CHEMICAL INDUCTION OF MITOTIC RECOMBINATION IN *ASPERGILLUS NIDULANS**

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DIPLOID strains of various fungi have been extensively used to study spontaneous and induced mitotic recombination. For such analyses it is important to have genetic markers available so that the four strands involved in an exchange can be easily recognized and recovered (ZIMMERMANN, SCHWAIER and VON LAER 1966; WOOD and KÄFER 1969), and to have a synchronous population of cells accessible for treatments with agents influencing recombination at different stages of DNA replication (HOLLIDAY 1964; PARRY and COX 1968; ESPOSITO 1968).

Our system in *Aspergillus nidulans* makes use of the two recently developed pairs of color markers on the right arm of linkage group VIII (chartreuse either with fawn or the dark-colored alkaline phosphatase mutant, WOOD and KÄFER 1967; WOOD 1967) which permit *visual* detection of the reciprocal products of mitotic crossing over as twin sectors of mutant color ("twin spots"). Thus, when the uninucleate conidia are treated during germination, such exchanges, if induced during the first, second, or third nuclear division produce half, quarter, or eighth colonies of mutant colors. If both products of a reciprocal exchange survive, two such color segments of complementary segregant genotypes are found. In addition, in *Aspergillus*, it is possible to place genetic markers on all chromosomes and to recognize visually or by genetic tests other segregant types which may be induced. These include aneuploids resulting from nondisjunction or disturbance of the nuclear division (KÄFER 1961; POLLARD, KÄFER and JOHNSTON 1968) and abnormal colonies heterozygous for semidominant lethal mutations which spontaneously produce mutation-free segregant sectors (recently described in detail, KÄFER 1969). However, in our system synchrony of cell division is only partial. During the incubation time which is needed to permit most of the quiescent conidia to reach the first mitosis, growth rates vary sufficiently to produce a fraction of the hyphae already in the second or third division (WOOD 1967; KESSEL and ROSENBERGER 1968).

The purpose of the present experiment was to compare the effects of different types of chemical agents with those observed previously with ultraviolet light (UV) in the same system. The chemicals chosen were N-methyl-N'-nitro-N-nitrosoguanidine (NG), 5-fluorodeoxyuridine (FUDR), and *p*-fluorophenylalanine (FP). The first two chemicals have been shown to increase mitotic crossing over in several species of fungi (ZIMMERMAN, SCHWAIER and VON LAER

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1966; ESPOSITO and HOLLIDAY 1964; BECCARI, MODIGLIANI and MORPURGO 1967), while treatment with FP appears to induce mainly chromosomal segregation (MORPURGO 1961; LHOAS 1961, 1968; McCULLY and FORBES 1965; GUTZ 1966; STRÖMNAES 1968).

The effects of these agents on mitotic crossing over were visually determined by identifying twin spots. Concurrently, estimates of mutagenic activity and of effects on chromosomal segregation were obtained by an examination of the percentage survival following treatment and from the frequency and type of abnormally growing colonies (mainly semidominant lethal mutations and aneuploids).

MATERIALS AND METHODS

Strains: Five different diploids heterozygous for various markers were used in these experiments. For the origin of mutant strains and details of the meiotic map, see DORN (1967). Diploid Y (Table 1) was used initially to determine the frequency of induced reciprocal crossing over, since in this diploid the twin products of a mitotic exchange between *fw2* and its centromere, i.e., paired fawn and chartreuse sectors, are expected to be equally viable—in contrast to twin sectors from *palB*-containing diploids (as explained below).

LINKAGE GROUPS

	I	II	III
DIPLOID D	$\frac{+ \quad + \quad \text{an} \quad + \quad + \quad + \quad \text{ad20} \quad \text{bi}}{\text{su} \quad + \quad + \quad + \quad \text{paba} \quad \gamma \quad \text{ad20} \quad +}$	$\frac{\text{Acr} \quad + \quad +}{+ \quad \text{w3} \quad +}$	$\frac{+ \quad \text{s4} \quad +}{+ \quad + \quad +}$
DIPLOID X	$\frac{+ \quad + \quad + \quad + \quad + \quad + \quad \text{ad20} \quad \text{bi}}{\text{su} \quad \text{ribo1} \quad \text{an} \quad \text{lu} \quad \text{paba} \quad \gamma \quad \text{ad20} \quad +}$	$\frac{\text{Acr} \quad + \quad +}{+ \quad \text{w2} \quad \text{ni3}}$	$\frac{\text{phen2} \quad + \quad +}{+ \quad + \quad \text{s0}}$

GROUPS	IV	V	VI	VII	VIII
D	$\frac{+ \quad +}{+ \quad \text{pyro4}}$	$\frac{+ \quad + \quad \text{p2}}{+ \quad + \quad +}$	$\frac{+ \quad \text{lac}}{+ \quad +}$	$\frac{\text{nic8} \quad +}{+ \quad \text{cho}}$	$\frac{\text{fw2} \quad + \quad + \quad + \quad \text{s15}}{+ \quad \text{ni21} \quad \text{ribo2} \quad + \quad \text{cha} \quad +}$
X	$\frac{+ \quad \text{pyro4}}{\text{meth} \quad +}$	$\frac{+ \quad \text{lys5} \quad +}{\text{nic2} \quad + \quad +}$	$\frac{\text{s3} \quad +}{+ \quad \text{lac}}$	$\frac{\text{nic8} \quad +}{+ \quad \text{cho}}$	$\frac{+ \quad \text{ni21} \quad + \quad \text{palB7} \quad + \quad +}{+ \quad + \quad + \quad + \quad \text{cha} \quad +}$

FIGURE 1.—Complete genotypes of diploids D and X used for analysis of NG- and FP-induced “abnormal” colonies. Conidial color markers: *cha* = chartreuse; *fw2* (allele of *fw1*) = fawn; *p2* (allele of *p1*) = pale; *w2* or *w3* (alleles of *w1*) = white; γ = yellow. Biochemical markers: *Acr* = resistance to acriflavine, semidominant; *ad20* (allele of *ad8*) = adenine; *an* = aneurin (thiamine); *bi* = biotin; *cho* = choline; *lac* = lactose; *lys5* = lysine; *lu* = leucine; *meth* = methionine; *ni3* and *ni21* (allele of *ni50*) = nitrite; *nic2* and *nic8* = nicotinic acid; *paba* = *p*-aminobenzoic acid; *palB7* = lacking alkaline phosphatase; *phen2* = phenylalanine; *pyro4* = pyridoxine; *ribo1* and *ribo2* = riboflavine; *s0* (= *s12*), *s3*, *s4* (allele of *s1*), and *s15* (new gene, GRAVEL 1969; NIKLEWICZ, unpublished) = sulphite; *su* (*su1ad20*) = recessive suppressor of *ad20*. All unnumbered mutants have isolation number 1.

To determine the distribution of induced mitotic crossover events along one chromosome arm and coincident events in several arms, NG and FUDR treatments were also performed on diploids E, F, and D. In diploids E and F, three nutritional markers of VIII R are set up in different arrangements in addition to fawn and chartreuse which are in coupling (diploid E) or repulsion (diploid F; see Table 2). Of these nutritional markers, only *palB7* shows viability effects when standard CM is used. (Addition of extra phosphate will increase viability, but also reduce phenotypic differences.) The effect of this mutant is so extreme in this case that *palB7* becomes a visible marker, since *palB* colonies are dark and grow slowly on complete medium. Thus *palB* sectors are always reduced in size, and often are completely outgrown so that they cannot be detected. Diploid D (Figure 1) was set up with markers in all eight linkage groups (on nine different chromosome arms) to examine more closely the distribution of exchanges in different arms and in different nuclei and to facilitate analysis of the "abnormal" colonies induced by NG. For treatment and analysis of "abnormals" induced with FP, diploid X with markers on all 16 homologues was used (Figure 1).

Treatments: Germinating diploid conidia were used in all experiments. Survival after treatment was calculated as a percentage of the corresponding values obtained from untreated germinating conidia (which is set at 100%). For NG treatment, germinating conidia (obtained by incubating quiescent conidia for 4½ hr in liquid minimal media at 37°C) were incubated with NG at a concentration of 0.5 mg/ml in Tris-maleate buffer (CLUTTERBUCK and SINHA 1966). To terminate this treatment at the end of 3 min, this solution was diluted 100-fold into phosphate buffer (CHANG and TUVESUN 1967). Control suspensions were treated identically, except the NG was omitted.

In the FUDR treatments, both the control and treated conidia were incubated in liquid minimal media at 28°C until they showed the slight elongation typical for germinating spores in the first division. Control suspensions of conidia needed 8 hr incubation at this temperature, while experimental suspensions (containing 100 µg/ml FUDR and 20 µg/ml uridine, ESPOSITO and HOLLIDAY 1964) required 12 hr.

The treatments with *p*-fluorophenylalanine were similar to those with FUDR but were carried out at 37°C. Conidia were incubated in liquid minimal media containing 0.02M or 0.04M FP for 10 or 12 hr, respectively—the times at which germ tubes were formed (MORPURGO 1963).

Genetic analysis: For the analysis of induced crossing over, twin spots or single-color sectors originating from the centers of normal colonies were recorded after 2 day's incubation at 37°C and a third day at room temperature. Sectors not originating from the center of the colony were found equally frequently among experimental and control samples and are therefore not included in these results. All sectors were isolated by restreaking, analyzed for ploidy, and tested for nutritional requirements. A few cases of twin types were analyzed for genotype by selecting and testing mitotic haploids from the diploid segregants.

To analyze colonies having abnormal growth, which frequently formed normal sectors of mutant color, conidia were replated from their centers. In these platings most of the "abnormals" showed repeated spontaneous segregation in a regular pattern which in simple cases resembled that of the original abnormal colony. Successful replatings were photographed for comparison and classification of phenotypes. To deduce the genotype of the induced "abnormal," 26 such sectors were isolated initially and tested for segregation of genetic markers. From complicated types (about half of all cases), 26–130 further sectors were analyzed. These could usually be classified as diploid or haploid on the basis of appearance and presence of heterozygous markers. In addition, an attempt was made to identify the type of mitotic recombination which produced the frequent diploid sectors. This was only possible for linkage groups having many heterozygous markers, preferably on both arms; namely, groups I, II, and VIII, which also carried the color markers. Consistent simultaneous segregation of all markers in a linkage group was classified as segregation of whole chromosomes, while segregation of only some of the markers, usually on one chromosome arm only, was taken as evidence for mitotic crossing over.

RESULTS AND DISCUSSION

Frequencies of reciprocal crossing over induced by NG and FUDR: Table 1

TABLE 1

Frequencies of color sectors from diploid Y after treatment with NG and FUDR as compared to UV

Linkage Groups: I

II

VIII

Diploid Y:

+

+

+

y

+

+

+

oha

Intervals between visible markers on VIII R:

1

2

3

		Control Number %	NG Number %	FUDR Number %	UV* Number %
Survival		(100)	72.5	102.4	9.2†
Total number of colonies		2389	1431	425	
Abnormals		5 0.2	187 13.1	26 6.1	
Normals		2384	1244	399	525†
Among normals:					
Without sectors		2370	1069	368	444

With any sector:	Total	14 0.6	175 14.1	31 7.8	81 15.4
Phenotypes of sectors	Exchange interval on VIII				
fawn/chartreuse	1	- -	32 2.6	4 1.0	17 3.2
fawn	1	3 0.1	32 2.6	2 0.5	14 2.7
chartreuse	1,2 or 3	4 0.2	71 5.7	16 4.0	27 5.1
yellow	-	7 0.3	40 3.2	9 2.3	23 4.4

* From WOOD and KÄFER (1969).

† Normal colonies only included; total survival approximately 20%.

shows the frequencies of diploid color segregants in colonies grown from conidia of diploid Y following treatments with NG and FUDR. These are compared to the corresponding control values, and earlier results obtained with UV are included (WOOD and KÄFER 1969). All color segregants segregated for linked markers only, as expected from mitotic crossing over; paired sectors of a reciprocal twin-type were found frequently and recorded as single events. Similar results are given in Table 2 for diploids E and F and in Table 3 for diploid D.

Large *spontaneous* twin spots are rarely observed. None was observed among over 7000 control colonies examined in these experiments. Using additional available data (Woon 1967) it is estimated that spontaneous reciprocal crossing

TABLE 2

Frequencies of single and multiple exchanges in color segregants induced by NG and FUDR; pooled data from diploids E and F

Linkage Groups:	I	II	III	IV	V	VI	VIII	IX	X
	y	+	fw2	+	ni21	+	ribo2	+	cha
Diploid E:	—○—	—○—	—○—	—○—	—○—	—○—	—○—	—○—	—○—
	+	w2		+	ni21	+	palB7	+	
Diploid F:	—○—	—○—	—○—	—○—	—○—	—○—	—○—	—○—	—○—
	+	w3		+	ni21	+	ribo2	+	cha
Intervals between visible markers on VIII R:			1			2		3	

	Control		NG		FUDR			
	Number	%	Number	%	Number	%		
Total number analyzed	4141		1420		692			
Abnormals	21	0.5	156	11.0	21	3.0		
Normals	4120		1264		671			
<u>Among normals:</u>								
Without sector	4073		1060		592			
With any sector	47	1.1	204	16.2	79	11.7		
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<u>Single exchanges:</u>	Total	45	1.1	193	15.3	76	11.3	
Phenotypes of sectors	from Diploid	Interval on VIII R						
fawn// <u>palB</u> or fawn <u>palB</u> //chartreuse	E F	1	-	-	14	1.1	7	1.0
fawn or fawn <u>palB</u>	E F	1	6	0.2	22	1.7	10	1.5
<u>palB</u> //chartreuse	E and F	2	-	-	7	0.6	2	0.3
<u>palB</u>	E F	1 or 2 2	1	0.1	10	0.8	1	0.2
chartreuse	E F	2 or 3 1,2 or 3	14	0.3	66	5.2	31	4.6
yellow	E and F	-	16	0.4	49	3.9	17	2.5
white	E and F	-	8	0.2	25	2.0	8	1.2
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Multiple exchanges in VIII R			-	-	5	0.4	-	-
Coincident exchanges in two chromosome arms			2	0.1	6	0.5	3	0.4

over in one of the first three nuclear divisions, which produces large fawn-chartreuse twin sectors, occurs with a frequency of about 1/10,000.

From the data in Tables 1-3, it is clear that treatments both with NG and

TABLE 3

Frequencies of NG-induced single and multiple events for eight marked chromosome arms of diploid D

	Control		NG	
	Number	%	Number	%
Total number analyzed	537		462	
Abnormals	2	0.4	60	13.0
Normals	535		402	
<u>Among normals:</u>				
Without sectors	529		368	
With any sectors	6	1.1	34	8.4

<u>Single exchanges in one arm:</u> Total	5	0.9	31	7.7
Phenotypes of sectors	Linkage group and arm			
fawn//chartreuse	VIII R	-	7	4.7
fawn		-	5	
chartreuse		2	7	
yellow	I R	2	8	2.0
white	II L	1	4	1.0

Coincident exchanges in two chromosome arms	1	0.2	3	0.7

FUDR increased the frequency of mitotic recombination 10- to 20-fold without reducing the survival of conidia to the same extent as UV. Since about half of the fawn sectors were isolated from "twin spots," even though NG reduced survival while *palB* reduced viability, it can be concluded that NG- and FUDR-induced mitotic crossing over is regularly of a reciprocal type. That is, we assume that in cases where only a fawn sector could be found, the reciprocal chartreuse product either contained an induced lethal mutation or was too small to be detected. On the other hand, since *cha* is far distal to *fw2*, chartreuse sectors without fawn twins are expected and are found more frequently. One of these twin spots from diploid D was analyzed for genotype and as expected, it was found to be completely homozygous for all markers distal to the exchange.

Of the two chemical agents used here, NG, a mutagen shown to induce mitotic crossing over in *Saccharomyces cerevisiae* by ZIMMERMANN, SCHWAIER and VON LAER (1966), was found to be the more potent recombinogenic agent, producing a 20-fold increase in color segregants over the control values. The frequencies of the various types of NG-induced color sectors are very similar to those obtained with UV (Table 1). However, since the survival following NG was much higher

than after UV, NG appears to increase reciprocal crossing over more effectively. With FUDR, the highest increase in the frequency of crossover color segregants was only about 10-fold over the control values. However, since it did not reduce the survival of conidia at all and did not produce many abnormal colonies, FUDR appears to be more specifically recombinogenic than NG. Unfortunately, the effective dose of FUDR could not be increased. Using higher concentrations of the FUDR had no apparent effect on the frequency of mitotic crossing over, while prolonging the treatment time only led to clumping of conidia. These results agree with the evidence for increased intergenic crossing over obtained in *Ustilago* (ESPOSITO and HOLLIDAY 1964) and in *Aspergillus* (BECCARI, MODIGLIANI and MORPURGO 1967).

Distribution of induced crossing over: The distribution of NG or FUDR-induced mitotic exchanges along the well-marked right arm of linkage group VIII in diploids E and F is shown in Table 2.

The induced crossover events were not localized to any specific portion of the right arm of linkage group VIII, but were randomly located, as far as these experiments could determine. An analysis of the biochemical and visible markers confirmed that the majority of the color sectors were of the type expected from single mitotic exchanges: Only five out of about 280 cases listed in Table 2 seem to have resulted from multiple exchanges, and two of these, which were classified with reference to both products of an apparent twin spot, could possibly have resulted from two independent single exchanges in sister nuclei. The coincidence of exchanges in two chromosome arms, that is, segregation of an unlinked marker in addition to the color mutant used for selection, is indicated in Table 2 for diploids E and F and in Table 3 for diploid D. The induced frequencies of homozygosis for specific color markers varied from 1 to 6%, increasing with the distance of the color mutant from its centromere. Therefore, the observed frequencies of segregation for a marker in an additional chromosome arm (0.4–0.5% in diploids E and F with markers in three further chromosome arms; 0.7% in diploid D with seven additional marked arms) are well within the range of those expected from random distribution of induced exchanges. This indicates that germinating cells were uniformly affected by both chemicals, even though synchrony of DNA replication was incomplete.

NG and FP-induced "abnormal" colonies: In Table 4 the results from the analysis of some of the NG-induced abnormal-looking survivors of diploid D are summarized. Since in diploid D only the three linkage groups containing the color mutants *y*, *w*, and *cha* are well marked (Figure 1), abnormal colonies with frequent color sectors were analyzed preferentially and only these could be classified with certainty. As described above, each "abnormal" colony was replated from the center and most of them formed large normal sectors, repeatedly and spontaneously. From the pattern of marker segregation in such normal sectors it is usually possible to deduce the genotype of the original abnormal center and, in simple cases, the likely effects of the treatment. About half of the NG-induced "abnormals" appeared to be of the type found most frequently after UV or γ irradiation (KÄFER 1969, 1963); viz., the abnormal growth seems

TABLE 4
Classification of induced abnormal colonies

	Treatment	
	NG Diploid D	FP Diploid X
Total replated	55	79
Normal on replating	10	1
Without normal sectors	1	1
Abnormal center lost, stable segregants recovered	0	15
With normal sectors on replating	44	62
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Among sectoring types: Total	44	62
1) Without segregation of markers in sectors	24*	3
a) all parental	15	3
b) all mutant	6	0
c) "mosaic"	3	0
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2) With marker segregation in sectors	20	59
a) Diploid segregants mainly of crossover type (eliminating semi dominant lethals)	8	1
centers of original type	6	1
centers mutant	2	0
b) Segregation of chromosomal type		
Typical aneuploids	10	6
$2n + 1$ or 2	9	6
$n + 1$ or more	1	0
With various types of sectors, including secondary aneuploids	2	52
$2n + 1$ and $2n$ sectors	0	14
$2n$ and $n + 1$ sectors	0	17
$2n$ and n sectors	2	10
$n + 1$ and n sectors	0	11

* Five chromosomes unmarked, see Figure 1.

to be caused by induced semidominant lethals or chromosomal aberrations which occasionally can become eliminated by mitotic recombination. When the resulting segregant is normal, diploid or haploid, it has a selective advantage and often shows up as a large sector. Such diploid normal sectors are usually of a specific crossover type, and in the haploid segregants from the same center only one homologue of the linkage group involved in this crossover is recovered.

The other NG-induced abnormalities unexpectedly turned out to consist of aneuploids of a relatively simple type, mainly $2n + 1$, which is the most frequent type found spontaneously. Sectoring segregants were classified as aneuploids if they showed the following three features typical for standard aneuploids (KÄFER

1961; POLLARD, KÄFER and JOHNSTON 1968): (i) chromosomal type of marker segregation in all sectors; (ii) 1:1 segregation of alleles in haploid sectors from disomics ($n + 1$) or 2:1 segregation in diploid sectors from trisomics ($2n + 1$); (iii) phenotypes indistinguishable from characteristic patterns of sector formation in corresponding spontaneous aneuploids. Using all three characteristics this classification is unequivocal. But while it is quite clear when an aneuploid product has been isolated, it is not always certain whether this aneuploid is the primary induced product. The NG-induced frequency of such aneuploids was about 7%, which is 3 to 5 times higher than that occurring spontaneously in quiescent conidia (KÄFER 1961). No reliable estimates of the spontaneous frequency of aneuploids are available for germinating conidia, but it must have been less than 0.5% in diploids Y, E, and F (Tables 1 and 2), while the average from several small control samples of diploid X was 2.6 ± 1.1 . Since the number of identified aneuploids is rather small, larger samples would have to be analyzed to decide whether NG treatment of germinating conidia increases the frequency of trisomics and therefore, of nondisjunction. Such primary aneuploids have been obtained with high frequency after treatment with acridines, which apparently increase nondisjunction considerably (UPSHALL and CROFT 1967).

On the other hand, since very few FP-induced abnormals are primary aneuploids, it is clear that FP does not increase the frequency of nondisjunction. The wealth of different aneuploids produced by most FP-induced abnormals indicates that FP increases haploid segregants by a mechanism which is different from that producing the spontaneous haploid segregants. Two main phenotypic types of abnormals have been observed after FP treatment; namely, tiny nonconidiating colonies which produce various aneuploid and euploid segregants after transfer and poorly conidiating, fairly well-growing colonies which produce bunches of conidia of similar segregant types (exactly as described by MORPURGO 1963). Detailed analysis of a sample of both types revealed no clear difference in their genotypes. In practically all cases marker segregation was of a chromosome type but most aneuploids were isolated as secondary segregants, often of more than one type, together with diploid and haploid segregants of many kinds from the same abnormal colony (Table 4). This indicates that the main effect is not to cause nondisjunction but more likely to disturb nuclear division producing surviving nuclei having various chromosome complements, usually at least diploid. No evidence for FP-induced stepwise loss of chromosomes was obtained (as postulated by LHOAS 1968), but such loss of chromosomes occurs spontaneously in abnormals after FP treatment under the conditions of our experiments. This may explain the lack of haploids among FP-induced segregants in yeast which does not form haploid segregants spontaneously (GUTZ 1966; STRÖMNAES 1968).

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SUMMARY

Fluorodeoxyuridine (FUDR) and nitrosoguanidine (NG) are both shown to

induce reciprocal mitotic crossing over in germinating diploid conidia of *Aspergillus nidulans*, since treatments with these chemicals greatly increased the frequency of "twin spots." In all experiments, the majority of induced color segregants was due to single mitotic exchanges. Multiple exchanges were no more frequent than expected from the coincident occurrence of two independent events. FUDR increased color segregants 10-fold, although the survival of conidia remained unaffected and few abnormal colonies were produced. This agrees with earlier evidence that FUDR is specifically recombinogenic and does not produce dominant and semidominant lethal mutations. On the other hand, NG, a mutagen which did produce abnormal colonies and reduce the survival of conidia, increased the frequency of mitotic crossing over 20-fold. The frequencies of the various types of color sectors induced by NG were similar to those previously observed for ultraviolet light, another potent mutagen; however, survival following NG was much higher than after UV. But unlike UV, NG not only increased the frequency of abnormalities resulting from semidominant mutations, but apparently also produced aneuploids. Since most of these were of a $2n + 1$ type, NG may increase mitotic nondisjunction of single chromosomes. The analogue *para*-fluorophenylalanine (FP) did not increase mitotic crossing over significantly but FP produced many abnormal colonies which spontaneously formed segregant sectors of various types, many of them aneuploid. The results agree best with the hypothesis that FP interferes with the normal distribution of chromosomes in mitosis; differences in viability and spontaneous stepwise loss of chromosomes then lead to the formation of haploid sectors.

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