INTRAGENIC RECOMBINATION OF ASCOSPORE COLOR MUTANTS IN ASCOBOLUS AND ITS RELATIONSHIP TO THE SEGREGATION OF OUTSIDE MARKERS[1]

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STUDIES of intragenic recombination in Neurospora and in Ascobolus have led to conflicting conclusions about the basic nature of this event. (The term "recombination" is used here to mean the event which produces a recombinant; it may be either an exchange of strands between the two segregating sites or—probably more often—a 3:1 segregation *at* one of the sites.)

MURRAY (1963) studied recombination in pairwise crosses involving 19 mutant alleles at the *me-2* locus of Neurospora. She constructed a map of the locus based on recombination frequencies, and she observed the segregation of outside markers in the selected recombinants. She noted a polarized pattern of marker segregation which led her to propose that recombination took place in "fixed pairing regions." The probability of recombination for a site near the end of such a region was different from that for a site near the middle. This explained the marker polarity, assuming that the markers revealed which of the two mutants was more frequently the site of the event. Thus she concluded that the recombination rates for the various mutant alleles at *me-2* were dependent on their *positions* in the pairing region.

KRUSZEWSKA and GAJEWSKI (1967) studied recombination tetrads from Ascobolus crosses involving seven allelic ascospore color mutants. They determined the frequency of gene conversion (3:1 or 1:3 segregation) of each mutant crossed to wild type, and they compared these results with the recombination in the mutant × mutant crosses. They concluded that the frequency of recombination did not show a simple relationship to the distance separating the mutant sites but was instead a function of the intrinsic frequencies of gene conversion of the two mutants. These conversion frequencies did *not* show a simple relation to physical position; the mutant with the most frequent conversion was very close to a mutant showing no conversion. Such a system of recombination should not generate the kind of regular polarity observed by MURRAY.

ROSSIGNOL (1969) studied 15 ascospore color mutants at another locus in Ascobolus. When frequencies of gene conversion for different mutant alleles were plotted against their positions on the map, no simple polarity was revealed. However, the author pointed out that the mutants could be grouped into several classes on the basis of their ratios of 3:1 *vs.* 1:3 segregation; within any single class, a

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polarity of conversion frequency *was* shown. He suggested that a class might include all those mutants which resulted from the same kind of change in base sequence.

The comparison of these studies is complicated by differences in the materials and the techniques. The Neurospora work included no analysis of tetrads, so it was not possible to ascertain whether (or how often) gene conversion had occurred at one or another mutant site. The Ascobolus systems had no outside markers.

The present work was started in an attempt to reconcile these two observations by getting both kinds of information in the same system. This paper reports the isolation of a set of allelic ascospore color mutants in *Ascobolus immersus*, the identification of markers outside this locus, and the preliminary analysis of recombination at this locus in crosses segregating for the markers.

MATERIALS AND METHODS

Strains: Wild-type strains P5(—) and K5(+) of the Pasadena collection of *Ascobolus immersus* were kindly supplied by Dr. CLARE C. C. YU-SUN and Dr. STERLING EMERSON. All strains used in this work were descendants of the cross between these two wild-type cultures.

Media: Minimal, complete, and crossing media were prepared according to the directions of YU-SUN (1964). Ascospores were germinated on the surface of 1.25% Bacto-Peptone containing 4% agar (LISSOUBA 1960).

Crosses: The two parent strains were inoculated at opposite edges of a 50 mm Petri dish containing 10 ml of crossing medium. These plates were incubated for 24 hr in darkness at 25°C, and then in day–night cycles at 22°C until mature ascospores were beginning to eject from the apothecia (about 12 days). Light-dark cycles were provided either by daylight (the cross plates were near a window but out of direct sunlight) or by a constant-temperature incubator with alternating 12-hr cycles of light and dark.

Octads of ascospores were collected by inverting open plates of 4% agar over the open cross plates. Collecting plates were changed as often as necessary to get the desired density of ascospores. At its peak, a fertile cross may eject several hundred asci in a few minutes.

The octads were scored for color patterns. (Clusters containing more or less than eight spores were ignored throughout this work). Those octads selected for further study were isolated on germination medium and dissected with a glass needle. The ascospores were activated in a 50°C oven for 75 min and then incubated at 25°C. Germination required about 6–10 hr and the spores continued to grow, forming small mycelia. Agar blocks (about 1 mm^3) were cut from these mycelia and transferred to culture tubes or to growth plates for classification of segregating mutants. (*Ascobolus immersus* does not produce asexual spores, therefore all vegetative transfers were made with mycelial samples.) Plates containing minimal medium plus 4% sorbose were used to classify colonial mutants, and the same medium plus 15 μg/ml *para*-fluorophenylalanine was used to score resistance to this substance.

Isolation of mutants: Ascospore color mutants were detected by scanning collecting plates of asci from wild-type crosses for octads containing four colorless spores. Single spores from such asci were isolated, germinated and crossed to wild type to demonstrate one-gene segregation: consistent 4:4 patterns in the progeny. Pairwise crosses between newly isolated colorless mutants were used to test for allelism. Mutants were assumed to represent the same gene if the cross between them gave more than 99% parental ditype asci (0 colored:8 colorless). All new mutants were crossed to a representative allele of the *w17* locus, and 26 alleles at this locus have been identified.

The eight spores of an ascus remain together when ejected from the apothecium because they are contained in a transparent, gelatinous coat. In order to isolate growth mutants (nutritional

or morphological), the spores were separated before activation and germination. The gelatinous coat was digested by placing a few drops of 0.1% pronase-B on the collecting surface (containing about 1000 asci) for ten minutes. The surface was then flooded with 1–2 ml of water and the ascospores (now separated) were drawn up in a Pasteur pipette and spread on germination medium. (Ascospores will not germinate on minimal or complete medium.) They were activated at 50°C and incubated five hours at 25°C. At this time the spores were about to germinate and were capable of germination even when transferred to minimal medium (by flooding with water, pipetting, and spreading). These plates were incubated for several hours at 37°C and examined microscopically for growth mutants.

Induced mutants: The origins of the 26 mutant alleles at *w17* are as follows:

Spontaneous: *c,g,s*
UV: *a,b,d,e,f,h,i,j,l,m,n,o,p,q,r*
X ray: *k*
ICR: *u,v,w,x*
NMG: *t,y,z.*

For UV or X-ray treatment, the two wild-type parents were inoculated at opposite edges of a 50 mm Petri dish of crossing medium and allowed to grow until they had nearly reached the center of the plate (one or two days). The UV treatment was 90 sec under a GE germicidal lamp with an output of about 500 ergs/mm^2/second. The X-ray treatment was approximately 68,800 roentgens. The plates were incubated in the dark for 24 hr after treatment and then in light–dark cycles until mature ascospores appeared.

Mycelia growing on the surface of sheets of cellophane were treated with the chemical mutagens 2-methoxy-6-chloro-9- [3-(ethyl-2-chloroethyl) aminopropylamino] acridine dihydrochloride (ICR) and N-methyl-N′-nitro-N-nitrosoguanidine (NMG) in the following manner. A single layer of cellophane was placed on the surface of a plate of crossing medium and inoculated at the center with the wild-type strain. This was allowed to grow until a mycelium with a diameter of about 20 mm was produced. The cellophane was then peeled off the medium and floated (mycelium up) in a dish of mutagen solution. The ICR solution was 10 μg per ml in pH 7 phosphate buffer, and the treatment lasted 120 minutes in darkness. NMG concentrations of either 10 μg or 50 μg per ml were used for treatments lasting either ten or 120 min, all with similar results.

After treatment the material was rinsed three times in liquid minimal medium and placed on a fresh crossing plate. At the same time, untreated wild type of opposite mating type was inoculated at the edge of the plate. The plates were then incubated in light–dark cycles until maturity. Mycelia for the control crosses were exposed to the same procedure, with buffer substituting for mutagen solution.

The mutants which were isolated from UV-treated or X-ray-treated material may include an unknown fraction which were spontaneous. No treated control crosses were run in parallel with these treatments to determine the frequency of spontaneous mutants. The experiment with ICR and NMG did include an untreated control, and it showed a frequency of new colorless mutants (4:4 patterns) of one per 2500 asci. The NMG-treated crosses had frequencies five to ten-fold higher, and the ICR treatment resulted in ten to 100-fold enhancements of the control frequency of mutants.

Our knowledge of the lethality of the mutagen treatments is very limited. The mycelia had to survive to complete the sexual cycle, and this was always accomplished. However, we have no information about what fraction of the nuclei in the mycelia survived.

RESULTS

Tetrad analysis—assumptions: In the analysis of tetrads segregating for ascospore color mutants in Ascobolus, we derive the frequencies of recombination events from the frequencies of certain patterns among the clusters of eight spores.

The use of these frequencies to develop hypotheses about the mechanism of recombination involves several assumptions, three of which we shall examine.

1st assumption: An aberrant spore pattern represents an aberrant genetic segregation.

In a cross between a colorless spore mutant and wild type the common ascus type is 4:4. Nonreciprocal recombination at the segregating site may yield 6:2 or 2:6, and an event which results in a heterozygous spore pair may give 5:3 or 3:5 patterns. (Note: our segregation ratios state wild type first, mutant second; thus a 6:2 ascus has six wild-type spores and two mutant spores.) There are several spurious ways of producing these other patterns even when a 4:4 genetic ratio occurs at the segregating site:

a) phenocopy: one or more spore develops pigment even though it carries the mutant allele;

b) new mutation: a spontaneous *w* mutation at some other locus is present in one or more nuclei of one parent before the cross; this may produce 2:6 and 0:8 patterns;

c) false cluster: spores from two or more asci land on the collecting surface in the same cluster of eight.

Phenocopies can be detected by crossing the spores in the majority class to determine their genotypes.

New mutations in 2:6 and 0:8 asci are detected by crossing the colorless spores to the mutant parent. The crosses involving one of the three mutant spore pairs in a 2:6 ascus should give wild-type recombinants. Two spore pairs from the spurious 0:8 ascus give recombinants in this test.

False clusters are detected by coincident aberrant segregation at other loci. The best check is a segregating locus which is linked to the spore color locus. The false cluster which shows an aberrant pattern for spore color will very likely show a coincident pattern for the linked marker.

The cross test to detect phenocopies and new mutations is slow, so we decided

TABLE 1

Aberrant segregation in crosses of colonial-white mutants; numbers of asci with various patterns (colored: colorless)

Strain crossed to wild type	4:4	6:2	5:3	3:5	2:6	0:8
cw 24	7,070	3(2/2)	2(1/1)	7	6(0/5)*	2(0/2)
cw 213	830	7(6/6)	3(3/3)	3(0/3)	0	0
cw 221	2,740	9(6/7)	13(1/5)†	12(0/8)‡	3(1/2)	1(0/1)
cw 302	750	6(4/4)	2(0/1)	2(0/1)	2(0/1)	0

Figures in parentheses refer to aberrant asci which were subjected to further tests. The denominator is the number of asci tested, and the numerator is the number of those in which the aberrant ratio was confirmed.

* Three of these false 2:6 asci were tested by crosses, and all were shown to result from the spontaneous occurrence of new *w* mutations at other loci.

† Three of the four false 5:3 asci each contained one colored, colonial ascospore which was fused to a colored, noncolonial ascospore.

‡ Each of these asci contained one colorless, noncolonial ascospore; four of them were crossed and shown to be genetically w^+.

to seek spore color mutants which also had a detectable effect on vegetative growth. This would permit us to check the aberrant pattern of spore phenotype by the vegetative phenotype. Spores of about 60 newly isolated *w* mutants were germinated and grown overnight. Six of the mutants showed the restricted growth characteristic of colonial mutants and were designated *cw* (colonial white).

Two of these mutants were infertile, but the other four were crossed to wild type, and the results are shown in Table 1. Fifty-two of the 83 aberrant tetrads were germinated and grown overnight to score for colonial. This revealed that 28 of them were really segregating 4:4 and were, therefore, spurious. (In several cases the same ascospores were further tested by crossing, and the result invariably confirmed the conclusion from the vegetative growth test).

The 6:2 asci were nearly all valid, but all the other aberrant classes included numerous cases of spurious patterns. Several 5:3 phenocopies contained pairs of pigmented spores which were visibly fused. (They were separated with a glass needle before germination.) The protoplasmic bridge had permitted the passage of pigment or precursor into a *cw* cell.

Table 2 lists the aberrant segregations in crosses to wild type of eighteen different mutant alleles at the *w17* locus. Vegetative growth habit could not be used to check these asci, but 174 of them were checked by crossing. The result was similar to that observed with the *cw* mutants in that the 6:2 asci were nearly all valid, while the 5:3 and 2:6 asci were frequently spurious. Some of the false 5:3

TABLE 2

Aberrant segregation in crosses heterozygous for white ascospore mutants at the w17 *locus*

w17 allele crossed to w^+	Total asci	6:2	5:3	3:5	2:6	0:8	6:2 per 10^3
a(7)*	24,500	94(19/20)†	44(1/8)	43(12/14)	24(7/8)	14	3.8
b(5)	9,950	49(10/10)	47(3/4)	37(13/13)	36(0/1)	8	4.9
c(4)	13,610	2	2	2	2	28	0.2
d(3)	3,310	0	0	2	2	0	0
e(4)	3,490	3	6(0/2)	2	2	0	0.9
f(4)	4,140	0	2	3	5(0/2)	27	0
h(3)	2,560	1	2(0/1)	0	0	8	0.4
i(2)	1,600	4(2/2)	2(1/2)	1(0/1)	1	0	2.5
j(3)	4,270	14(1/1)	1	0	9(6/7)	78(1/1)	3.3
k(4)	2,660	3	4	4	6	0	1.1
l(3)	6,790	16(1/1)	8(1/1)	23(1/1)	6(1/2)	148(0/1)	2.4
m(3)	2,470	0	0	0	0	6	0
n(5)	17,160	29(11/11)	16(4/6)	13(2/2)	18(0/2)	10	1.7
o(1)	1,650	0	1	0	1(1/1)	0	0
p(3)	1,870	2	6	3	3	0	1.1
q(3)	9,020	8(4/4)	11(7/7)	7(2/2)	3	2	0.9
s(4)	8,920	1	3	6	37(5/5)	7	0.1
z(2)	20,850	6(4/4)	24(14/14)	5(1/1)	27(2/11)	30	0.3

* Number in parentheses following allele is the number of crosses analyzed.
† The denominators of fractions in parentheses are the numbers of the putative aberrant asci which were tested and the numerators are the numbers of those which proved to represent true aberrant genetic segregation.

TABLE 2a

Comparisons of 6:2 segregation frequencies for the same w17 *allele in different crosses or in different platings of the same cross*

Cross	Total asci	6:2	6:2 per 10^3	Cross	Total asci	6:2	6:2 per 10^3
*a*1	5,850	16	2.7	*l*1	4,070	11	2.7
*a*2	4,140	11	2.7	*l*2	850	0	0
	850	2	2.4	*l*3	1,870	5	2.7
*a*3	3,160	9	2.8				
*a*4	3,690	9	2.4	*n*1	3,780	2	0.5
*a*5	480	0	0	*n*2	970	1	1.0
*a*6	1,830	19	10.4	*n*3	680	2	2.9
	2,160	14	6.5		4,370	11	2.5
	770	7	9.1	*n*4	1,550	1	0.6
*a*7	1,000	4	4.0	*n*5	5,810	12	2.1
	560	3	5.3				
				*q*1	3,660	1	0.3
*b*1	4,210	15	3.6	*q*2	2,720	3	1.1
*b*2	1,210	16	13.2		1,210	4	3.3
	400	6	15.0	*q*3	1,100	0	0
*b*3	250	1	4.0		330	0	0
*b*4	2,340	5	2.1				
*b*5	1,150	5	4.3	*z*1	4,630	0	0
	390	1	2.6		6,120	2	0.3
				*z*2	3,200	2	0.6
*j*1	3,310	14	4.2		4,090	2	0.5
*j*2	630	0	0		2,810	0	0
*j*3	330	0	0				

This table includes the crosses to w^+ of all those *w17* alleles from which six or more 6:2 asci have been recovered.

asci resulted from fusions of spores, and some false 2:6 asci resulted from new *w* mutations. The results differed from those with the *cw* mutants in that the 3:5 asci in *w17* crosses were mostly valid.

Spurious color patterns are a problem in the analysis of mutant × wild type crosses because the normal asci have equal numbers of colored and colorless spores. The recombination event is not signaled by any unique spore type, but only by an unusual combination of the two types which are in abundance. This is not true in the mutant × mutant crosses. The normal (nonrecombinant) asci have only colorless spores. Therefore, recombination is signaled by the rare pigmented spores on the cross plate. Of the three kinds of spurious events listed earlier, only phenocopies could pose a problem here, and they apparently do not occur. Numerous cross tests of the pigmented ascospores from crosses between different *w17* alleles have consistently verified their w^+ classification.

2nd assumption: Aberrant segregation frequency is genetically determined. The possibility that uncontrolled environmental factors have some influence can be checked in those cases in which the same cross has been made and analyzed two or more times. Table 2a lists ten such crosses (*a*2, *a*6, *a*7, *b*2, *b*5, *n*3, *q*2, *q*3,

TABLE 3

Recombination between alleles at the w17 *locus*

Parents	Total asci	2:6 asci	2:6 per 10^3 asci	Sum of 6:2 segregation frequencies per 10^3 asci (Table 2)	2:6 frequencies in separate crosses
a x b	27,800	19	0.7	8.7	6/2,410 4/7,000 1/2,460 2/5,010 6/10,930
a x c	44,850	179	4.0	4.0	11/1,010 17/4,020 15/1,940 47/10,310 11/7,140 78/20,430
a x d	18,160	87	4.8	3.8	7/1,600 65/13,980 15/2,580
a x e	18,660	58	3.1	4.7	3/500 2/710 3/960 50/16,490
a x f	36,140	122	3.4	3.8	1/420 45/15,040 0/1,020 76/19,660
a x g	6,240	36	5.8		31/4,870 3/1,160 2/210
a x h	13,580	60	4.4	4.2	
a x i	3,610	8	2.2	6.3	3/2,000 1/780 4/830
a x j	9,490	26	2.7	7.1	1/1,000 24/7,860 1/630
a x k	29,420	76	2.6	4.9	8/3,300 4/1,000 29/10,800 35/14,320
a x l	20,700	77	3.7	6.2	4/1,000 66/18,670 2/530 5/500
a x m	6,890	23	3.3	3.8	12/4,350 11/2,540
a x n	17,830	123	6.9	5.5	25/6,530 97/10,840 1/460
a x o	14,520	99	6.8	3.8	42/8,380 2/680 35/4,300 20/1,160
a x p	1,810	5	2.8	4.9	1/960 4/850
a x q	22,450	130	5.8	4.7	
a x r	5,780	11	1.9		7/4,230 4/1,550
a x s	11,490	66	5.7	3.9	32/5,700 34/5,790
b x c	9,550	118	12.4	5.1	95/5,410 21/3,870 2/270
b x d	24,980	136	5.4	4.9	14/950 0/160 65/12,760 57/11,110
b x e	2,320	23	9.9	5.8	15/1,270 8/1,050
b x f	7,430	61	8.2	4.9	22/3,290 39/4,140
b x g	2,110	25	11.9		
b x h	13,550	109	8.1	5.3	19/2,790 18/3,140 27/2,620 45/5,000
b x i	2,950	32	10.8	7.4	
b x j	3,860	28	7.3	8.2	

TABLE 3—Continued

Parents	Total asci	2:6 asci	2:6 per 10^3 asci	Sum of 6:2 segregation frequencies per 10^3 asci (Table 2)	2:6 frequencies in separate crosses
b x k	12,310	66	5.4	6.0	22/4,260 44/8,050
b x l	4,430	19	4.3	7.3	19/4,160 0/270
b x m	10,200	48	4.7	4.9	42/4,800 6/5,400
b x n	14,540	38	2.6	6.6	
b x q	1,680	2	1.2	5.8	
b x r	1,340	11	8.2		3/470 8/870
b x s	5,410	40	7.4	5.0	40/4,570 0/540 0/300
c x d	1,240	0	0	0.2	0/920 0/320
c x e	12,160	0	0	1.1	
c x f	2,040	0	0	0.2	
c x g	13,510	0	0		0/6,150 0/7,360
c x h	1,140	0	0	0.6	
c x j	15,530	68	4.4	3.5	
c x k	19,650	54	2.7	1.3	
c x l	15,470	124	8.0	2.6	75/8,180 49/7,290
c x n	12,590	30	2.4	1.9	
c x o	26,220	7	0.3	0.2	1/10,370 6/15,850
c x r	1,160	0	0		
c x s	7,320	0	0	0.3	0/1,070 0/6,250
d x e	4,190	1	0.2	0.9	
d x g	10,650	2	0.2		2/5,900 0/4,750
d x i	4,840	6	1.2	2.5	0/690 6/4,150
d x j	2,780	6	2.2	3.3	

*z*1, *z*2). The frequencies of 6:2 asci are seen to be fairly homogeneous in duplicate crosses. (Other patterns are not considered here because of the uncertainty about the frequencies of true aberrant segregations.) Table 3a lists the frequencies of recombination (2:6) asci in those mutant × mutant crosses which were made and analyzed more than once. Again the homogeneity appears to be satisfactory.

3rd assumption: The frequency of recombination events at a particular locus

TABLE 3—Continued

Parents	Total asci	2:6 asci	2:6 per 10^3 asci	Sum of 6:2 segregation frequencies per 10^3 asci (Table 2)	2:6 frequencies in separate crosses	
d x k	2,620	5	1.9	1.1		
d x m	6,250	0	0	0		
d x o	4,060	1	0.2	0		
d x q	3,740	1	0.3	0.9	1/2,170	0/1,570
d x r	3,220	1	0.3			
d x s	2,050	0	0	0.1		
e x f	2,100	12	5.7	0.9		
e x g	2,650	0	0		0/390	0/2,260
g x h	1,330	0	0			
g x o	4,130	4	1.0			
g x p	1,810	3	1.7			
h x s	5,380	0	0	0.5		
k x s	1,100	4	3.6	1.2		
o x q	5,530	14	2.5	0.9	2/910	12/4,620
o x s	5,260	3	0.6	0.1		

This table includes all pairwise combinations of *w17* alleles for which at least 1,000 asci have been scored.

is determined entirely by the genetic composition at that locus; it is not influenced by other parts of the genome.

This assumption is tested by comparing crosses involving the same alleles at the *w17* locus in different genetic backgrounds. Most of the mutant alleles give consistently very low frequencies of 6:2 asci. However, the frequencies for *w17a* and *w17b* are high enough to permit some comparisons between different crosses. Among the seven crosses of *w17a* $\times$ w^+ (Table 2a), six have similarly low frequencies of 6:2 asci, while the frequency in cross *a6* is three-fold higher. Similarly, the frequency in cross *b2* is much higher than in the other four crosses of *w17b* $\times$ w^+. Table 3 gives several examples of different crosses between the same pairs of *w17* mutant alleles in which there are significant fluctuations of recombination frequency. It is clear that there are genetic elements outside the *w17* locus which influence these frequencies.

It is not known whether a single factor is responsible for these differences; nor is it known whether such a factor is linked to *w17*. We do not know whether the

TABLE 3a

Recombination between alleles at the w17 *locus; frequencies of 2:6 asci in different platings of the same cross*

Cross	Total asci	2:6	2:6 per 10^3
a x b	1,340	1	0.7
(#3)	1,120	0	0
a x n	5,100	48	9.4
(#2)	5,740	49	9.5
a x o	2,530	10	4.0
(#1)	2,750	14	5.1
	3,100	18	5.8
b x d	8,190	45	5.5
(#3)	2,650	12	4.5
	1,920	8	4.2
b x d	5,680	29	5.1
(#4)	2,240	11	4.9
	3,140	19	6.0
b x k	2,050	9	4.4
(#2)	3,600	24	6.7
	2,400	11	4.6

same determinants have similar effects on the frequencies of 6:2 segregation for different alleles at *w17* or for mutants at other loci. Answers to these questions may give important information about the genetic control of recombination and about the basic nature of the recombination process. Furthermore, until these recombination determinants are known and properly assessed, mapping by recombination frequencies can be done only tentatively. However, problems of sterility and inviability have frustrated our efforts to answer these questions.

An earlier study of ascospore color mutants of Ascobolus (EMERSON and YU-SUN 1967) reported dramatic differences of aberrant segregation frequency for the same colorless spore mutant in different crosses, and there have been numerous reports of influences of genetic background on recombination frequency in Neurospora (for references, see CATCHESIDE 1968; STADLER and KARIYA 1969).

We conclude that 6:2 spore patterns do represent aberrant genetic segregation, while other aberrant patterns must be studied further. The frequencies of these aberrant segregations are not significantly affected by environmental fluctuations under the conditions used in this study. Certain alleles (*w17a*, *w17b*) exhibit 6:2 segregation frequencies which are consistently higher than those of other alleles; however, one or more separate genetic elements which have marked effects on these frequencies are segregating in our stocks.

Recombination frequency map of the w17 *locus:* Aware of the pitfalls of map-

ping by recombination frequency, let us nevertheless examine the data of Tables 2 and 3 to see what arrangement of the mutant sites seems most reasonable. We should like to know whether the frequency of nonreciprocal (6:2) segregation is determined by the position of the segregating site. If so, the sites might be arranged in order of increasing frequency of 6:2 segregation, and the data from Table 2 would generate the following order:

$$(d, f, m, o)\text{–}s\text{–}c\text{–}z\text{–}h\text{–}(e, q)\text{–}(k, p)\text{–}n\text{–}l\text{–}i\text{–}j\text{–}a\text{–}b.$$

The frequencies of recombination in mutant $\times$ mutant crosses (Table 3) may be examined to see if they appear to be compatible with this (or any) order. We may attempt to order the sites by the simple frequency $\propto$ distance relationship: the order of three sites α–β–γ must be such that the frequencies of recombination for the internal segments (α–β and β–γ) are both lower than the frequency in the cross between the outside sites (α–γ). There is no single order of the mutant sites for which all the data of Table 3 can fit this rule. However, it is notable that the cross $a \times b$ gives a lower recombination frequency than any cross of either of these mutants to any other mutant. This suggests that these two mutants which show the highest 6:2 segregation frequencies are close to each other and more distant from the other mutant sites.

KRUSZEWSKA and GAJEWSKI (1967), in a study of allelic ascospore color mutants of Ascobolus, concluded that the recombination frequency between allelic mutant sites is not in simple proportion to the distance separating them, but is complicated by their intrinsic frequencies of 6:2 segregation. They concluded that their seven mutants fell into two clusters. Any cross between two mutants in the same cluster gave a very low frequency of recombination, while a cross between mutants in separate clusters gave a frequency similar to the sum of the frequencies of 6:2 segregation for the two participating mutants. The idea was that the 6:2 event (gene conversion) extended over a segment longer than the length of a cluster, and co-conversion at the two segregating sites would not yield a recombinant. A direct demonstration of the high frequency of co-conversion for sites very close together has been made in yeast by FOGEL and MORTIMER (1969).

We apply the cluster-mapping method by comparing the recombination frequencies to the sums of the conversion frequencies for the participating mutants (Table 3). According to KRUSZEWSKA and GAJEWSKI, if the recombination frequency is markedly lower than the conversion sum, the two mutants are clustered. This method emphasizes the evidence for tight linkage between a and b, because their conversion rates are the highest of all. It also suggests clustering of a and j, which would be consistent with the order based on increasing conversion rates.

The cluster-mapping method is of no help in placing the low-frequency conversion mutants ($d,f,m,o,r,s,c,q,p,e,k,n,h$) with respect to each other, because the observed low frequencies of recombination are expected whether or not the two mutants are clustered.

KRUSZEWSKA and GAJEWSKI found no cases in which the recombination frequency was markedly *higher* than the sum of conversion frequencies, and they offered no basis for expecting such a result. The several cases of this type observed in our results ($b \times c$, $c \times l$, $e \times f$) may indicate the presence of extragenic ele-

TABLE 4

Linkage of w17 *to C* (colt) *and R* (fpr)

Cross	Total asci	Non-crossovers	Single crossovers C–w	Single crossovers w–R	Multiple crossovers	Total crossovers C–w	Total crossovers w–R
Ca+ × ++*R*	104	81	16	5	1 3SD–I,II 1 4SD–I,I	19	6
+*a*+ × *C*+*R*	30	21	7	2		7	2
+*b*+ × *C*+*R*	42	29	9	3	1 2SD–I,II	10	4
+*c*+ × *C*+*R*	4	3	0	1		0	1
+*d*+ × *C*+*R*	50	42	7	0	1 3SD–I,II	8	1
+*e*+ × *C*+*R*	12	9	1	1	1 2SD–I,II	2	2
+*g*+ × *C*+*R*	20	13	5	1	1 3SD–I,II	6	2
+*h*+ × *C*+*R*	22	18	3	1		3	1
+*i*+ × *C*+*R*	26	16	6	3	1 4ST–I,II,II	7	5
+*j*+ × *C*+*R*	11	6	4	1		4	1
+*k*+ × *C*+*R*	17	15	1	1		1	1
+*l*+ × *C*+*R*	21	17	3	1		3	1
+*m*+ × *C*+*R*	23	18	3	2		3	2
+*n*+ × *C*+*R*	22	15	5	2		5	2
+*oR* × *C*++	28	24	4	0		4	0
+*p*+ × *C*+*R*	25	22	3	0		3	0
+*s*+ × *C*+*R*	8	7	1	0		1	0
Ct+ × ++*R*	49	31	15	2	1 3SD–I,II	16	3
Cu+ × ++*R*	48	35	11	2		11	2
Totals	562	422	104	28		113	36

Abbreviations: 3SD–1,II: 3-strand double crossover in regions I and II. 4ST–I,II,II: 4-strand triple crossover with one exchange in region I and two in region II.

The cross parents are abbreviated by using capital letters to represent the mutant alleles of the markers: *C* represents *colt* and *R* represents *fpr*. The lower case letters represent *w17* alleles.

Calculation of coincidence of crossing over in both regions shows no chiasma interference. The expected (excluding multiples *within* a region) is 111/562 × 34/562 × 562=6.7. The observed is seven.

ments which raise recombination frequencies, like those present in crosses *a*6 and *b*2 of Table 2.

It may be concluded that the frequencies of recombination from the two-point crosses do not provide us with precise information about the arrangement of the mutant sites, but the broad trends are compatible with an order based on increasing frequency of gene conversion.

Markers linked to w17: Ascospores from a UV-treated cross between wild-type strains were screened on minimal medium containing an inhibitory level (15 μg/ml) of *p*-fluorophenylalanine. A resistant strain (*fpr*) was isolated and shown to represent a single gene mutation by its consistent 4:4 segregation in asci from a cross to a sensitive (wild-type) strain. The *fpr* strain was then crossed to representative spore color mutants for about 20 different loci. It showed close linkage (3% recombination) to *w17*, one of the loci for which we had already isolated several mutant alleles.

A systematic search for more *w17* alleles was made with progeny from wild-type crosses which had been treated with mutagens (UV, X ray, ICR, NMG),

and there are now 26 mutant alleles at this locus available for study. At the same time, a search was begun for a linked marker on the other side of the *w17* locus. Separated ascopores from UV-treated crosses were screened for growth on minimal medium at 37°C. Those which germinated but grew slowly or abnormally or not at all beyond germination were isolated on complete medium and incubated at 18°C. This procedure was designed to detect both nutritional and morphological mutants whether they were mutant at all temperatures or only at high temperature. However, the only types recovered were colonial mutants which did not respond to complete medium. Some were colonial at both high and low temperatures, but these tended to give poor crosses, so further study was limited to the colonial-temperature (*colt*) mutants. Twenty-five of these were crossed to the double mutant *w17 fpr*. Three showed linkage, but two of these were difficult to score in some of the progeny of the crosses. The one remaining *colt* mutant was found to be ten map units from *w17*, on the side opposite *fpr*.

Table 4 shows the analysis of 562 asci from 19 different crosses which were segregating at these three loci. There is no evidence of crossing over interference between the two marked regions. Other crosses to a centromere marker have shown that *w17* is loosely linked to its centromere, but it is unlikely that the centromere intervenes between *w17* and either of the markers.

Mapping the w17 *locus by the segregation of outside markers:* A popular method for ordering mutant sites within a gene depends on the segregation of outside markers in asci with intragenic recombination. In the cross *A m–1 B* × *a m–2 b*, the m^+ recombinants are selected and scored for the outside markers. If the order of sites were *A–m-1–m-2–B*, a single exchange would yield *a* m^+B, while a triple exchange would be required to produce the reciprocal marker combination *A* m^+b. Assuming that single exchanges are more likely than triple exchanges, one concludes that the more frequent of these two nonparental marker combinations among the recombinants reveals the order of the mutant sites in gene *m*.

Table 5 summarizes the results of crosses between different alleles at *w17* in which markers on both sides were segregating. In only a few crosses are there sufficient data to show a significant difference between the two nonparental marker combinations. However, a pattern is apparent. The analysis to date of all crosses of *a* or *b* to other mutants indicates that *a* and *b* are at the right end of the locus. Only one cross not involving *a* or *b* has sufficient results to determine order; it suggests that *l* is to the right of *c*. Many more recombinants must be scored before a complete map of the *w17* locus can be constructed and tested for internal consistency. The results to date are compatible with an order of sites based on increasing gene conversion frequencies (Table 2).

Fogel and Hurst (1967) performed a monumental analysis of 1081 yeast tetrads with recombination at the *hi-1* locus. Linked markers were segregating on both sides. They tested the three mutant members of each tetrad to determine whether the recombination event had been reciprocal or nonreciprocal, and they discovered an interesting relationship to outside markers. The hi^+ recombinants which resulted from nonreciprocal recombination might have any of the four

TABLE 5

Segregation of outside markers accompanying recombination between alleles at the w17 *locus*

Cross	Total asci	Total 2:6	Marker segregation in 2:6 asci								Irreg. asci*
			P-1		P-2		R-1		R-2		
			PD	T	PD	T	T	NPD	T	NPD	
Ca+ x +bR	10,930	6	1	0	1	1	0	0	1	0	
Ca+ x +cR	20,430	78	27	1	0	0	19	0	6	0	
+aR x Cd+	13,980	65	30	5	1	2	12	1	3	0	
+a+ x CdR	2,580	15	0	0	0	1	0	1	0	0	
Ca+ x +eR	16,490	50	10	8	0	1	10	1	4	0	
Ca+ x +fR	19,660	76	27	3	0	0	14	1	1	0	
Ca+ x +iR	830	4	0	0	0	1	2	0	0	0	
Ca+ x +jR	630	1	1	0	0	0	0	0	0	0	
Ca+ x +kR	14,320	35	14	2	4	1	2	0	1	0	2
Ca+ x +lR	530	2	1	0	1	0	0	0	0	0	
+aR x Cl+	500	5	1	0	0	0	0	0	0	0	
+a+ x CoR	4,300	35	11	1	4	0	9	1	2	0	1
	1,160	20	7	0	2	1	2	0	1	0	
Ca+ x +sR	5,790	34	14	3	3	0	5	0	1	0	
+b+ x CdR	12,760	65	11	7	0	0	20	1	3	0	
+bR x Cd+	11,110	57	33	3	0	0	11	0	1	0	
Cb+ x +mR	5,400	6	4	1	0	0	1	0	0	0	
+cR x Cl+	7,290	49	0	0	14	3	1	0	11	1	
CdR x +i+	4,150	6	0	0	0	0	0	0	0	1	
CdR x +j+	2,780	6	0	0	2	0	0	0	0	1	
CdR x +k+	2,620	5	0	0	0	1	1	0	0	3	
CdR x +l+	260	3	0	0	2	0	0	0	0	0	
CoR x +q+	4,620	12	0	0	2	1	1	0	2	0	2

Explanation of symbols: column headed P-1 refers to those 2:6 asci in which the colored spore pair had the markers of the first parent and P-2 refers to those with the markers of the second parent. The colored spores in an R-1 ascus had the left-hand marker from the first parent and the right-hand marker from the second parent; R-2 asci had the reverse combination. PD, T, and NPD refer to asci which were parental ditypes, tetratypes, and nonparental ditypes, respectively, with regard to the outside markers. The cross parents are abbreviated as in Table 4. Only a sample of the 2:6 asci from each cross have been scored for the segregating markers, therefore they do not add up to the total shown at the left.

* The two colored spores in an irregular 2:6 ascus are members of different spore pairs, as shown by the segregation of the markers.

TABLE 6

Backcross analysis of asci with interallelic recombination at the w17 *locus; crosses segregating for one linked marker* (fpr)

Cross	Total asci	2:6	2:6 per 10^3	w^+ pair R	R^+	Analysis of asci with w^+ R pair	Analysis of asci with w^+ R^+ pair
$a+ \times kR$	10,800	29	2.7	4	19	....	5 N*a*, 1 N*k*/RR
$a+ \times lR$	18,670	66	3.5	15	21	8 N*l*	11 N*a*
$a+ \times mR$	2,540	11	4.3	0	5	....	5 N*a*
$a+ \times nR$	10,840	97	8.9	7	14	1 RR, 1 N*a*, 1 N*n*	9 N*a*, 1 N*n*
$a+ \times oR$	8,380	42	5.0	6	3	[illegible] N*a*, 4 N*o*/RR	3 N*a*
$aR \times p+$	850	4	4.7	2	2	2 N*a*	1 N*p*, 1 N*p*/RR
$a+ \times qR$	22,450	130	5.8	32	63	2 RR, 7 N*a*, 5 N*q*, 3 N*q*/RR	28 N*a*
$bR \times k+$	8,050	44	5.5	26	7	10 N*b*	....
$bR \times n+$	14,540	38	2.6	20	7	14 N*b*, 1 N*n*	1 RR, 2 N*b*

Cross parents are abbreviated as in Table 4. Other abbreviations: RR: reciprocal recombination; N*a*: nonreciprocal (3:1) segregation at site *a*; N*a*/RR: incomplete analysis revealed that the ascus resulted either from nonreciprocal segregation at *a* or from reciprocal recombination.

Relative frequencies of the three kinds of recombination events (NRR at either site and RR) may be distorted in some cases, because backcrosses to one parent were infertile. For example, in several cases the crosses of mutant segregants back to *a* were infertile while the crosses to the other allele were fertile; this tends to give firm classification of conversions of *a* but fails to distinguish conversion of the other allele from reciprocal recombination. The same thing is true of some of the analyses reported in Table 7.

Only a sample of the 2:6 asci from each cross were scored for *fpr* segregation, and only a sample of these were further analyzed by backcrosses. Therefore they do not add up to the totals shown at the left.

marker combinations, while nearly all (97 of 101) of the reciprocal recombination products had the same nonparental marker combination. The authors concluded that reciprocal recombination was a single exchange event and revealed the order of sites.

Some of the recombination tetrads from crosses between different *w17* alleles have been analyzed by the backcross test (MITCHELL 1955) to determine whether the event was reciprocal or nonreciprocal (Tables 6 and 7). The results confirm the finding of previous investigators that the large majority of the intragenic recombinations result from nonreciprocal events. It is also apparent that the frequencies of nonreciprocal segregation in these asci show some correlation with the frequencies shown by the same *w17* alleles in crosses to w^+; thus most of the nonreciprocal events are seen to occur at *w17a* in crosses of this mutant to other alleles which had shown lower frequencies of 3:1 segregation.

Table 7 shows the backcross analysis for crosses which were segregating for both outside markers. This permits us to compare marker segregation in asci with reciprocal and nonreciprocal recombination. In crosses of this type only ten cases of reciprocal recombination have been found. Eight of these occur in tetrads in which the w^+ recombinant has nonparental markers, and in each case it is the more frequent of the nonparental combinations. This indicates that ordering sites by frequencies of marker combinations gives a valid map.

Correlation of intragenic events with outside marker recombination: There have been extensive studies of intragenic recombination events in two situations:

TABLE 7

Backcross analysis of asci with interallelic recombination at the w17 *locus; crosses segregating for both linked markers*

Cross	P-1		P-2		R-1		R-2	
	PD	T	PD	T	T	NPD	T	NPD
Ca+ x +cR	27 (1RR, 5 Na, 1 Na/RR, 3 Nc/RR)	1 (Na)	0	0	19 (1RR, 3 Na, 1 Na/RR, 4 Nc/RR)	0	6 (3 Na, 1 Nc/RR)	0
+a+ x CdR	0	0	0	1	0	1 (RR)	0	0
+aR x Cd+	30	5	1	2	12 (3 Na, 1 Nd/RR)	1 (Na)	3	0
Ca+ x +eR	10	8 (1 Na)	0	1	10 (2 RR, 2 Na)	1 (Na)	4 (2 Na)	0
Ca+ x +fR	27 (6 Na)	3 (1 Na)	0	0	14 (3 Na, 6 Nf/RR)	1 (Na)	1 (Na)	0
Ca+ x +kR	14 (8 Na)	2 (1 Na)	4 (2 Nk, 1 Nk/RR)	1	2	0	1 (Na)	0
Ca+ x +sR	14 (1 RR, 5 Na)	3 (2 Na)	3 (1 Ns, 1 Ns/RR)	0	5 (4 RR, 1 Na/RR)	0	1 (Na)	0

Column headings refer to the segregation of the markers and are used as in Table 5. Cross parents are abbreviated as in Table 4. Other abbreviations are used as in Table 6.

1) crosses between allelic mutants which produce nonmutant recombinants; 2) crosses of mutant × wild type which produce tetrads with nonreciprocal segregation ratios. These may represent two different kinds of recombination event, or they may be two ways of revealing the same event. In the latter case, both should have common characteristics. Recombination between two allelic mutants has been shown to correlate strongly with outside marker recombination in Neurospora, Aspergillus, and yeast. There is little information in these organisms on the correlation of nonreciprocal segregation (in mutant × wild type crosses) with marker recombination. In Sordaria, KITANI and OLIVE (1967, 1969) have shown a strong correlation of this kind, using ascospore color mutants. Using the *w17* system we can test this correlation in Ascobolus.

The recombination asci from mutant × mutant crosses which have been scored for segregating outside markers are listed in Table 5. We can tell which asci had crossing over between the outside markers, and, in these cases, we can determine whether or not the chromatid with intragenic recombination at *w17* ("recombinant chromatid") was involved in the crossover. The asci in classes R1 and R2 are those in which the recombinant chromatid had nonparental markers and thus

TABLE 8

Segregation of markers in asci with gene conversions at w17

Cross	Noncrossover	Crossover of converted chromatid	Crossover of nonconverted chromatid		Crossover of all chromatids		Double crossover of nonconverted chromatid	Total asci	Total crossovers		
			C–w	*w–R*	*C–w*	*w–R*			Converted chromatid	Nonconverted chromatid *C–w*	Nonconverted chromatid *w–R*
6:2 asci:											
$+a+ \times C+R$	17	8	1	2	2	0	1	31	10	4	3
$Ca+ \times ++R$	5	3	2	0	0	0	0	10	3	2	0
$+t+ \times C+R$	16	17	3	2	1	0	0	39	18	4	2
ten others*	9	12	4	3	1	0	0	29	13	5	3
subtotal	47	40	10	7	4	0	1	109	44	15	8
2:6 asci:											
$+v+ \times C+R$	9	5	3	0	0	0	0	17	5	3	0
$+w+ \times C+R$	7	4	0	0	1	0	0	12	5	1	0
$+x+ \times C+R$	1	4	0	0	0	0	0	5	4	0	0
$+y+ \times C+R$	10	1	0	1	1	0	0	13	2	1	1
$+z+ \times C+R$	11	16	4	0	1	0	1	33	17	6	1
ten others*	12	8	0	0	0	0	0	20	8	0	0
subtotal	50	38	7	1	3	0	1	100	41	11	2
Total	97	78	17	8	7	0	2	209	85	26	10

In the nonconverted chromatids, crossovers to the left of the *w17* locus (*C–w*) can be distinguished from those to the right (*w–R*). The asci in the double crossover class have two crossovers in the nonconverted chromatid (one in each region). Crossovers in the converted chromatids produce the same genotype whether they are located left or right of the *w17* locus. The cross parents are abbreviated as in Table 4.

* Crosses of the form $+w+ \times C+R$ involving the *w17* alleles *b,h,j,k,l,n,p,q,s* and *z* or *a* (6:2 includes *z* and 2:6 includes *a*).

was involved in a crossover; this gives a total of 159 out of 434 asci, or 36.6%. The nonrecombinant sister chromatid was involved in all other crossovers: tetratypes in the P1 and P2 classes and nonparental ditypes in the R1 and R2 classes. The total is 59 out of 434 asci, or 13.6%. These frequencies compare with 13%, which is the proportion of chromatids in the general population showing crossing over between these markers.

Table 8 lists the asci from $w \times w^+$ crosses in which there was gene conversion (3:1 or 1:3 segregation) and in which outside markers were segregating. In this case we can ask whether or not a given crossover involved the converted chromatid. In a 3:1 ascus we cannot tell which one of the three pigmented spore pairs is the product of conversion. However, we know the one mutant pair was not converted, and we know that it was the sister to the converted chromatid. Each reciprocal crossover involves one or the other of each pair of sister chromatids. Therefore, when the unique pair (mutant in 6:2 or wild type in 2:6) is recombinant for the markers, we conclude that the nonconverted chromatid was involved in the crossover. In a tetratype ascus in which the unique pair has parental markers, the crossover must have involved the converted chromatid. In a nonparental ditype both the converted and nonconverted chromatids are involved in crossing over. Results of this analysis show 85 out of 209 asci or 40.7% crossing over for the converted chromatid and 36 out of 209 or 17.2% for the nonconverted

chromatid. In this situation we can also tell whether a crossover involving the nonconverted chromatid occurred to the left or right of *w17*. There were 26 in the *colt–w17* region and 10 between *w17* and *fpr*. This is close to the ratio of ten to three found in the general population.

We conclude that the crossing over pattern in gene conversion asci from mutant × wild type crosses is strikingly similar to that found in asci with intragenic recombination in mutant × mutant crosses. In both cases the chromatids which are involved in the events at the middle locus have greatly enhanced frequencies of crossing over for the outside markers (3× normal in this situation). The other chromatids in the same tetrads show crossover rates which are similar to those of the general population.

SUMMARY

Twenty-six *w17* alleles causing colorless ascospores have been isolated in *Ascobolus immersus*. Tetrads have been analyzed for recombination at this locus in two situations: frequencies of gene conversion (3:1 or 1:3 segregation) have been determined in crosses of the various mutants to $w17^{+}$, and the frequencies of wild-type recombinants have been measured in pairwise crosses between the mutants.—Linked markers have been isolated on both sides of the *w17* locus. *fpr* (fluorophenylalanine resistance) is 3 map units to the right of *w17*, and *colt* (colonial at 37°C) is 10 units to the left. No chiasma interference was shown in 562 asci from 3-point crosses, although the two regions are in the same chromosome arm.—Preliminary mapping information on the mutant sites at *w17* is compatible with an order based on increasing rates of gene conversion. Attempts have been made to order the sites on the basis of both recombination frequencies and marker combinations accompanying intragenic recombination. However, the interpretation of recombination frequencies was limited by the finding that our stocks were segregating for genetic elements which have pronounced effects on these frequencies.—Similar patterns of segregation of outside markers were found in gene conversion asci from mutant × wild type crosses and in recombination asci from mutant × mutant crosses; this indicates that both situations involve the same basic intragenic event.

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