Pedigree Analyses of Yeast Cells Recovering from DNA Damage Allow Assignment of Lethal Events to Individual Post-Treatment Generations

Franz Klein, Anneliese Karwan and Ulrike Wintersberger

Department of Molecular Genetics, Institute of Tumourbiology and Cancer Research, University of Vienna, A-1090 Wien, Austria

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ABSTRACT

Haploid cells of *Saccharomyces cerevisiae* were treated with different DNA damaging agents at various doses. A study of the progeny of individual such cells (by pedigree analyses up to the third generation) allowed the assignment of lethal events to distinct post treatment generations. By microscopically inspecting those cells which were not able to form visible colonies we could discriminate between cells dying from immediately effective lethal hits and those generating microcolonies (three to several hundred cells) probably as a consequence of lethal mutation(s). The experimentally obtained numbers of lethal events (which we call apparent lethal fixations) were mathematically transformed into mean probabilities of lethal fixations as taking place in cells of certain post treatment generations. Such analyses give detailed insight into the kinetics of lethality as a consequence of different kinds of DNA damage. For example, X-irradiated cells lost viability mainly by lethal hits (which we call 0-fixations); only at a higher dose also lethal mutations fixed in the cells that were in direct contact with the mutagen (which we call 0-fixations), but not in later generations, occurred. Ethylmethanesulfonate (EMS)-treated cells were hit by 0-fixations in a dose dependent manner; 0-fixations were not detected for any dose of EMS applied; the probability for fixation of lethal mutations was found equally high for cells of the first and second post treatment generation and, unexpectedly, was well above control in the third post-treatment generation. The distribution of all sorts of lethal fixations taken together, which occurred in the EMS-damaged cell families, was not random. The small but significant surplus of pedigrees without any lethal fixation on the one hand and of pedigrees with more than one lethal fixation on the other hand may indicate the existence of unequally sensitive subpopulations. For comparison analyses of cells treated with methyl methanesulfonate, N-methyl-N'-nitro-N-nitrosoguanidine and nitrous acid are also reported.

During our studies on the effects of DNA damaging agents on haploid yeast cell populations we became interested in the timing of induced lethal events in relation to post treatment cell divisions. This problem has attracted interest before, e.g., in connection with proposed explanations for the development of pure mutant clones from cells assumed to contain DNA with single strand lesions (James and Werner 1966a, b; Nasim and Auerbach 1967; Haefner 1967a, b; Hannan, Duck and Nasim 1976; Eckardt and Haynes 1977; Nasim, Hannan and Nestmann 1981). One hypothesis then proposed that such lesions caused mutations exclusively by miscoding during DNA replication. The extinction of the nonmutated progeny descending from the complementary DNA strand without the particular miscoding lesion (lethal sectoring) would have been a plausible reason for the existence of pure mutant clones.

We encountered the question of possible delayed cell death when we determined the fraction of colony-forming cells (CFCs) in populations proliferating after DNA damage. We observed that such cell populations after several population doublings may contain more non colony-forming cells (NCFCs) than expected from the fraction of such cells present in the treated population itself (Klein, Karwan and Wintersberger 1989). This behaviour was especially pronounced in cell populations recovering from DNA damage exerted by low doses of the alkylating drug ethyl methanesulfonate (EMS). We concluded from our results that during cell proliferation NCFCs could originate either from residual divisions of lethally mutated cells or (and in addition) from lethal events not occurring in the treated cells but in part of their progeny. These events could be severe alterations in DNA which make further replication impossible (lethal hits) or lethal mutations, in turn leading to residual divisions of these newly arisen NCFCs.

Because of the asynchronous duplication of individual cells and the considerable amplification of this asynchrony under the influence of DNA damaging agents (Wintersberger and Karwan 1987) assignment of lethal events to particular generations after the injury to DNA is very difficult with population
studies. Therefore we had to approach the problem of delayed death by studying the progeny of individual cells with the aid of pedigree analyses. Similar experiments had been carried out earlier by other workers with UV or X-irradiated cells (JAMES and WERNER 1966a, b; HAENNER 1967a; HANNAH, DUCK and NASSIM 1976; JAMES and KILBEY 1977; JAMES, KILBEY and PREFONTAINE 1978) but not, to our knowledge, with EMS treated cells which, in our population studies were those most extremely prone to the phenomenon of delayed death. Furthermore, several of the previous studies for the pedigrees used diploid cells (JAMES and WERNER 1966a, b; JAMES and KILBEY 1977, JAMES, KILBEY and PREFONTAINE 1978) or haploids of which an unknown fraction was in the G2 phase of the cell cycle (HAENNER 1967a). The presence of two genomes in these cells, although advantageous for certain questions (JAMES and KILBEY 1977; JAMES, KILBEY and PREFONTAINE 1978), together with the induction of recombination events would impair the establishment of an exact genealogical order of lethal events after mutagenic treatment. Therefore we have analyzed three generations of haploid cells which had been treated with mutagens during the G1 phase.

In addition a mathematical model has been worked out (explicitly in the APPENDIX) which, by comparing the experimental data to it, allowed us to address the following issues: firstly, a quantitative calculation of the probabilities for lethal events in cells suffering from DNA damage and in three generations of their progeny; secondly, the dependence of these probabilities on varying doses of different mutagens; and thirdly, an estimation of the contribution of lethal hits vs. lethal mutations (leading to cell death only after exhaustion of the respective gene products) to the number of lethal events.

**MATERIALS AND METHODS**

Materials and the strain VY5 were as described previously (KLEIN, KARWAN and WINTERSBERGER 1989). For comparison some of the experiments were repeated with the haploid *S. cerevisiae* strain RC43 (a, his1-7, hom3-10, ade2-1, trp5-48, arg1-17, lys1-5) which was obtained from F. Fabre.

**Experimental procedures and pedigree analyses:** Mutagen treatment of stationary cells was as described previously (KLEIN, KARWAN and WINTERSBERGER 1989). After treatment the cells were washed three times with 1 mm potassium phosphate buffer (pH 7.0) and samples were streaked onto slabs of solid YPGlu for micromanipulation. Pedigree analyses were started with nonbudded cells (i.e., cells in the G1 phase of the cell cycle) and separations of daughter from mother cells were carried out for three generations. The slabs were incubated at 30°C and were inspected on the following days for macrocolonies by eye and for microcolonies under the microscope.

**RESULTS**

**Experimental data of pedigree analyses:** We performed pedigree analyses of cells treated with different doses of EMS and other DNA damaging agents up to the third generation (Table 1). The results were documented as "binary trees" (Figure 1A) in which large and small circles represent mother and daughter cells, respectively. The trees consist of mother/daughter pairs that each emerge from a single budding event. A subset of NCFCs descending from each other by division can always be traced down to the earliest NCFC which is either the treated cell itself or part of a CFC/NCFC pair, i.e., a pair of one colony forming cell and one noncolony forming cell (see Figure 1B). We will refer to this type of event as an "apparent lethal fixation." The word "apparent" pays regard to the fact that what we find experimentally is, that this NCFC does not proliferate or its complete progeny dies out. This must either be due to a single lethal fixation in a certain generation which commits one cell of a mother/daughter pair and therefore all its progeny to death, or to several lethal fixations taking place during the following generations in different cells, but likewise exterminating all the progeny of that cell which itself had not been lethally fixed.

Before studying the results in detail two predictions were tested: first, a branch dying out as a consequence of a lethal fixation should arise with equal probability from a mother or a daughter cell. Second, because of the budding mechanism for cell division, usually mother cells would receive more of the remaining product of a formerly intact and now mutated gene. Therefore we expected for a pair of microcolonies that most frequently the colony originating from the mother cell might grow to a larger size than that originating from the daughter. Data are shown in Table 2. Dying out branches, dating back to the first post treatment generation were compared for originating from the mother or the daughter cell. When data were pooled for all EMS-experiments no difference between the frequencies of non-viable mothers

**TABLE 1**

Summary of pedigree experiments

<table>
<thead>
<tr>
<th>Experiment (% survivors)</th>
<th>Strain</th>
<th>Dose % (min)</th>
<th>Number of cells put on for pedigree analyses</th>
</tr>
</thead>
<tbody>
<tr>
<td>EMS (95)</td>
<td>VY5</td>
<td>2.0 (40)</td>
<td>20</td>
</tr>
<tr>
<td>(85)</td>
<td></td>
<td>2.0 (60)</td>
<td>20</td>
</tr>
<tr>
<td>(75)</td>
<td></td>
<td>2.0 (60)</td>
<td>20</td>
</tr>
<tr>
<td>(75R)</td>
<td>RC43</td>
<td>2.0 (40)</td>
<td>20</td>
</tr>
<tr>
<td>(62)</td>
<td>VY5</td>
<td>4.0 (60)</td>
<td>50</td>
</tr>
<tr>
<td>(20)</td>
<td></td>
<td>8.0 (60)</td>
<td>50</td>
</tr>
<tr>
<td>MMS (80)</td>
<td>VY5</td>
<td>0.01 (60)</td>
<td>20</td>
</tr>
<tr>
<td>(44)</td>
<td></td>
<td>0.25 (60)</td>
<td>50</td>
</tr>
<tr>
<td>X-ray (38)</td>
<td></td>
<td>5 krad</td>
<td>50</td>
</tr>
<tr>
<td>(15)</td>
<td></td>
<td>14 krad</td>
<td>81</td>
</tr>
<tr>
<td>NaNO2 (29)</td>
<td></td>
<td>2.5 mg/l (30)</td>
<td>45</td>
</tr>
<tr>
<td>MNNG (17)</td>
<td></td>
<td>30 mg/l (30)</td>
<td>36</td>
</tr>
</tbody>
</table>

* Survivors expressed as percentage of treated cells giving rise to at least one cell able to form a colony.
and daughters was detectable (left side of Table 2). However, in all experiments more cases of “bigger mother than daughter microcolony” compared to those of “bigger daughter than mother microcolony” were observed (right side of Table 2). Therefore both of the former expectations were fulfilled by the experiments.

When analyzing the data obtained earlier from population studies of EMS-treated cells (KLEIN, KARWAN and WINTERSBERGER 1989) we had asked whether the unexpected high number of NCFCs found during posttreatment proliferation resulted from residual divisions of lethally mutated cells or from lethal fixations occurring in later than the first post treatment generation. The pedigree analyses reported here enables us to qualitatively answer that question even at first sight: Both mechanisms must contribute to the increase of NCFCs during post treatment proliferation. Residual divisions were observed as microcolonies (two to about a few hundred cells), unable to grow into a visible macrocolony. Such microcolonies frequently developed at the end of a “dead” branch. On the other hand CFC/NCFC pairs were found to arise in the first, but also in the second and third generation after treatment. When sizes of microcolonies of all available EMS experiments were taken together and those originating in different generations were compared to each other, two observations became evident.

First, for every posttreatment generation the majority of apparent lethal fixations produced microcolonies except for generation zero, where many cells stayed undivided or with a single bud. Second, for generations zero, one and two the majority of the microcolonies consisted of more than 15 cells, whereas strikingly none of the 14 apparent lethal fixations from generation three developed into more than 13 cells (data not shown). For a quantitative estimation of the contribution of the two mechanisms, residual divisions and late lethal fixations, a mathematical analysis of the experimental data turned out to be necessary (see later).

In Table 3 the numbers of apparent lethal fixations (i.e. the number of CFC/NCFC pairs) found for different generations, as well as expression of these numbers as percent of total fixations observed per experiment, are shown. We discriminated between cells which did not divide at all after treatment and supposedly died from a lethal hit without having finished one round of DNA replication (00-fixations), and those which produced a limited number of progeny but not a single branch developing into a macrocolony (0-fixations or also referred to as “primary lethal fixations”). A first, second and third generation apparent lethal fixation denotes a pair of CFC/NCFC cells in the respective generation (collectively called “secondary lethal fixations”). Table 3 indicates that cells treated with moderate doses of EMS yielded most lethal fixations from the first generation on, whereas all other agents were effective predominantly within the cells that were in direct contact with the mutagens (compare Table 3 upper vs. lower part). For EMS-treated cells, the percentage of 00-fixations and that

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**TABLE 2**

Comparison of mother and daughter branches

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Number of nonviable cell lineages</th>
<th>Relative sizes of microcolonies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$m^*$</td>
<td>$d^*$</td>
</tr>
<tr>
<td>EMS (95)</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>(85)</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>(75)</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>(75R)</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>(62)</td>
<td>5</td>
<td>11</td>
</tr>
<tr>
<td>(20)</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Sum</td>
<td>22</td>
<td>25</td>
</tr>
</tbody>
</table>

*$^*$ Cell lineage descending from a mother cell of the first generation.

$^*$ Cell lineage descending from a daughter cell of the first generation.

$^*$ Pairs of nonviable branches for which the mother microcolony is definitely larger than the daughter microcolony.

$^*$ Pairs, for which microcolony sizes are about equal.

$^*$ Not determined.
of apparent 0-fixations increased with dose, and the corresponding initial fraction of CFCs (i.e., the conventionally determined surviving fraction) decreased as expected. Remarkably however, the number of secondary lethal fixations decreased at higher doses. We will explain this by showing that Table 3 states only the numbers of experimentally observable CFC/NCFC pairs per generation without taking into account the fact that the increasing number of 00- and apparent lethal fixations reduced the cells available for lethal fixations in later generations. For the estimation of the probability for a cell, descending from a not lethally fixed cell to become lethally fixed, we have to find an algorithm to convert "apparent lethal fixations" into "real lethal fixations."

Corrected probabilities for lethal fixation are largely dose dependent for all three generations inspected after EMS treatment: As described in Appendix A the numbers of CFC/NCFC pairs from Table 3 could be used to calculate the probabilities of real lethal fixations per total cells of a certain generation which had not previously been lethally fixed (Table 4). All probabilities, except that for the directly observable 00-fixations, had to be corrected because, on the one hand, varying sample sizes, and on the other hand, multiple lethal fixations that extinct the progeny had to be accounted for. For the third generation Table 4 shows the probabilities for apparent lethal fixations because data of a fourth generation needed for correction were not available. However, assuming that lethal fixations are rare after the third generation, the difference between apparent and real lethal fixations for the third generation is probably negligible.

Comparison of Table 3 with Table 4 shows that the probabilities of 00-fixations (lethal hits) are proportional to their numbers. Interestingly, 0-fixations seemed to arise dose dependently from EMS treated cells according to Table 3, but the corresponding probabilities for lethal 0-fixation were found comparably small and, as statistical considerations revealed, were not even significant (marked by an asterisk in the table). In other words, for EMS-experiments all the lethal 0-fixations of Table 3 might well be just "apparent" and due to multiple lethal fixation events in later generations. A high number of lethal events happened in generations one and two (Table 3). Table 4 uncovers that, in contrast to the experimentally observed numbers, the calculated probabilities for these lethal fixations rise dose dependently. More than that, probabilities for secondary lethal fixations increased about fourfold in all three generations tested. According to Table 4 probabilities for lethal fixations in generations one and two were about equal and even slightly higher than probabilities for lethal hits. In generation three extinction probabilities are by no means zero, but reduced to about 30% of lethal fixation probabilities in generation two. Thus, in EMS treated yeast cell populations descendants are more at risk than the cells that were in direct contact with the mutagen!

Comparison of EMS effects to those of other DNA damaging agents: The distributions of probabilities for lethal fixations differ enormously for cells treated with various DNA damaging agents. The probability for real 0-fixations was significantly greater than zero in cells treated with the other agents, X-ray, methyl methanesulfonate (MMS), N-methyl-N’-nitro-N-nitro-
TABLE 4
Distributions of corrected mean probabilities for lethal fixations over the generations

<table>
<thead>
<tr>
<th>Experiment</th>
<th>00*</th>
<th>0*</th>
<th>p00</th>
<th>1*</th>
<th>2*</th>
<th>3*</th>
</tr>
</thead>
<tbody>
<tr>
<td>EMS (95)</td>
<td>&lt;0.05*</td>
<td>&lt;0.05*</td>
<td>0.68</td>
<td>0.157</td>
<td>0.068</td>
<td>0.026</td>
</tr>
<tr>
<td>(85)</td>
<td>0.1</td>
<td>&lt;0.05*</td>
<td>0.43</td>
<td>0.086</td>
<td>0.105</td>
<td>0.037</td>
</tr>
<tr>
<td>(75)</td>
<td>0.15</td>
<td>&lt;0.05*</td>
<td>1.23</td>
<td>0.382</td>
<td>0.27</td>
<td>0.047</td>
</tr>
<tr>
<td>(75R)</td>
<td>0.1</td>
<td>0.11*</td>
<td>0.48</td>
<td>0.235</td>
<td>0.284</td>
<td>0.087</td>
</tr>
<tr>
<td>(62)</td>
<td>0.22</td>
<td>0.06*</td>
<td>0.85</td>
<td>0.294</td>
<td>0.335</td>
<td>0.107</td>
</tr>
<tr>
<td>(20)</td>
<td>0.46</td>
<td>0.18*</td>
<td>0.57</td>
<td>0.598</td>
<td>0.406</td>
<td>0.096</td>
</tr>
<tr>
<td>MMS (80)</td>
<td>0.15</td>
<td>0.05*</td>
<td>0.28</td>
<td>0.065</td>
<td>0.034</td>
<td>&lt;0.01*</td>
</tr>
<tr>
<td>(44)</td>
<td>0.5</td>
<td>0.07</td>
<td>0.012</td>
<td>0.047</td>
<td>0.051</td>
<td>0.041</td>
</tr>
<tr>
<td>(15)</td>
<td>0.704</td>
<td>0.148</td>
<td>0.000012</td>
<td>0.04</td>
<td>&lt;0.02*</td>
<td>&lt;0.01*</td>
</tr>
<tr>
<td>X-ray (38)</td>
<td>0.58</td>
<td>0.04</td>
<td>0.005</td>
<td>&lt;0.02*</td>
<td>&lt;0.01*</td>
<td>0.01</td>
</tr>
<tr>
<td>NaN02 (29)</td>
<td>0.4</td>
<td>0.31</td>
<td>0.00023</td>
<td>0.13</td>
<td>0.022</td>
<td>&lt;0.01*</td>
</tr>
<tr>
<td>MNNG (17)</td>
<td>0.28</td>
<td>0.532</td>
<td>0.024</td>
<td>0.322</td>
<td>0.125</td>
<td>&lt;0.025*</td>
</tr>
</tbody>
</table>

* See footnote of Table 3.
* Mean probability of lethal fixations occurring in generation 0, 1, 2 and 3, respectively.
* p00 is the ratio between two probabilities. The numerator is the probability that none of the apparent 0-fixations was a real one, calculated from our results. The denominator is the probability that none of the apparent 0-fixations was a real one, assuming equal probabilities for any number of real 0-fixations among the apparent 0-fixations (i.e., assuming no information). The smaller p00, the better is the evidence that there is a real 0-fixation among the apparent ones (see Table 3).

For 294 mother/daughter pairs, the control showed two lethal fixations (see Table 3); therefore background fixation probability is approximately 0.007.

Long-Term Lethal Effects of EMS

TABLE 5
Characterization of the DNA damaging agents tested

<table>
<thead>
<tr>
<th>Agent</th>
<th>00-Fixations (lethal hits)</th>
<th>0-Fixations</th>
<th>1, 2-Fixations</th>
<th>3-Fixations</th>
</tr>
</thead>
<tbody>
<tr>
<td>X-ray</td>
<td>XXX</td>
<td>X</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MMS</td>
<td>XXX</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>NaN02</td>
<td>XX</td>
<td>XX</td>
<td>X</td>
<td>0</td>
</tr>
<tr>
<td>EMS</td>
<td>XX</td>
<td>(X)</td>
<td>XXX</td>
<td>XX</td>
</tr>
<tr>
<td>MNNG</td>
<td>X</td>
<td>XXX</td>
<td>XX</td>
<td>0</td>
</tr>
</tbody>
</table>

XXX denotes the generation where the clear majority of the lethal fixations occur; XX denotes generations where lethal fixations occur frequently; X denotes generations where lethal fixation are detectable but rare; (X): lethal fixations may occur very rarely, but were not detected; 0: lethal fixation was not significantly higher than background.

Distribution of lethal fixations over the different pedigrees is not completely random: Finally, we inspected the data with respect to the question of whether there existed a fraction of especially sensitive cell families (pedigrees) accumulating CFC/NCFC pairs more frequently than the rest (nonrandom distribution). Assuming random and independent occurrence of lethal fixations, one can calculate the probabilities for finding certain tree configurations (see Figure 1 and APPENDIX B) which then can be compared with the experimentally obtained distribution of tree shapes. In total, 18 different tree configurations are possible. In Table 6 numbers of experimentally found pedigrees exhibiting no, one, two, or three apparent lethal fixations are listed for different experiments and are compared to the numbers expected according to the calculation. Though experimental
and theoretical values are similar, a bias can be observed, as in every single experiment there are too few pedigrees with only one apparent lethal fixation. When data of all experiments listed are taken together, the experimental value is more than two standard deviations smaller than the theoretical value. In biological terms this means that apparent lethal fixations tend to cluster in some pedigrees, while in others none are found. Therefore the probabilities listed in Table 4 represent mean probabilities per generation, considering that pedigrees with no apparent lethal fixation in previous generations tend to have lower probabilities for apparent lethal fixations in later generations. Thus, in our experiments the tendency was observed that a subpopulation accumulated more apparent lethal fixations than the rest of the population.

**DISCUSSION**

We have presented a detailed study on the timing of the lethal action of various DNA damaging agents with the aid of pedigree analyses. Treatment was carried out with haploid stationary phase cells and unbudded cells were selected for the analyses which included three post treatment generations. Thus, we were dealing with cells which at the time of treatment were in the G0 or G1 phase of the cell cycle and most probably contained just one complete copy of the genome. A probabilistic model to estimate the probabilities for the members of the pedigrees to become lethally fixed during post treatment proliferation was designed (see *APPENDIX*). It was based on the assumption that the lethal effects of the agents in question were genetic in nature and that other causes of cell death at the doses employed were negligible.

In the pedigree analyses of X-irradiated cells we found that practically all lethal events occurred directly in the treated cells (Tables 3 and 4). This result fits well with our earlier observation that the decrease of nonviable cells from X-irradiated populations during proliferation proceeded according to a simple concept of dilution of killed cells by proliferating survivors (KLEIN, KARWAN and WINTERSBERGER 1989). Comparing the two experiments with cells having received different doses of X-ray (5 and 14 krad) shows that, according to expectation, the probability of 00-fixations (the synonym of lethal hits) increased with dose. We suggest that the 00-fixed cells predominantly died from DNA double strand breaks, directly produced by the radiation (FRIEDBERG 1984). Remarkably, the percentage and the calculated probabilities of 0-fixations (which we interpret as lethal mutations fixed directly in the treated cells before DNA-replication and affecting both strands of DNA) rose more steeply than that of the 00-fixations. The number of secondary lethal fixations was not higher than in the pedigrees of untreated cells; thus in the small sample of cell families analyzed we were not able to observe any long term lethal effects from X-irradiation.

During our population studies on MMS treated cells we did hardly notice any difference to X-irradiated cells (KLEIN, KARWAN and WINTERSBERGER 1989). The pedigree analyses, however, uncovered a small but significant probability of secondary lethal fixations among the progeny of MMS affected cells (Tables 3 and 4). These data illustrate how rare effects on individuals may remain undetected in populations.

Sodium nitrite was once called a "direct mutagen," indicating the idea that this chemical may convert amino groups of DNA bases into hydroxyl groups and thus may cause mutations by mispairing during DNA replication (DRAKE and BALTZ 1976). Lethal mutations arising by this mechanism could account for the first and second generation lethal fixations in our analyses. However, these occurred at relatively low probability (Tables 3 and 4). We have recently reported, that cells which had survived nitrous acid treatment were extremely delayed in their progress through the first post treatment cell cycle but accelerated during the following cycles (WINTERSBERGER

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**TABLE 6**

Comparison between actual and calculated frequencies of tree configurations

<table>
<thead>
<tr>
<th>Experiment</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>EMS (95)</td>
<td>9 (7.7 ± 2.1)</td>
<td>7 (9.5 ± 2.2)</td>
<td>3 (1.8 ± 1.5)</td>
<td>0 (0.1 ± 0.3)</td>
</tr>
<tr>
<td>(65)</td>
<td>8 (6.5 ± 2.1)</td>
<td>8 (10.9 ± 2.2)</td>
<td>4 (2.4 ± 1.4)</td>
<td>0 (0.2 ± 0.4)</td>
</tr>
<tr>
<td>(75)</td>
<td>4 (0.9 ± 0.9)</td>
<td>7 (13.8 ± 2.1)</td>
<td>8 (4.8 ± 1.9)</td>
<td>1 (0.5 ± 0.7)</td>
</tr>
<tr>
<td>(75/8)</td>
<td>3 (1.7 ± 1.2)</td>
<td>11 (12.5 ± 2.2)</td>
<td>4 (5.0 ± 1.9)</td>
<td>2 (0.8 ± 0.9)</td>
</tr>
<tr>
<td>(62)</td>
<td>3 (0.9 ± 0.9)</td>
<td>28 (33.5 ± 3.3)</td>
<td>13 (12.4 ± 3.1)</td>
<td>6 (3.1 ± 1.7)</td>
</tr>
<tr>
<td>(20)</td>
<td>0 (0.3 ± 0.2)</td>
<td>42 (46.6 ± 1.9)</td>
<td>7 (2.7 ± 1.6)</td>
<td>1 (0.6 ± 0.8)</td>
</tr>
<tr>
<td>Sum</td>
<td>27 (18 ± 4)</td>
<td>103 (127 ± 6.1)</td>
<td>39 (29 ± 4.9)</td>
<td>10 (5 ± 2.3)</td>
</tr>
</tbody>
</table>

Tree shapes are classified according to the number of nonviable branches they harbor. Cases of four dead branches per tree (expected to be rare) were not observed. Positive coupling shows up in Table 6 as in every single experiment there are too few pedigrees with only one apparent lethal fixation. The probabilities for apparent lethal fixations in later generations tend to have lower probabilities for apparent lethal fixations in later generations.
and Karwan 1987). Together with the finding of nearly 80% of all lethal mutations happening in the cells that were in direct contact with the mutagen (0-fixations, this study) this indicates that in NaNNO damaged cells pre-replication repair mechanisms may be very active.

In the work presented here we mainly concentrated on the lethal effects of EMS. It turned out that EMS from all agents tested (at doses resulting in comparable conventionally determined survival rates) had the strongest potential to induce secondary lethal fixations (Tables 3 and 4). Therefore one question posed earlier (Klein, Karwan and Wintersberger 1989), namely whether delayed lethal fixations (in addition to residual divisions of lethally mutated cells) had occurred in the EMS treated populations (in which the fraction of colony forming cells had decreased during proliferation) can now be answered definitely: The probability for being lethally mutated is significant only from the first post treatment generation on, is about equally high in the second and is not at all zero in the third generation! That means, even if the whole progeny of a treated cell died out (an apparent 0-fixation) this was achieved most probably by more than one lethal fixation among the descendents of that cell. These secondary lethal fixations can lead to a decrease in the fraction of not lethally fixed cells in populations proliferating after mutagenic treatment, in spite of the doubling of cell numbers in each generation. From our pedigrees studied up to the third generation we have no information in which generation the emergence of newly fixed NCFCs among the descendents of EMS treated cells finally would stop. The significant decline from the second to the third generation in all the experiments with various doses of EMS will probably continue in further generations, ultimately leading to background levels of lethal fixations. The further increase of the number (not the fraction!) of NCFCs observed in the growing population (Klein, Karwan and Wintersberger 1989) can be explained by residual divisions of lethally fixed cells.

Comparing the experiment carried out with the highly mutagenic N-nitroso-compound, MNNG, with those employing EMS and MMS, uncovers an interesting fact which could not have been detected by conventionally determining the fraction of noncolony forming cells: Although the total fraction of NCFCs directly after the mutagenic treatment is similar for the experiments EMS (20) and MNNG (17), Table 4 impressively shows that the causes of death brought about by the two agents are very different. EMS, at the high molecular concentration necessary to kill 80% of the treated cells, causes lethal hits (00-fixations) in about half of these cells and multiple secondary lethal fixations in the progeny of the rest. MNNG, which at a much lower molecular concentration kills a comparable fraction of treated cells, produces mainly primary lethal fixations (i.e., mutations) and only a smaller fraction of lethal hits. MMS, if used at a dose leading to a similar probability of lethal hits (00-fixations) as in the two experiments discussed (compare experiment MMS (44) with experiments EMS (20) and MNNG (17) of Table 4) does hardly cause any lethal 0-fixations. A semiquantitative comparison of the lethal effects exerted by the DNA damaging agents studied can be found in Table 5.

In a population of yeast cells, even though descending from a recently cloned individual, cells more or less sensitive to a certain treatment (a certain kind of stress) might exist. We asked whether lethal events (all sorts of lethal fixations) were evenly distributed over all pedigrees descending from our EMS treated cells. As documented in Table 6 we found a small but significant deviation from the distribution calculated under the assumption of complete randomness. More cell families than expected were found "completely healthy" (no nonviable member within three generations); more families than expected gave rise to more than one nonviable descendent ("sick families"); and families with just one nonviable member were found underrepresented. Whatever the mechanisms underlying such heterogeneity among genetically closely related cells might be, our finding clearly indicates how subtle information on biological properties of individuals is lost by determining such properties from averaged data only.

In conclusion then, by employing our mathematical model for the interpretation of data from pedigree analyses of DNA damaged haploid yeast cells, one gets insight into the kinetics of cell killing by genotoxic agents. Such a study makes it possible to assign "death" (defined as the loss of the ability to produce a lineage of viable descendants) to certain cells of discrete post treatment generations. In addition one can discriminate between cells killed by immediately effective lethal hits and those suffering from lethal mutations which allow several residual cell divisions. We have shown that DNA-damaging agents differ widely concerning the mechanisms by which they bring about cell death. Moreover the distribution between different kinds of cell death caused by one agent varies with the dose. If we assume that nonlethal mutations may arise by the same molecular processes as do lethal mutations (which seems to be a reasonable assumption) our analysis gives information in general about the kinetics of mutation fixation. In this sense the results of our analysis concerning fixation of lethal mutation after DNA-damage with EMS, are consistent with earlier findings of Lementt (1977) and Kilbey and Hunter (1983). These authors reported that nonlethal mutations caused by EMS increased in num-
ber if treated cells had the chance to replicate their DNA and to divide before mutants were selected. Our results do not give any clue to the molecular mechanisms underlying the genesis of lethal hits, lethal or nonlethal mutations. Nevertheless, hypotheses about such mechanisms triggered by various agents (as, e.g., pre- or post-replication mutagenic repair of various DNA lesions) can be dismissed if their predictions are at variance with the kinetic data reported herewith.

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LITERATURE CITED

**APPENDIX**

A method to estimate the rate of genetic fixations from pedigree data

From observations of cell populations, as well as from the pedigree analyses it is obvious, that lethally mutated cells may still be able to perform a limited number of divisions. This circumstance prevents direct interpretation of the results of the pedigree analyses and prompted us to introduce mathematical corrections to assess the number and the genealogical points of occurrence of the primary genetic events. In order to sort the raw data emerging from pedigree analyses, we made two basic assumptions.

1. Cells that are not able to develop into visible colonies under optimal growth conditions, especially after DNA-damaging treatment, suffer from genetically determined alterations.

2. Probabilities for lethal fixations may differ in the course of successive generations, but will be assumed equal for all cells belonging to the same generation, irrespective of whether they descend from the same mother cell or not.

Whereas Assumption 1 seems evident from the understanding of the action of mutagens, Assumption 2, the independence of lethal fixations, has to be examined. If it is falsified, we have to interpret the corrected fixation probabilities as mean probabilities per generation. With the data at hand we cannot investigate whether there exists a mechanism like “curing” if we understand “cured cells” as those descendants of sick families which themselves do no longer produce inviable progeny.

Provided the validity of assumption 1 it follows that:

i. A cell, producing as little as one viable branch (compare Figure 1B, f) can itself not be regarded as lethally fixed, we call it a CFC.

ii. The first NCFC, that occurs in a cell lineage, will usually be the one in which the lethal fixation had happened. If, however, several lethal fixations destroy all the progeny of a cell independently in later generations, the result will at first sight be indistinguishable from the previous case.

A. Calculating the corrected probabilities for lethal fixations for the different generations

Declaration of variables:

\[ \hat{a}_i \] number of pairs of cells in the ith generation, one of which is apparently not lethally fixed, while the other is.

\[ \hat{b}_i \] number of pairs of cells in the ith generation, both of which are apparently not lethally fixed.

\[ a_i \] corrected number of pairs of cells in the ith generation with one lethal fixation.

\[ b_i \] corrected number of pairs of cells in the ith generation without lethal fixation.

\[ \hat{G}_i \] total number of pairs in the ith generation

\[ G_i \] total number of pairs in the ith generation, not lethally fixed before then.

\[ p_i \] probability of lethal fixation of a cell in the ith generation or of all of its progeny.

\[ \hat{p}_i \] probability of lethal fixation of a cell in the ith generation.

Relation between \( p_i \) and \( \hat{p}_i \):

\[
\hat{p}_i = p_i + (1 - p)\hat{p}_{i+1}.
\] (A1)

This recursive formula takes into account that extinction may be caused by immediate lethal fixation (\( p_i \)) or, alternatively, by simultaneously dying out of both daughter branches (\( \hat{p}_{i+1} \)). Relations between \( p_i \) and \( \hat{a}_i \) and \( \hat{b}_i \):

**LITERATURE CITED**


**HAEFNER, K., 1967b.** A remark to the origin of pure mutant clones observed after UV treatment of Schizosaccharomyces pombe. Mutat. Res. 4: 514-516.


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\[ \hat{a}_i = 2\hat{p}_i(1 - \hat{p}_i)G_i \]  \hspace{1cm} (A2)

\[ \hat{b}_i = (1 - \hat{p}_i^2)G_i \]  \hspace{1cm} (A3)

Equation A2 describes pairs of branches, one of which dies out \((\hat{p}_i)\), whereas the other \((1 - \hat{p}_i)\) does not. Regard, that there are two possible arrangements (factor 2). Multiplication with \(G_i\) converts the probability into a number. Note that \(\hat{a}_i + \hat{b}_i \leq G_i\). Equation A5 describes pairs of colony forming cells \((1 - \hat{p}_i^2)\) times \(G_i\).

By eliminating \(G_i\) from (A2) and (A3) we get:

\[ \hat{p}_i = \hat{a}_i/(\hat{a}_i + 2\hat{b}_i) \]  \hspace{1cm} (A4)

When \(\hat{p}_i\) is computed explicitly from (A1) and \(\hat{p}_i\) is replaced by (A4), we get the relation:

\[ \hat{p}_i = (\hat{a}_i/(\hat{a}_i + 2\hat{b}_i) - \hat{p}_i^2)/(1 - \hat{p}_i^2) \]  \hspace{1cm} (A5)

Equation A5 yields the corrected probabilities from directly countable numbers obtained by the pedigree analyses. Remark: We are not able to correct the \(\hat{p}_i\) for the last observed generation, because for that case \(\hat{p}_{i+1}\) is, of course, not available. However, as \(\hat{p}_i\) was found lower than 0.11 for all experiments, correction of \(\hat{p}_i\) becomes unnecessary, if we assume that \(\hat{p}_i\) is not greater than \(\hat{p}_0\) (compare Equation A1).

B. Frequencies of configurations of trees

Here we present all possible combinations of apparent lethal fixations within a three generation binary tree: \(F, F_0, F_1, F_2, F_3, F_{12}, F_{13}, F_{23}, F_{125}, F_{135}, F_{235}, F_{223}, F_{335}, F_{325}\). Each number following the \(F\) (for fixation) represents one apparent lethal fixation, the number denoting the generation of its occurrence. Thus, for example \(F\) is a tree without any apparent lethal fixations, \(F_{325}\) is one with four apparent lethal fixations in the third generation (for easy comprehension, compare Figure 1B, a).

To test whether Assumption 2, namely that lethal fixation events are distributed randomly over the progeny of different cells, is justified, we will calculate the frequencies under that assumption and will then compare them with the experimental data. We are especially interested in comparing the proportion of trees with one single lethal fixation event only, to the rest of the trees. If there was positive or negative coupling between the events (e.g., inhomogeneity in sensitivity to the agent (positive) or protection after the first lethal fixation (negative)) the calculated proportions would differ from the experimental ones.

For the sake of clearness of representation the following abbreviations will be introduced:

\[ \hat{q}_j = 1 - \hat{p}_j \hspace{1cm} j = 0, 1, 2, 3, \ldots, N \]  \hspace{1cm} (B1)

\(\hat{q}_j\) simply denotes the probability for a cell not to seem to be lethally fixed in generation \(j\).

\[ F_0^1 \times 2^2 \times 3^3 \times \ldots \times N^N \]  \hspace{1cm} (B2)

\((i_0 = 0 \text{ or } 1), (0 \leq i_j \leq 2^{j-1} \text{ for } j \geq 1)\)

shall symbolize a binary tree with \(i_0\) apparent lethal fixations \((i.e., \text{CFC/NCFC pairs})\) in the \(j\)th generation. Two trees are topologically equivalent if all their \(i_j\) are the same \((i.e., \text{they harbour the same number of CFC/NCFC pairs in every generation})\).

\[ M_{i+1} = 2M_i - i_{i+1} \hspace{1cm} \text{and} \hspace{1cm} M_0 = 1 - i_0 \]  \hspace{1cm} (B3)

Given a certain tree shape, \(M_i\) is a number (multiplier) that counts how many cells are not apparently fixed in the \(j\)th generation.

\[ F_j = \binom{M_{j-1}}{i_j} \]  \hspace{1cm} (B4)

\(F_j\) is a factor of multiplication, that counts the possible arrangements of the two kinds of distinguishable cell pairs per generation.

Now we write the probability of the general \(N\)-generation tree with apparent lethal fixations occurring at random:

\[ p(F_0^1 \times 2^2 \times 3^3 \times \ldots \times N^N) = \prod_{j=0}^{N} F_j(2\hat{p}_j)^{i_j}(\hat{q}_j)^{M_j} \]  \hspace{1cm} (B5)

Remark: In the case of trees up to the third generation, only 18 configurations have positive probabilities.

For example, the probabilities of trees \(F_3\) (see Figure 1B, a) and \(F_{125}\) (see Figure 1B, f) are:

Tree \(F_3\): first calculate \(M_j\) and \(F_j\)

\(M_0 = 1, M_1 = 2, M_2 = 4, M_3 = 7\)

\(F_0 = 1, F_1 = 1, F_2 = 1, F_3 = \binom{4}{1} = 4\)

\[ p(F_3) = p(F_0^1 \times 2^2 \times 3^3) = 4 \cdot (2\hat{p}_3)^{i_3}(\hat{q}_3)^{M_3}. \]

Tree \(F_{125}\):

\(M_0 = 1, M_1 = 1, M_2 = 1, M_3 = 1 F_0 = 0, F_1 = 1, F_2 = 1, F_3 = 1\)

\[ p(F_{125}) = (2\hat{p}_3)^3(\hat{q}_3)^{M_3}(\hat{p}_3) \cdot \hat{q}_3 \cdot \hat{q}_3. \]

The values for \(\hat{p}_i\) and \(\hat{q}_i\) are calculated from the data using relation (4A). For Table 6 the calculated frequencies of trees with 0, 1, 2 or 3 CFC/NCFC pairs were pooled and compared to the experimental frequencies.