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Neurospora Prototroph Selection System for Studying Aneuploid Production

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Various environmental agents have been tested for their ability to produce aneuploid products of meiosis in a Neurospora cross. The cross was between two multiply marked strains designed specifically for this purpose. These parental strains were heterozygous for four auxotrophic mutations on chromosome 1. Prototrophic disomics could be selected by plating ascospores on minimal medium. Out of 48 agents tested, at least 10 increased the aneuploid frequency significantly above control levels. Some of these positive-testing agents have also been found active in other aneuploid detection systems. It is suggested that if it should become necessary to perform widespread testing of agents in the human environment for their ability to generate aneuploids at meiosis, the Neurospora system could be successfully applied for this purpose.

Introduction

In a recent survey of human genetic disease, Carter (*1*) estimated the incidence of autosomal trisomies to be 0.14% and sex chromosome aneuploidies to be about 0.2% of live births. This total aneuploid frequency of about 0.34% is only one quarter of the estimated frequency of disorders due to mutant single genes of large effect, but nevertheless represents a large and significant proportion of the human genetic load.

The causes of this large amount of human meiotic nondisjunction are unknown, but, like gene mutation, an environmental component has been suggested (*2*). The more optimistic aspect of this suggestion is that it holds out, if true, a hope of epidemiological control: the environmental components could be identified and removed. The frequencies of aneuploids are maintained in populations almost wholly by new mutations, and could theoretically respond very quickly. This situation contrasts with that for gene mutations, since these are probably maintained not only by new mutation but in many cases by mutation and selection equilibria established over many generations. Such equilibrium frequencies would respond sluggishly to changes in mutation rates.

The present experiments were initiated to establish a detection system for environmental agents which can cause aneuploidy. A microbial system was chosen because of the convenience and short life cycles of microbes. *Neurospora crassa* was selected for the following reasons. (1) This species has been studied extensively both genetically and cytologically. In both these regards it appears to have a classical meiosis, with relatively large and easily-studied typical eukaryotic chromosomes (*3, 4*). (2) Aneuploids are regularly produced in the form of $(n + 1)$ disomic ascospores, which subsequently haploidize to form heterokaryotic cultures whose nuclei differ by virtue of which of the disomic chromosome pair they carry (*5*). These heterokaryotic cultures are easily detected by prototroph selection systems involving complementing auxotrophic mutant combinations in the parental strains. The frequency of these relatively rare "pseudo-wild type" (PWT) heterokaryotic cultures is an index of aneuploid frequency. Aneuploids can also be detected by tetrad analysis using appropriate selective markers such as those affecting ascospore color. In such a system Threlkeld and associates (*6, 7*) obtained results which suggested that aneuploidy can be caused by nondisjunction at the first or second meiotic division, by precocious division of the centromeres, or by some form of extra chromosome replication.

A Neurospora prototroph selection system was developed (*8*) and used to study aneuploid production in response to externally-added environmental agents (*8, 9*). It has been shown that several agents

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increase the mean frequency of PWT colonies over background in this test system. The possible application to large-scale surveys of agents from the human environment is considered.

Materials and Methods

The Selective System

The strains and the selective system have been described in detail elsewhere (*8*). Here they will be merely summarized. Figure 1 shows the genotypes of the two strains which are used as parents in a standard cross. In an attempt to achieve constancy in these stocks they are maintained on silica gel and a fresh vegetative culture is generated for each cross.

The functions of the mutant alleles were as follows. The alleles *arg-1*, *ad-3A*, *ad-3B*, and *nic-2* are tight auxotrophic mutants which served as selective agents for the detection of prototrophic PWT colonies arising from aneuploidy (disomy) for chromosome 1. It has been demonstrated (*8*) that the prototrophs are heterokaryons bearing complementary nuclear types reflecting either parental or recombinant chromosomes 1, and that no $arg^+ad^+nic^+$ triple recombinants ever arise. The background frequency of PWT colonies in the standard cross is low, suggesting a normal meiosis. The alleles *leu-3* and *un-3* are redundant in this study; leucine supplementation was routine but *un-3* requires no supplementation. The *tol* gene is a recessive suppressor of the mating-type incompatibility reaction and permits vigorous growth of the PWT heterokaryon. The complementing *al-1* and *al-2* alleles were originally extra chromosome markers but served no function in the present studies. The heterokaryon incompatibility genes *C/c*, *D/d*, and *E/e* served to prevent the formation of multiple disomics (*10*) to make the system more closely mimic human trisomic aneuploidy. These genes also served to contraselect against the formation of heterokaryons by the chance fusion of the germ tubes of adjacent ascospores. As a further

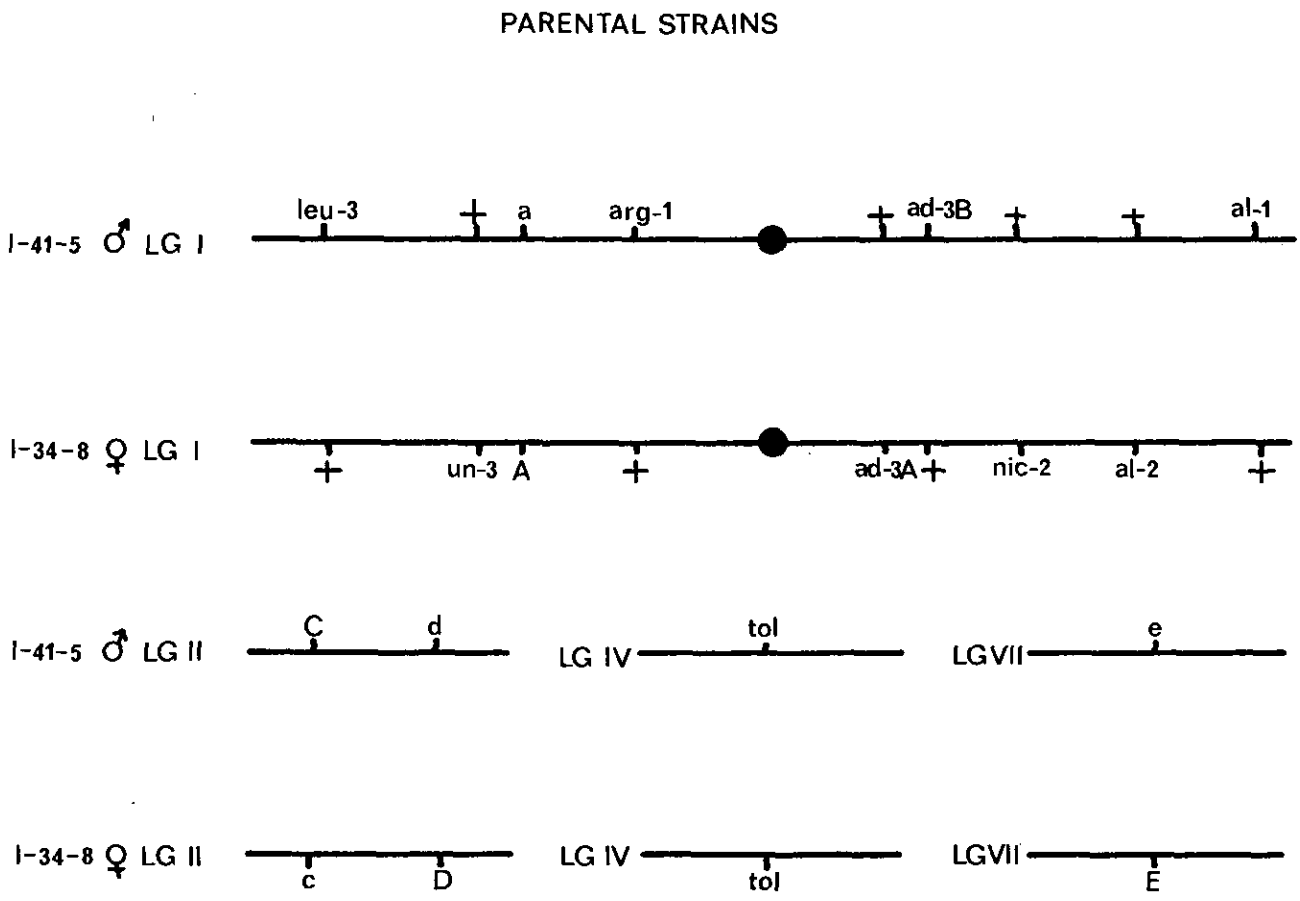

FIGURE 1. Genotypes of parental strains used in the standard cross. (I-41-5 and I-34-8 are the parental strain designations. LG stands for linkage group. Linkage relations shown are only approximate.)

guard against scoring such fusions as aneuploids, the prototrophs were checked at 2 days after plating to see if growth emanated from one ascospore.

Protocol

Day 1. The crossing medium was prepared and dispensed into 140 mm diameter plates. The protoperithecial parent was inoculated. Incubation was at 25°C.

Day 8. Conidia of the now-established protoperithecial parent were removed by vacuum, in a vertical laminar-flow hood. A dense conidial suspension of the male parent was added and spread over the surface of the cross plate. Several hours (usually 6 hr) later, the plates were exposed to the environmental agent. A wide range of doses was utilized in five or six crosses; the highest dose compatible with adequate fertility was eventually analyzed.

Day 30. Plating medium was prepared. Ascospores were harvested, suspended in 0.1% agar, heatshocked, and about 10^5 were plated on selective medium. Simultaneously, a diluted suspension was plated on supplemented medium to estimate total viable ascospores.

Day 32: Young prototrophic colonies were examined to check for single ascospore origin.

Day 34/35: Colonies were counted and recorded. Aneuploid frequency was determined as number of PWT colonies/total viable ascospores.

The cross showing the appropriate optimum dose was repeated to provide a minimum total of three replicate values for each agent tested. (Experience with the system has shown that five is probably a safer minimum and this is now aimed for.) With each treated cross, a control cross was run and analyzed simultaneously. Often, one control cross served for several treated crosses run the same day with the same medium batch.

Statistical Analysis

A full statistical analysis is currently in preparation. The statistical statements made in this paper should be regarded as tentative and are based on individual one-way analyses of variance performed on those ten agents whose PWT frequencies were consistently well above control values. Significance levels of 5% were used.

Costs

Each cross and its respective control are handled for a total time of approximately 2 days. Since at least five replicate tests of each agent are aimed for, one agent requires a total time of about 10 days. Approximately 25 agents can therefore be tested per year by one full-time technician. The total cost per agent tested is approximately $850 US, including technician's salary, supplies, and service overhead.

Results

Mean frequencies of PWT production are shown in Table 1, together with the number of replicate tests performed in each case. In all these treatments the

Table 1. Pseudo-wild type (PWT) frequencies in control and in treatment crosses, X10^5 ascospores.

Agent	Mean PWT frequency $\times 10^5$	No. of tests
Control	5.5	128
Actidione	2.0	3
Alloxan	8.1	9
Amethopterin	22.9	11
Ametryne	7.3	6
m-Aminophenol	36.5	3
Aminopterin	10.1	5
Atrazine	10.3	19
Bromacil	7.6	6
Caffeine	24.8	7
4-Chlororesorcinol	4.5	3
Cyanazine	6.5	12
Cyclic AMP	6.9	8
2,4-Diaminoanisole sulfate	21.1	6
2,5-Diaminoanisole sulfate	7.3	13
2,4-Diaminotoluene	12.2	5
Dimethyl sulfoxide	9.3	3
Enterovioform	5.8	3
Eptan	6.1	5
DL-Ethionine	7.9	5
Ethylene glycol	10.0	6
Fluorodeoxyuridine	10.8	3
p-Fluorophenylalanine	28.7	34
β-Fluoropyruvic acid	6.0	4
5-Fluorouracil	3.4	5
γ-Rays	14.3	18
8-Hydroxyquinoline	16.3	4
Maleic hydrazide	10.4	12
Mercuric chloride	11.4	3
Mitomycin C	5.2	4
l-Naphthol	6.5	6
p-Nitrobiphenyl	7.5	4
4-Nitro-*o*-phenylenediamine	4.2	4
Nitroquinoline	5.5	5
Nystatin	9.0	7
Orthene	6.2	8
Potassium fluoride	10.7	4
Resorcinol	7.5	11
Saccharin	7.2	3
Sarcosine	8.0	3
Simazine	5.7	11
Sodium fluoride	9.9	3
Stilphosterol	7.2	12
Sulfacetamide	29.9	3
Sulfanilamide	7.8	4
Terbacil	11.0	6
Trifluralin	19.6	51
Trimethoprin	9.5	47
Vinblastine sulfate	8.3	5

Table 2. Complete listings of PWT frequencies (X10^5 ascospores) obtained for selected agents showing typical results.

PWT frequency ×10^5		Dose, mg/l. (min)	Mean
Agent treated	Control		
Amethopterin			
17.1	7.6	32	
37.3	7.6	32	
20.6	7.6	32	25.0
18.4	9.2	109	
7.2	9.2	109	
5.1	9.2	109	10.2
33.3	9.2	218	
43.8	9.2	218	
40.0	9.2	218	39.0
12.9	9.2	325	12.9
16.7	9.2	435	16.7
22.9	8.4		
Cyanazine			
11.6	7.3	10	
2.5	7.5	10	
2.8	8.7	10	5.6
7.1	7.3	100	
5.5	7.5	100	
6.2	8.7	100	6.3
12.5	8.1	250	
6.7	4.4	250	
7.9	2.9	250	9.0
2.8	8.1	500	
5.3	4.4	500	
7.1	2.9	500	5.1
6.5	6.5		
Trifluralin			
13.0	2.9	1	13.0
8.4	0.4	10	
10.5	0.9	10	
11.0	2.9	10	
5.5	1.8	10	8.9
25.0	5.6	13	
24.1	4.7	13	
4.6	8.7	13	
10.0	6.6	13	
10.3	9.1	13	14.8
5.2	8.7	25	5.2
23.5	5.6	50	
14.5	4.7	50	
3.9	6.6	50	
7.1	9.1	50	
11.6	6.1	50	
3.4	9.1	50	10.7
11.9	4.7	63	
13.6	4.7	63	
9.7	5.6	63	
10.9	5.6	63	11.5
7.4	5.6	75	
8.3	5.6	75	

PWT frequency ×10^5		Dose, mg/l. (min)	Mean
Agent treated	Control		
Trifluralin			
3.2	4.7	75	
12.5	4.7	75	
8.5	8.7	75	
32.3	8.7	75	
15.9	6.6	75	
14.2	9.1	75	
13.3	7.1	75	
17.1	7.1	75	
16.7	7.1	75	
8.9	8.5	75	
9.8	8.5	75	
28.6	10.1	75	
36.0	10.1	75	
18.6	10.1	75	
3.9	10.1	75	
8.6	11.9	75	
4.6	11.9	75	14.1
217.6	1.8	104	
162.1	3.3	104	
18.1	3.3	104	
5.4	0.9	104	
9.2	2.9	104	
8.2	0.4	104	
3.0	11.9	104	60.5
19.0	11.9	122	
22.7	8.5	122	
11.5	8.5	122	
18.8	7.1	122	18.0
19.6	5.8		
γ-Rays			
3.0	1.2	5	
2.9	1.2	5	
12.4	1.2	5	
7.9	1.2	5	
4.0	1.2	5	
13.3	1.2	5	
3.8	6.2	5	
4.9	6.2	5	6.5
17.7	6.2	15	
8.5	3.1	15	
16.3	6.2	15	
14.7	6.0	15	
10.5	3.1	15	
14.6	6.2	15	
15.4	6.2	15	
30.3	6.2	15	
55.6	6.2	15	
21.4	6.2	15	20.5
14.3	4.1		

great majority of doses analyzed were those showing reduced fertility, or the maximum doses for which adequate ascospore numbers could be harvested. In a small number of cases, crosses showing normal fertility were also analyzed. A complete listing of frequencies and doses has been given elsewhere (*8, 9*). Some idea of the kind of variability normally observed can be obtained from Table 2 which shows the complete listings for amethopterin, cyanazin, gamma rays and trifluralin. These four agents were selected for display partly because of their representative nature, and partly because they have been studied at several different doses. These different doses are shown in Table 2, together with means for the individual doses. In all cases not listed fully, correlations between different treatment doses and PWT frequency were either not testable, or, where testable, not found.

Discussion

Agents showing major consistent and significant effects are amethopterin, atrazine, caffeine, 2,4-diaminoanisole sulfate, 2,4-diaminotoluene, *p*-fluorophenylalanine, γ-rays, sulfacetamide, trifluralin, and trimethoprin. Higher means are also found for several other agents (for example, *m*-aminophenol, 8-hydroxyquinoline, mercuric chloride, terbacil), but in these cases the individual values are generally low with one abnormally high value. Although abnormally high values have never been obtained in 128 control crosses performed to date, these results are viewed with less confidence. (No statistical tests were performed on them.)

The system is therefore demonstrably sensitive to environmental modification by a wide spectrum of agents. Positive results for some of these agents have been found in other systems. Some examples follow. Parry (*11*) reported that sulfacetamide and trimethoprin were active in producing monosomics in a yeast mitotic system, and that *p*-fluorophenylalanine was active in producing disomics in a meiotic prototroph selection system. Murnick (*12*) has shown that trifluralin increases meiotic nondisjunction in Drosophila. Caffeine is a well-known point mutagen, but chromosomal action has also been claimed; see Timson for a review of the genetic effects of caffeine (*13*). γ-rays have been shown to be active in a yeast mitotic system (*14*). The confirmation of some of the present results in other systems increases the likelihood that it is the same biological processes which are being affected by the agents studied.

Of the agents showing less pronounced, or no effects, some have proven positive in other aneuploid detection systems. Quinoline compounds, including enterovioform, and sulfanilamide, have been found active in an Aspergillus mitotic system (*15*). Grant (16) has reviewed the ability of simazine to cause mitotic abnormalities in higher plant tissues. Mitomycin C causes increased monosomic production in the yeast system (*11*). Alloxan induces aneuploidy in mouse tissue culture cells (*17*).

In the present system, the significance of the negative results is not clear. It is difficult to prove that the meiotic apparatus has been challenged by an adequate concentration of chemical agent. Although the maximum concentration compatible with fertility is routinely used, the loss of fertility could result not from high exposure of the meiotic cells but from lethality of the fertilizing conidia. A period of 6 hr is left between the addition of male conidia and the addition of chemical in an attempt to give the fertilizing nuclei time to enter the protoperithecium. A longer delay is possible, but meiosis begins soon after. Another possibility is that perithecia in Neurospora may be relatively impervious to externally added chemicals. Incorporation into the medium is not advisable since this interferes with morphogenetic development of the sexual apparatus. Experiments are currently under way to try to increase the accessibility of the meiotic cells with DMSO.

In this connection, two other aspects of the results may have relevance. First, there is very high variability of values between repeats. Notable is the tendency to recover "jackpots" of PWT colonies in some treated crosses. Trifluralin shows some good examples of this, but the phenomenon is common in most treatments. No jackpots have ever been recovered from controls, where the maximum frequency ever obtained is $15.3/10^5$. The jackpots could be caused by perithecia which have by chance been penetrated with a high concentration of the chemical. It seems significant that the variation at a given dose is less marked in the case of γ-rays, where the actual dose at the meiotic target site must be more directly related to the exposure. Second, the general lack of correlation of dose with PWT frequency is possibly another aspect of this same problem, although again γ-rays are the exception. The lack of overall consistency between repeats means that larger numbers of crosses have to be performed to obtain statistically meaningful data. This is an area where improvements are necessary.

A dose effect is observed for γ-rays, but none for amethopterin or trifluralin. A suspicion of an optimum concentration is seen for amethopterin (218 mg/l.) and this has been reported previously for *p*-fluorophenylalanine in the present system (*8*) and for mitomycin C in the yeast mitotic system (*11*).

Of 48 agents tested, at least 10 showed consistent large increases over control values and these were

found to be significant increases. Although the agents were not selected at random, this is still a high proportion and suggests that agents which can produce aneuploidy are quite common. Furthermore, the list of positive agents contains compounds which are not on record as causing gene mutations (amethopterin, *p*-fluorophenylalanine, sulfacetamide, trifluralin, and trimethoprin). Several of the agents are prevalent in the human environment. Caffeine is a well-known stimulant, atrazine and trifluralin are widely-used herbicides, sulfacetamide is used in medicine in topical antimicrobial treatment, amethopterin (methotrexate) is an antineoplastic agent, and 2,4-diaminoanisole sulfate and 2,4-diamino toluene are used in hair dyes.

Human populations are exposed to such a vast and heterogeneous array of environmental agents, that any large-scale test system for aneuploid production would of necessity have to be based on organisms having short life cycles, and aneuploids which are easily studied and whose incidence is demonstrably sensitive to a wide spectrum of treatment. The Neurospora system described here fits all these requirements and could play an important role in such a screening program, should one become necessary.

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