unknown in Neurospora, the two divisions in these asci are most simply interpreted as being meiotic divisions and the missing divisions as the two postmeiotic mitoses. The two-spored asci from *Fsp*; $Sk^S \times Fsp$; Sk^K were examined cytologically to determine if one of the two ascospores degenerates as expected. In 23 out of 48 two-spored asci, one ascospore aborted (Figure 10e). In the remaining 25 asci, both asco-

spores developed through the rib-formation stage and formed some pigment (Figure 9d). In these asci, ascospore death due to *Sk* is much delayed, if it occurs at all.

The 1:1 asci would be expected as a result of first-division segregation of *Sk*, whereas both ascospores would be expected to develop (2:0 asci) if *Sk* segregated at the second division. Such a high frequency of 2:0 asci might suggest a high crossover rate between *Sk* and centromere in the *Fsp* genotype. This suggestion is, however, untrue, because *Sk* always showed the normal first-division segregation in the four-spored and eight-spored asci of *Fsp*. Progeny tests of appropriately marked crosses were not made on the two-spored asci, because of their rarity and poor ascospore germination.

The Four spored mutant has also been used in a manner similar to *Ban* to show that Sk^S nuclei are not killed when they are included in the same ascospore with Sk^K. Only three ascospores are formed in some *Fsp*; *Sk-2*^{S}*/Fsp*; *Sk-2*^{K} asci; usually the middle ascospore is the largest (Figure 9c). Both Sk^K and Sk^S nuclei are usually present in the abnormally large middle ascospore, because *Sk-2* almost always segregates at the first meiotic division. A cytological examination of iron-hematoxylin-stained asci showed that all four nuclei in the large ascospores are morphologically alike, without any signs of nuclear degeneration. In these three-spored asci, two ascospores mature, the middle larger ascospore (having both *Sk-2*^{K} and *Sk-2*^{S} nuclei) and one of the two smaller ascospores (*Sk-2*^{K}). The remaining smaller ascospore that contains the *Sk-2*^{S} nucleus degenerates (Figure 9c).

Cultures from 16 randomly obtained, abnormally large ascospores were crossed to both standard testers for determining their nuclear types. These are known to be mostly from three-spored asci in which the middle spore is the largest. Five tests contained both *Sk-2*^{K} and *Sk-2*^{S}, whereas eight showed only *Sk-2*^{K} and three showed only *Sk-2*^{S}. These results are similar to those obtained from Sk^K*/Ban* giant ascospores that were described earlier, except that with *Fsp*/Sk^K all tests are scorable for *Sk*, whereas with the Banana ascospores, only the Ban^+ tests could be scored for *Sk*. The *Fsp* experiment provides further proof that *Sk*-sensitive nuclei are not affected while they are together with Sk^K nuclei in the large ascospores.

Tests using cycloheximide and temperature treatments: The Spore killer system in Neurospora does not involve a component that behaves like the RNA-containing cytoplasmic factor in the killer strains of Saccharomyces (Fink and Styles 1972; Wickner 1974) or Ustilago (Koltin and Day 1976). In exploratory experiments, either *Sk-2*^{K} strains or *Sk-2*^{K} × *Sk-2*^{S} crosses were subjected to chemical and temperature treatments. The effect of cycloheximide was studied in two crosses that were heterozygous for *Sk-2* alleles: (1) A Spore killer strain was grown on synthetic crossing medium (SC) containing cycloheximide (0.12, 0.25, and 0.50 μg per ml of medium), and these cultures were used to fertilize a spore-killer-sensitive strain that was grown on SC without cycloheximide. (2) The same spore-killer cultures that were grown on cycloheximide were fertilized with an untreated Sk^S strain. In the first case, normal numbers of perithecia

were formed and produced 4:4 asci. In the second case, the cycloheximide in the crossing medium greatly inhibited perithecial development. The few perithecia that matured nevertheless produced 4:4 asci as usual, showing that cycloheximide did not prevent the expression of *Sk*. High-temperature (34°) treatment of the Sk^K parent before it was used for fertilizing crosses, or of $Sk^K \times Sk^S$ crosses during ascospore delimitation, had no effect on *Sk* expression. Similarly, when crosses were carried out at low temperature (15°), the expression of *Sk* was not affected.

DISCUSSION

Time of expression of Sk *and of other spore- or gamete-killing systems*

Precise timing of the expression of *Sk* is critical for probing its possible modes of action. The evidence presented in this paper indicates that the lethal effect of *Sk* is expressed some time after the killer and sensitive alleles have been sequestered into separate ascospores, although the primary effect may occur before the ascospores are delimited. The visible course of events leading to the death of Sk^S ascospores superficially resembles that suffered by deficiency ascospores in a rearrangement heterozygote. In the latter, one or more nuclei may become deficient during meiosis, but these nuclei will complete both postmeiotic mitoses and normal ascospore delimitation without exhibiting any effects of deficiency. Thus, the time of visible degeneration in the doomed Sk^S ascospores does not necessarily bear any direct relationship to the time of primary lethal action by Sk^K upon the Sk^S allele.

Normally, two postmeiotic mitoses occur in Neurospora asci before *Sk* is expressed. These mitoses are not required for the expression of *Sk*, however. This is amply demonstrated in the four-spored asci of $Sk^K \times Fsp$, where one mitosis is omitted, yet two of the four ascospores regularly degenerate. Furthermore, in the rare two-spored asci from $Sk^K \times Fsp$, where both postmeiotic mitoses are believed to be omitted, one ascospore degenerates in some, but not all, asci. The failure of *Sk* to be expressed in some two-spored asci might mean that the primary effect of Sk^K does not occur until considerably after the first meiotic division; thus, some asci that delimit ascospores before the second meiotic division may circumvent the critical stage.

In Podospora, PADIEU and BERNET (1967) have described a case of ascospore abortion that closely resembles *Sk* behavior in Neurospora. In crosses heterozygous for two unlinked ascospore abortion factors (*a* and *b*), meiosis and ascospore delimitation are normal, but certain ascospores abort depending upon what allele or combination of alleles of these two factors the ascospores receive (see TURNER and PERKINS 1979). All eight nuclei in these asci are normal until after ascospores are cut out. Thus, the time of *a*- or *b*-induced ascospore death in Podospora is comparable to that of *Sk* in Neurospora.

In Nicotiana (CAMERON and MOAV 1957), wheat (LOEGERING and SEARS 1963) and tomato (RICK 1966), the pollen-killing effect is expressed postmeiotically. In all three plant species, meiosis is normal in heterozygous anthers, except that the microspores not carrying the killer allele (or chromosome) begin

to degenerate shortly after microspore quartets are formed. (In tomato, megaspores are also affected similarly.) At least in wheat, it is known that the microspores degenerate prior to the first pollen mitosis.

In Drosophila, TOKUYASU, PEACOCK and HARDY (1977) have shown that Segregation Distorter (*SD*) is expressed during the latter half of spermiogenesis. All 16 primary spermatocytes in each cyst undergo meiosis simultaneously and form 64 spermatids with a single nucleus in each. No further mitoses intervene between meiosis and sperm differentiation. TOKUYASU, PEACOCK and HARDY (1977) have also shown that the nuclear chromatin in SD^+ spermatids fails to undergo a normal condensation process, thus leading to gradual degeneration of spermatids. MANGE (1968) used temperature treatments to show that the *SD* effect can be altered if treatments are given early in meiosis, even though *SD* expression occurs only during sperm differentiation.

Thus, in Drosophila and in the other gamete or spore-killer systems cited, the effects seem to be expressed after the completion of meiosis. At least in Drosophila and wheat, no postmeiotic mitosis intervenes before killing.

Absence of killing when nuclei are not sequestered

The present observations on Sk^K/Ban and Sk^K/Fsp clearly show that there is no damaging effect of Sk^K on Sk^S while they are together in a common cytoplasm. These observations also show that ascospore wall formation *per se* has no consequence on the expression of *Sk*, but that sequestering of Sk^K and Sk^S alleles into separate ascospores is essential for killing to occur. In Podospora, the ascospore abortion alleles (a_1/a_2 and b_1/b_2) interact similarly in the binucleate ascospores (PADIEU and BERNET 1967). In doubly heterozygous crosses, ascospores abort when both nuclei are a_1 or b_2, or a_1b_2. An ascospore survives if it contains at least one each of a_2 and b_1 alleles, either in the same nucleus or in different nuclei; the nonsister nucleus in the same ascospore is then unaffected, irrespective of its genotype.

In contrast, when both *SD* and SD^+ are enclosed in the same nucleus of a Drosophila spermatid, that spermatid does not become differentiated into a functional sperm (SANDLER and CARPENTER 1972). Similarly in wheat, LOEGERING and SEARS (1963) have reported that *Ki ki* microspores abort in *Ki Ki ki ki* tetrasomics and *Ki Ki ki* trisomics, but not in *Ki ki ki* trisomics. They suggest that killing is an effect of maternal tissue on microspores and that its severity depends on the relative dosage of *Ki* and *ki*. We have no way at present of varying the intranuclear dosage of *Sk* alleles in Neurospora. It should be possible, however, to obtain multinucleate ascospores containing various numbers of Sk^K and Sk^S nuclei and to determine whether killing is found with some ratios but not with others.

Hypotheses regarding the mechanism of Sk *action*

Light microscopy has not provided any indication whether killing is due to events in the cytoplasm, in the nucleus, or at the chromosomal level. No effect of *Sk* is visible in crosses of $Sk^K \times Sk^S$ until after ascospores have been formed. Meiosis and the two postmeiotic mitoses appear to be perfectly normal. No irre-

versible changes could have taken place in SK^S nuclei prior to ascospore delimitation, because Sk^S nuclei can still be rescued if they are not sequestered into separate ascospores, but are included in the same ascospore with Sk^K.

Killer and protective functions: A single-function model for Spore killer might be proposed, which assumes that Sk^K acts merely to turn off a gene (or set of genes) on its homolog whose function is essential for normal ascospore development. However, such a model is inadequate, because it would not explain why the same essential function is not turned off on the Sk^K chromosome. It would thus be necessary to assume a dual function for Sk^K in the Sk^S/Sk^K asci—to kill Sk^S and to protect itself. The latter function may also protect Sk^S from being turned off while in the common cytoplasm, but not long after sequestration.

Restriction-modification systems: The dual function for Sk^K might suggest a molecular model based on the restriction-modification systems of prokaryotes (reviewed by ARBER 1974). However, if protection is by a modification mechanism that involves methylation, that should be highly stable, contrary to what is required to render Sk^S ascospores vulnerable. The same difficulty stands in the way of applying restriction-modification models generally to eukaryote development (RIGGS 1975).

Chromosome breakage or inactivation: Preferential chromosome breakage has been observed in some cases of meiotic drive (e.g., NEWTON, WOOD and SOUTHERN 1976, in Aedes; ERICKSON 1965, in Drosophila), but not in wheat, tomato, Nicotiana, *SD* in Drosophila, or Neurospora. However, breakage that occurred after ascospore formation should not kill the ascospore unless it entailed inactivation.

Specific loci are known to mediate chromosome inactivation in other systems (CATTANACH 1975). Imprinting (CROUSE 1960), which leads to delayed inactivation or breakage of single chromosomes or chromosome sets at a specific developmental stage, is a widespread and well-documented phenomenon (BROWN and CHANDRA 1977).

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LITERATURE CITED

ARBER, W., 1974 DNA modification and restriction. Prog. Nucl. Acid Res. Mol. Biol. **14**: 1–37.

BROWN, S. W. and H. S. CHANDRA, 1977 Chromosome imprinting and the differential regulation of homologous chromosomes. pp. 109–189. In: *Cell Biology. A Comprehensive Treatise. I. Genetic mechanisms of Cells*. Edited by L. GOLDSTEIN and D. M. PRESCOTT. Academic Press, New York.

CAMERON, D. R. and R. MOAV, 1957 Inheritance in *Nicotiana tabacum*. XXVII. Pollen killer, an alien genetic locus inducing abortion of microspores not carrying it. Genetics **42**: 326–335.

CATTANACH, B. M., 1975 Control of chromosome inactivation. Ann Rev. Genet. **9**: 1–18.

CROUSE, H. V., 1960 The controlling element in sex chromosome behavior in Sciara. Genetics **45**: 1429–1443.

ERICKSON, J., 1965 Meiotic drive in Drosophila involving chromosome breakage. Genetics **51**: 555–571.

FINK, G. R. and C. A. STYLES, 1972 Curing of a killer factor in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. U.S. **69**: 2846–2849.

KOLTIN, Y. and P. R. DAY, 1976 Inheritance of killer phenotypes and double-stranded RNA in *Ustilago maydis*. Proc. Natl. Acad. Sci. U.S. **73**: 594–598.

LOEGERING, W. Q. and E. R. SEARS, 1963 Distorted inheritance of stem-rust resistance of Timstein wheat caused by a pollen-killing gene. Can. J. Genet. Cytol. **5**: 65–72.

MANGE, E. J., 1968 Temperature sensitivity of segregation-distortion in *Drosophila melanogaster*. Genetics **58**: 399–413.

NEWTON, M. E., R. J. WOOD and D. I. SOUTHERN, 1976 A cytogenetic analysis of meiotic drive in the mosquito, *Aedes aegypti* (L.). Genetica **46**: 297–318.

PADIEU, E. and J. BERNET, 1967 Mode d'action des gènes responsibles de l'avortement de certains produits de la méiose chez l'Ascomycète *Podospora anserina*. Comp. Rend. Acad. Sci. Paris Sér.D **264**: 2300–2303.

RAJU, N. B., 1978 Meiotic nuclear behavior and ascospore formation in five homothallic species of Neurospora. Can. J. Bot. **56**: 754–763. ——, 1979 A four-spored mutant of *Neurospora crassa*. Exp. Mycol. **3**: 270–280.

RAJU, N. B. and D. NEWMEYER, 1977 Giant ascospores and abnormal croziers in a mutant of *Neurospora crassa*. Exp. Mycol. **1**: 152–165.

RICK, C. M., 1966 Abortion of male and female gametes in the tomato determined by allelic interaction. Genetics **53**: 85–96.

RIGGS, A. D., 1975 X inactivation, differentiation, and DNA methylation. Cytogenet. Cell Genet. **14**: 9–25.

SANDLER, L. and A. T. C. CARPENTER, 1972 A note on the chromosomal site of action of *SD* in *Drosophila melanogaster*. pp. 233–246. In: *Proc. Int. Symp., The Genetics of the Spermatozoon*. Edited by R. A. BEATTY and S. GLUECKSOHN-WAELSCH.

SINGLETON, J. R., 1953 Chromosome morphology and the chromosome cycle in the ascus of *Neurospora crassa*. Am. J. Bot. **40**: 124–144.

TOKUYASU, K. T., W. J. PEACOCK and R. W. HARDY, 1977 Dynamics of spermiogenesis in *Drosophila melanogaster*. VII. Effects of segregation distorter (*SD*) chromosome. J. Ultrastruct. Res. **58**: 96–107.

TURNER, B. C. and D. D. PERKINS, 1979 Spore killer, a chromosomal factor in Neurospora that kills meiotic products not containing it. Genetics **93**: 587–606.

WICKNER, R. B., 1974 "Killer character" of *Saccharomyces cerevisiae*: curing by growth at elevated temperature. J. Bacteriol. **117**: 1356–1357.

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