CONCERNING THE MECHANISM OF ULTRAVIOLET MUTAGENESIS. A MICROMANIPULATORY PEDIGREE ANALYSIS IN *SCHIZOSACCHAROMYCES POMBE*

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A problem basic to molecular genetics involves the explanation of a mutation brought about by a base alteration in only one of the two DNA strands. Considering a semiconservative model of DNA replication (MESELSON and STAHL 1958) one would expect stable alterations to cause a mutation in one half the progeny of a cell carrying only one set of genetic information. However, frequently pure mutant clones have been found to arise from a variety of cells treated with different mutagens known to cause preferentially single-base alterations (e.g. GILLHAM and LEVINE 1962; KIMBALL 1965; KUBITSCHEK 1964; NASIM and CLARKE 1965; NASIM and AUERBACH 1967; WITKIN 1951, 1966a).

Various models have been put forward to explain these pure mutant clones (see also NASIM and AUERBACH 1967): (1) *Special alteration.* Mutations affecting the entire progeny of a mutagen-treated cell result from alterations other than single-strand base changes, which in some way affect both DNA strands (see e.g. REISSIG 1963; WITKIN 1966b). (2) *Simulation of pure mutant clones by lethal sectoring.* The mutagen results a base alteration causing a mutation in only one half of the progeny, but the unmutated half of the progeny is inactive by some reason. Such inactive descendents of colony forming cells (lethal sectors) have been found after ultraviolet- and X-irradiation in yeast (e.g. HAEFNER 1965; JAMES and WERNER 1966) and in *Escherichia coli* (HAEFNER and STRIEBECK 1967). (3) *Double mutation.* Two possibly independent single-base changes occur in opposite DNA strands at different nucleotide pairs but within the same gene causing two heteroallelic mutations. The resulting clone is phenotypically pure. (4) *Enzymatic repair.* The base alteration takes place primarily in only one DNA strand but is copied appropriately to the opposite strand by an enzymatic (repair) process which for example may take place in the same region on the opposite DNA strand (FREESE and FREESE 1966; SETLOW 1964; WITKIN 1966a). (5) *Master strand DNA replication.* When DNA replication takes place, both DNA strands do not act as independent templates. A single altered DNA base determines a change in the base sequence of both new double strands (KUBITSCHEK and HENDERSON 1966).

In this paper results are presented which were obtained by studying the descendents of phased mutagen-treated cells using micromanipulator techniques. Ultraviolet light was chosen as a mutagen which is believed to alter predomi-

nantly bases or base pairs on one strand, although other photoproducts have been found (for review see SMITH 1966). The yeast *Schizosaccharomyces pombe* was taken as an organism which is uninucleate and haploid, and allows pedigree analysis of the progeny of a large number of treated cells as well as a detailed genetic analysis of the mutants. Mutations from prototrophy to auxotrophy as well as from normal to slow growth rates were studied. The results obtained have been used to discuss the relevance of the proposed models. Explanations (2) and (3) seem very unlikely to account for the appearance of all pure mutant clones.

MATERIALS AND METHODS

Strains and media: The following strains of *Schizosaccharomyces pombe* were used: L972 (haploid, wild type, mating type h^-), L975 (haploid, wild type, mating type h^+) and the UV-sensitive strain U30/23-4d (haploid, mating type h^-). The later strain is genetically identical to strain U23-30-1b (for further information see HAEFNER and HOWREY 1967).

All experiments were carried out on YEP-agar (1% yeast extract, ½% peptone, 2% glucose, and 2% agar). To detect auxotrophic mutants, colonies were replica plated on a minimal medium (MMA, LEUPOLD 1955). The requirements of the mutants isolated were determined by replica plating on MMA supplemented with amino acids (100 mg/ml). All crosses leading to zygote formation and sporulation were made on malt extract agar (MEA, 3% malt extract, 2% agar).

Pedigree analysis: Pre-culture and cell phasing was done in the following way: Cells from the stock cultures were streaked out at the edge of a YEP-agar-layer (for the production of such layers, see HAEFNER 1967a), and 24 hours later, cells which just had finished the formation of the partition wall were isolated with a *de Fonbrune* micromanipulator, separated, and spaced equally in one line near the edge of the layer. Within ten minutes after separation, the cells were UV-irradiated on the medium (low pressure mercury lamp HNS12, OSRAM; dose rate 59 erg mm $^{-2}$ sec^{-1}, maximum output at 2537 Å). The number of cells placed in a row was chosen so as to have an average of three colony-forming cells among them. The cells were incubated at 30°C and observed microscopically. Those which started to divide were placed in specific positions. Descendents of these cells were transferred to other places resulting in one line of 16 cells in the fourth generation.

The colony-forming ability was examined after incubation for 72 hours at 30°C. A pedigree branch cell was classified as inactive if it did not give rise to a colony with a diameter > 0.1 mm. Lethal sectors were scored, disregarding possible residual divisions of the inactive pedigree cell. If for example, the inactive cell (lethal sector) shown in the first generation in pedigree S135-31d (Figure 1) would have two inactive descendents they are not taken into further consideration.

All illumination, including that for the microscope, was with yellow light, even though there is no photoreactivation or photoreactivating enyzme in *Schizosaccharomyces pombe* (HAEFNER and RUPERT, unpublished results).

Detection and characterization of mutations: All colonies obtained as descendents from irradiated cells in the pedigree experiments were transferred to MMA agar by replica plating. Forty-eight hours after transfer, phenotypically auxotrophic mutants could be detected easily on the MMA agar incubated at 30°C. (In some cases such auxotrophic colonies failed to show their character again later on. They have been disregarded, although possibly an interesting problem of instability might be involved.) Colonies growing slowly on the YEP layers were transferred to YEP agar to reconfirm their slow growth characteristics. Auxotrophic, as well as slow-growing strains, were crossed with strain L975. At least five asci were isolated from each cross and dissected with the micromanipulator. Only those phenotypic mutants which gave a 2:2 segregation for the phenotype under study, were considered to be genetic mutants and taken into further consideration. Only a small fraction of the phenotypic mutants isolated failed to give this 2:2 segregation.

Spontaneous mutations in control experiments: The rate of spontaneous mutations occurring in the pedigrees was estimated without micromanipulation by simulating a pedigree experiment in the following way: From a single prototrophic cell, a colony was grown on YEP. This colony was resuspended in M/20 KH_2PO_4 at a titer of 3 cells per 0.1 ml. About 300 petri dishes containing YEP, which were preirradiated with 1500 erg mm^{-2} to take care of possible induction of mutations by the UV-irradiated medium, were each inoculated with 0.1 ml of the cell suspension and were incubated at 30°C long enough to allow growth of 16-cell-microcolonies, on the average. At that point, the microcolonies were spread on the agar surface with 0.2 ml M/20 KH_2PO_4 and reincubated for 4 days. All colonies formed were replicated on MMA. Inspection of the replica spots after 36 hours incubation allowed detection of all spontaneous mutants which have appeared in the 16-cell-microcolonies, resembling fourth generation pedigrees.

Crosses between auxotrophic branches of a mutant pedigree: Strains resulting from auxotrophic pedigree branches were crossed with strain L975. Asci resulting from the crosses were dissected using micromanipulation, and auxotrophic spore cultures of mating type h^+ were selected. These spore cultures were crossed with strains resulting from other pedigree branches of the same pedigree. To test for intragenic recombination, about 10^8 spores from such a cross were spread on MMA. Absence of any prototrophic colony on this medium after 5 days at 30°C was taken as an indication that the crossed pedigree branches were homoallelic.

RESULTS

Mutations and lethal sectoring in control experiments: In control experiments on UV-irradiated medium but with *un*irradiated cells, the probability of spontaneous mutations and spontaneous lethal sectors respectively was studied. In the simulated pedigree experiments, no auxotrophic or slow growing mutant was found (Table 1). According to the calculation shown in Table 1, the frequency of spontaneous mutations in the pedigrees is less than 5×10^{-4} per cell.

To calculate the chance of having introduced a spontaneous mutation in the UV experiments, 7548 cells of strain L972 from the stock culture were plated on YEP and incubated for 4 days at 30°C. The colonies grown were replicated to MMA. One auxotrophic and no slow growing colony was found. This gives a probability of less than 10^{-3} for the introduction of a mutated pedigree mother cell in the UV experiments.

The frequency of noncolony-forming descendants (lethal sectors) in pedigrees derived from unirradiated cells of strains L972 and U30/23–4d was determined by pedigree analysis on UV-irradiated YEP medium. For both strains, fewer than 1.5% lethal sectors per generation were observed (Table 2). As known from

TABLE 1

Spontaneous mutations in simulated pedigrees derived from unirradiated cells of strain L972 on UV-irradiated YEP medium

No. of plates inoculated	Mean No. of cells per plate before resuspending	Mean No. of colonies per plate grown after resuspending	Total No. of colonies replicated on MMA (a)	No. of auxotrophic replica spots and slow growing colonies (b)	Mutation frequency per pedigree cell (b/a)
283	4.8	74	20,957	0	0/20,957 $< 5.10^{-4}$*

* Calculated as upper limit of confidence according to equation (18), page 173, in WEBER (1957) for $\epsilon = 5\%$.

TABLE 2

*Spontaneous lethal sectoring of unirradiated cells on UV-irradiated YEP-layers**

	No. of colony-forming cells (c) and lethal sectors (l) observed in generations I to IV†											
	I			II			III			IV		
Strain	c	l	$\frac{l}{c+l}$	c	l	$\frac{l}{c+l}$	c	l	$\frac{l}{c+l}$	c	l	$\frac{l}{c+l}$
L972	296	2	*0.6%*	585	8	*1.3%*	1098	4	*0.4%*	1868	6	*0.4%*
U30/23-4d	156	2	*1.3%*	301	1	*0.3%*	588	8	*1.3%*	757	9	*1.2%*

* The UV dose for the layers with strain L972 was 1500 ergs mm^{-2}, with strain U30/23-4d, 500 ergs mm^{-2}.
† In a complete pedigree analysis the sum of $c+l$ in one generation should always be two times the number of c in the preceding generation. Deviations from this expectation come from pedigrees which were not completed, owing to cell divisions delayed beyond the observation time.

control experiments this small amount of lethal sectors does not result from the UV-irradiated agar.

Occurrence of mutations in pedigrees after UV-irradiation: 1,592 pedigrees derived from phased cells of strain L972 irradiated with 885 erg mm^{-2} (average survival 36%) resulted in 23 pedigrees with at least one branch carrying a genetic mutation. Considering the low frequency of spontaneous mutations, a large majority of these mutants must be UV-induced.

Only those pedigrees displaying a mutated branch *and* at least one lethal sector are presented in Figure 1. In 19 of the 23 pedigrees, shown in Table 3, it was

TABLE 3

The pattern of occurrence of mutations observed in pedigrees derived from strain L972 after UV-irradiation with 885 ergs mm^{-2}

Pedigree No.	Mutation pattern	Type of lethal sectoring	Type of mutation
S16-11a*	1/1	1/4 and 1/16	methionine auxotrophy
S26-14c*	1/4	1/4, 2/8 and 1/16	auxotrophic†
S26-22a	1/4	no	auxotrophic†
S31-26e	1/1	no	adenine auxotrophy
S45-17d	1/2	no	leucine auxotrophy
S51-18b	1/2	no	slow growth
S53-25f	1/2	no	slow growth
S61-9c	1/2	1/4	lysine auxotrophy
S61-11b	1/2	no	threonine auxotrophy
S110-15a*	1/2	1/4	slow growth
S111-22a	1/1	no	adenine auxotrophy
S111-31b*	1/2	2/8	leucine auxotrophy
S112-1c	1/2	1/4 and 1/8	slow growth
S112-22b*	1/2	1/4	slow growth
S114-5a	1/1	no	slow growth
S132-17a	1/4	no	slow growth
S135-51c*	1/2	1/8	slow growth
S135-43a	1/1	no	slow growth
S135-58b	1/4	no	slow growth

* Shown in Figure 1.
† Does not grow on MMA + all supplements tested, grows on YEP.

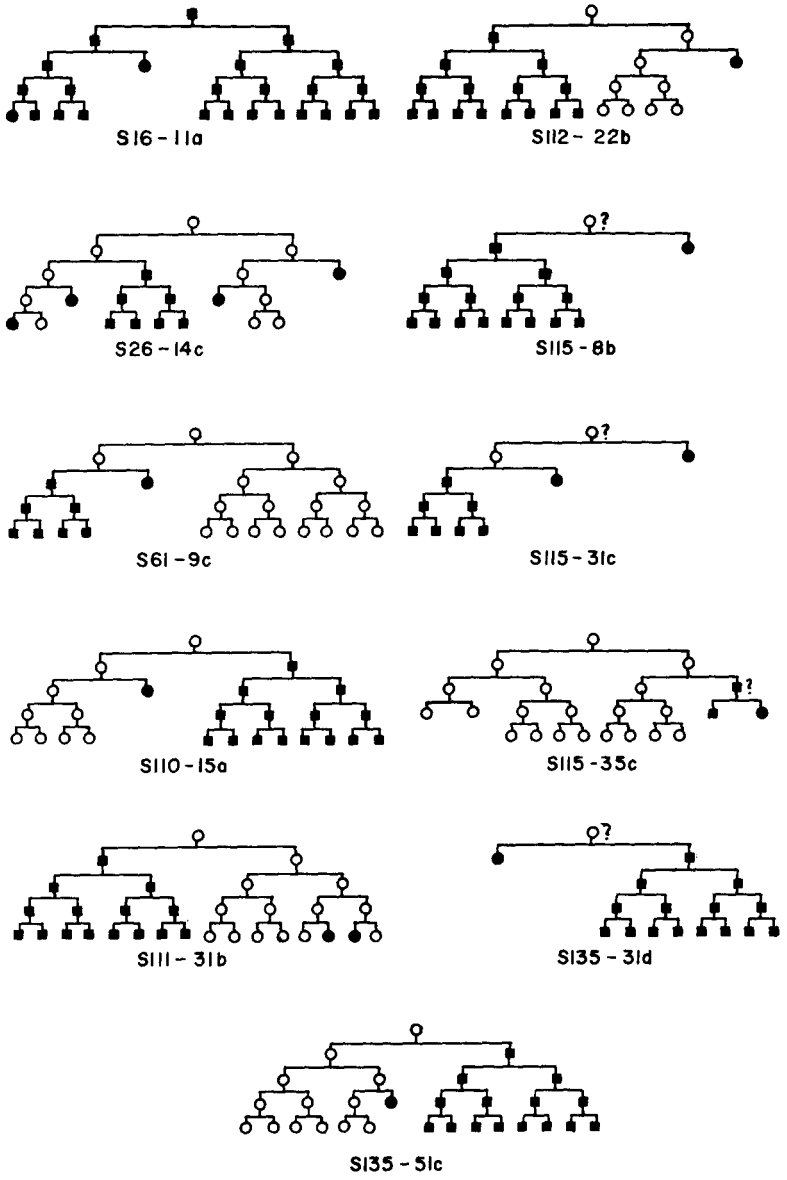

FIGURE 1.—Pedigrees containing mutants *and* lethal sectors. Strain L972, UV-dose: 885 ergs mm^{-2}. Notice pedigrees which possess only mutated and inactive cells. ○ = colony-forming branch, unmutated, ● = non-colony-forming branch (lethal sector), ■ = mutated colony-forming branch. A question mark indicates that the mutational stage of that particular cell is indeterminable.

possible to ascertain the generation in which the mutation has occurred. In five out of these 19 pedigrees, the mutation affects the entire progeny of the UV-irradiated cell. No case was observed in which an unmutated descendent occurred

within an otherwise mutated pedigree branch. This indicates that all mutations obtained show no reversions in the first postirradiation generations.

With the UV-sensitive strain U30/23–4d, only two pedigree experiments were done. No mutant pedigree was found in 136 pedigrees tested.

Recombination between mutant pedigree branches: To study how far all branches of a mutant pedigree contain the mutation at the same intragenic site, crosses were made between auxotrophic strains resulting from different branches. In several crosses between branches of pedigrees S16–11a, S31–26e, and S111–22a, no prototroph recombinant was observed. This suggests strongly that all branches within a mutant pedigree possess the mutation at the same intragenic site.

Appearance of lethal sectors after UV-irradiation: Table 4 shows the total numbers and the frequencies of non-colony-forming descendants (lethal sectors) observed in a large number of mutation experiments with the wild-type strain L972 and with the UV-sensitive mutant U30/23–4d. For strain L972 only data obtained from 975 pedigrees are presented, since our last four experiments resulting in 607 pedigrees have not been exploited in respect to lethal sectoring.

The frequencies of lethal sectors obtained for strain L972 are in good agreement with data reported earlier (HAEFNER 1966). The UV-sensitive strain U30/23–4d shows a remarkably high amount of lethal sectoring. At 14% survival all 22 pedigrees show one lethal sector in the first generation. At the lower dose, giving 59% survival, 89 out of 114 pedigrees have a lethal sector in the first generation, only 25 or 22% have two active first-generation descendants.

DISCUSSION

The results presented in Table 3 and Figure 1 demonstrate clearly that the entire progeny of a UV-irradiated *Schizosaccharomyces pombe* cell can carry a mutation. In addition, a number of pedigrees have been obtained in which mutations occur only in one half or one quarter of the progeny. To interpret the latter in genetic terms, it is important to take into consideration the relative DNA content of the cells at the time of UV-irradiation. All our experiments were done with cells which had just finished cell division. Such cells have been shown to be in late G_1- or S-phase (BOSTOCK *et al.* 1966), indicating that at the time of irradiation they possess between one and two complete sets of DNA[1]. Therefore, it is very likely that a fraction of the ½-mutant pedigrees results from cells containing at least part of the genome already doubled. These pedigrees cannot be taken as an example for a case in which a cell with one chromosome set gives rise to a mutated and an unmutated descendant ("mutation segregation"). The ¼-mutant pedigrees S26–14c, S26–22a, S132–17a, and S135–58b (see Table 3), however, can

[1] The division cycle of *Schizosaccharomyces pombe* makes it impossible to use exponentially growing cells with an unreduplicated set of DNA for this type of experiments, since nuclear division is followed immediately by DNA replication, which takes place simultaneously with cell division (BOSTOCK *et al.* 1966). This complication was not envisaged at the time our experiments were started. Cells immediately after cell division were used, since this stage is a very well defined one.—Experiments with UV-irradiated spores are now in progress. Spores have been found to contain only one set of DNA (BOSTOCK, private communication).

TABLE 4

Ultraviolet-induced lethal sectoring

Strain	UV-dose (ergs mm^{-2})	Survival	No. of pedigrees	No of colony-forming cells (c) and lethal sectors (l) in generations I to IV*											
				I			II			III			IV		
				c	l	$\frac{l}{c+l}$	c	l	$\frac{l}{c+l}$	c	l	$\frac{l}{c+l}$	c	l	$\frac{l}{c+l}$
L972	885	0.36	975	1627	323	*17%*	2880	312	*10%*	5306	192	*3.5%*	9912	138	*1.4%*
U30/23-4d	120	0.59	114	139	89	*39%*	244	32	*12%*	447	24	5%	817	22	*2.5%*
	180	0.14	22	22	22	*50%*	29	14	*33%*	48	12	*20%*	86	4	*4.5%*

* see † in Table 2.

be explained most readily by assuming mutation segregation in the progeny of a cell which had already duplicated its genome at least partly.

The use of cells in G_1- or S-phase, however, makes it impossible to determine the exact fraction of pure mutant clones from our data; however, this must equal or exceed $5/19 = 0.26$. This frequency is consistent with data reported by KIMBALL (1965), who showed with another individual-cell technique that recessive lethal mutations occur mainly in the entire progeny of UV-irradiated *Paramecium aurelia*. Similar results have been obtained with individual-cell techniques after X-irradiation (JAMES and WERNER 1966; KIMBALL 1964).

For further discussion we shall take as a basis that in *Schizosaccharomyces pombe* UV-induced mutations affect either one half or the entire progeny which descends from one chromosome set. Our data show no certain case of a further delayed mutation expression.

The observed mutations affecting one half of the progeny are consistent with the simple assumption that mutagenic photoproducts are restricted to one DNA-strand, causing a change in the genetic information of this strand. By semiconservative DNA replication, this alteration is transferred appropriately to only one of the two new DNA double strands. The observation of mixed mutant clones resulting from a single chromosome set indicates that in *Schizosaccharomyces pombe* DNA replication in general does not operate according to a master strand DNA replication mechanism (KUBITSCHEK and HENDERSON 1966), since this model would require that no mixed mutant clone should occur.

Our data show that mutant clones can occur which carry the mutation in all cells without showing lethal sectors (Table 3). Furthermore, the lack of intra-allelic recombination between the pedigree branches crossed suggests strongly that a pure mutant pedigree carries the same homoallelic mutation in all its branches. Therefore, models (2) and (3) from the Introduction ("simulation of pure clones by lethal sectoring" and "double mutation") are ruled out experimentally as an explanation for the real pure mutant clones obtained here. Lethal sectoring, however, causes quite frequently mutant clones which are phenotypically pure (see pedigrees S115–8b, S115–31c, S135–31d in Figure 1).

To gain information relevant to the "enzymatic repair" model, we attempted to determine the fraction of pure UV-induced mutant clones in the UV-sensitive strain (U30/23–4d) which probably lacks a repair system. This attempt failed, since the high frequency of lethal sectoring observed in this strain (Table 4) made it impossible to obtain a sufficient number of mutant pedigrees without lethal sectors in the first generation.

A similar high amount of lethal sectoring was found in *Escherichia coli* B_{s-1} (HAEFNER and STRIEBECK 1967). Therefore, it is believed that lethal sectoring in general will complicate studies of the influence of changed repair capacities on the fraction of pure mutant clones. A simple mutation assay system (color test, replica plating), as it is used frequently (e.g. WITKIN 1966a), might give a misleading figure, since the high amount of lethal sectors will simulate a higher fraction of pure mutant clones. Pedigrees S115–8b, S115–31c, and S135–31d in Figure 1 are examples of such a simulation of pure mutant clones. In a microscopic assay

system, they would be scored as pure mutant clones. Their classification, however, is in principal impossible, since there is no way of testing a lethal sector in respect to its mutational state (see also HAEFNER 1967b).

To what extent the "master strand DNA replication" explanation accounts for the pure mutants cannot be determined from our data. As discussed before, the ¼-mutant and presumably part of the ½-mutant pedigrees indicate that DNA replication normally requires both strands as templates. On the other hand, however, it is not impossible to assume that particular DNA alterations may occur after UV-irradiation which, at the time of the first DNA replication, influence both new double strands.

In agreement with conclusions drawn by NASIM and AUERBACH 1967, our results leave two possibilities to explain the real pure mutant clones in *Schizosaccharomyces pombe*: (a) UV induces special photoproducts which in some way affect both newly synthesized DNA double strands or (b) pure mutant clones result from single-strand alterations which are copied appropriately to the opposite strand, e.g. as a result of a repair process acting on the opposite strand. Whereas it seem technically very difficult to detect biochemically such rare "special alterations," it might be possible to determine the significance of model (b) by studying the frequency of UV-inducted real pure mutant clones in strains lacking repair systems using micromanipulator techniques, if these techniques are improved.

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SUMMARY

The pattern of occurrence of ultraviolet-induced auxotrophic and slow growth mutations in pedigrees derived from about 1600 individually irradiated phased cells was studied, using micromanipulation. In five out of 23 pedigrees, all fourth-generation branches of the pedigree were mutated (pure mutant clones). Within such pedigrees all branches carry the same homoallelic mutation. In 10 out of those 23 only one half of the progeny is mutated.—From our data it seems likely that real pure mutant clones either come from photoproducts affecting both DNA strands simultaneously, or result from single-strand alterations followed by a process copying the change in one strand onto the opposite stand. Several cases have been found in which a pure mutant clone occured from a cell which had only one active first generation descendent.

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