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Analysis of the genetic instability induced by nitrous acid in *Schizosaccharomyces pombe*

BY N. LOPRIENO, A. ABBONDANDOLO, S. BONATTI AND R. GUGLIELMINETTI
Institute of Genetics, The University, Pisa, Italy

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1. INTRODUCTION

When cells of *Schizosaccharomyces pombe* are treated with nitrous acid (NA), most induced mutations are scored as mosaic colonies (Guglielminetti, Bonatti, Loprieno & Abbondandolo, 1967). When cells from such mosaic colonies are replated on agar, new mosaic colonies are found among the colonies scored during thirty or more cell divisions after the mutagenic treatment (Abbondandolo, Bonatti, Guglielminetti & Loprieno, 1967)

In *S. pombe* this phenomenon is not limited to a given mutagen, since ethylmethanesulphonate (EMS), methylmethanesulphonate (MMS), hydroxylamine (HA) and nitroso-compounds have produced the same effect (Loprieno, Guglielminetti, Abondandolo & Bonatti, 1965). This behaviour is particularly clear when mutations are analysed which induce a change in the phenotype of the mutated cells: the genetic system we have been using for detection of mosaic colonies is forward mutation from wild type to adenine requirement at two loci (*ad-6*, *ad-7*) controlling two of the several steps of the adenine pathway in *S. pombe* (Leupold, 1956).

Similar results have been obtained in the same organism, but for a different genetic system, by Nasim (1965), using chemical mutagens and u.v.-light: this finding resembles the results obtained in *Drosophila* after treatments with chemical mutagens (Auerbach, 1951; Mathew, 1965). According to Auerbach (1967), 'chemical treatment must have created instabilities which can also replicate as instabilities'.

The present investigation is an analysis of five nitrous-acid-induced mosaics for mutations at the *ad-6* locus. For each of them, the incidence of mosaicism after repeated replating has been determined; four have been further analysed by tests of complementation and recombination and by physiological tests for adenine requirement at different temperatures and pH's.

2. MATERIALS AND METHODS

(i) *Genetic system*

Mutations from wild type to adenine requirement at the *ad-6* and *ad-7* loci of *S. pombe* can be recognized by their purple phenotype on yeast-extract agar

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medium (Leupold, 1956). Forward mutations at these two loci were induced by treating the wild-type *972,h*$^{-}$ strain of *S. pombe*. Mosaics formed colonies with purple and wild-type (white) sectors.

(ii) *Mutagenic treatment*

The treatment with nitrous acid (as $NaNO_2$ solution) was made according to a previously described method (Guglielminetti *et al.* 1967). A treatment of 6 min was used: the survival, evaluated from the number of colony-forming viable cells, was of the order of *c.* 50 % with a frequency of mutant colonies of 4×10^{-3}. In these conditions, the percentage of mosaic colonies among the total mutant colonies was 70–80 %.

(iii) *Detection of instability*

For several mosaics (first cell-colony generation), all the cells from each colony were suspended in 10 ml. of GSVB buffer (Loprieno & Clarke, 1965) and diluted samples (10^{-4}–10^{-5}) were replated on yeast-extract medium in amount allowing a density of 20–30 colonies per plate. After 4 days of incubation at 30 °C the second cell-colony generation was scored and the proportions of wild-type, purple, and new mosaic colonies were determined. The third cell-colony generation was obtained by replating cell from ten independent wild-type and ten whole purple colonies of the second cell-colony generation, as well as from newly formed mosaic colonies. It was analysed in the same way as the second generation. In some cases, the fourth and the fifth cell-colony generations were also analysed. The adenine independence of the white colonies and the adenine dependence of the purple colonies were confirmed by replica plating. White adenine-dependent colonies were not included in the analysis since they represent second mutations at other loci for adenine requirement (Heslot, 1961).

(iv) *Isolation and tests of mutant clones*

Mutant clones were isolated by picking several whole mutated colonies per cell-colony generation. Mutant clones deriving from the same original mosaic colony were analysed both physiologically and genetically. Their adenine requirement at different temperatures (25°, 30° and 35 °C), and at different pH-buffered media (pH 3, pH 4, and pH 5) were determined and they were crossed with the tester strains *UV, ad-7, 50,h*$^{+}$ and *UV, ad-6, 250,h*$^{+}$ in order to determine their genotype. Only *ad-6* mutants were further subjected to intragenic recombination and interallelic complementation analyses.

(v) *Intragenic recombination*

The two heteroallelic h^{-} and h^{+} strains were prepared for each mutant clone, and reciprocal crosses in all possible combinations among the clones deriving from one original mosaic were done. The recombination frequency was evaluated after killing the vegetative cells by alcohol treatment, according to the method developed by Leupold (1965).

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(vi) *Interallelic complementation*

An interallelic complementation analysis of the mutant clones was made in crosses with seven *ad-6* tester strains, kindly provided by Professor U. Leupold, i.e. *U V, ad-6: 566,h⁺, 445,h⁺, 670,h⁺, 271,h⁺, 256,h⁺, 442,h⁺, 588, h⁺*. These mutants were shown by Leupold & Gutz (1965) to be able to complement the majority of the complementing mutants induced by u.v.-light and nitrous acid.

(vii) *Intralocus mapping*

The location of the mutants inside the *ad-6* locus of *S. pombe* was determined from the recombination frequencies observed in the crosses of the mutant clones with four *ad-6* non-complementing tester strains (*U V, ad-6: 250,h⁺, 428,h⁺, 463,h⁺, 430,h⁺*).

3. RESULTS

Five out of some fifty mosaic mutant colonies were chosen at random for analysis.

Figures 1, 2, 3, 4 and 5 illustrate the incidence of wild-type, mosaic mutant, and complete mutant colonies among the succeeding cell-colony generations of these five mosaics (NA-529, -535, -1013, -1019, and -1023).

From the NA-529 colony (Fig. 1), four cell-colony generations were analysed: mosaics were present in the 2nd, 3rd and 4th generations. Mutant clones 1–13 were isolated from the four generations; their origin is shown in Fig. 1. All proved ad_6^-

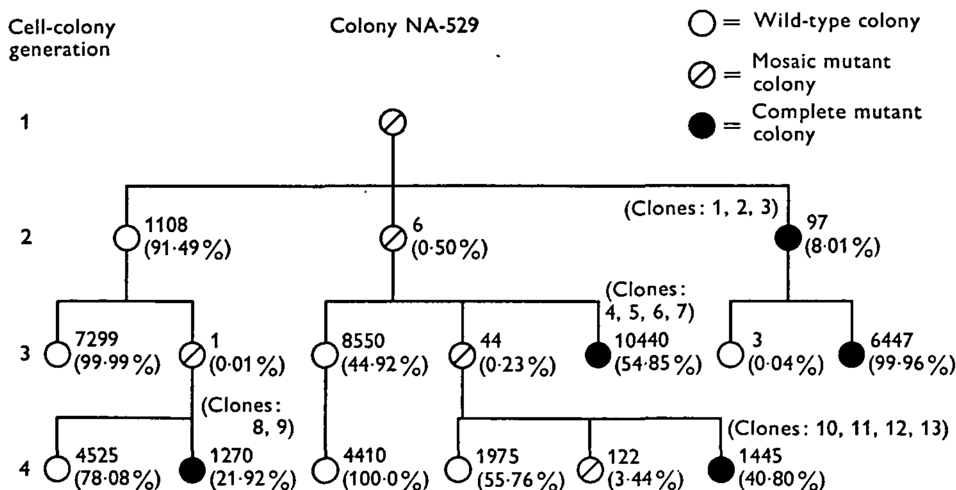

Fig. 1. Pedigree analysis of the NA-529 mosaic colony of *Schizosaccharomyces pombe* obtained after treatment with nitrous acid (NA). Actual numbers and percentages (in brackets) refer to wild-type, mosaic and complete mutant colonies scored after replating a sample of the buffered suspension of the whole colony of the previous cell-colony generation. Solid lines indicate the origin of the colonies in the generations following the original mosaic. Small numbers in brackets refer to subclones isolated and analysed both physiologically and genetically.

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and strongly leaky at 25 °, 30 ° and 35 °C; no further analysis was carried out on them.

The NA-535 mosaic (Fig. 2) gave new mosaics at the 2nd, 3rd and 4th cell-colony generations: subclones were ad_6^- and temperature adenine-dependent (they required adenine only at 35 °C).

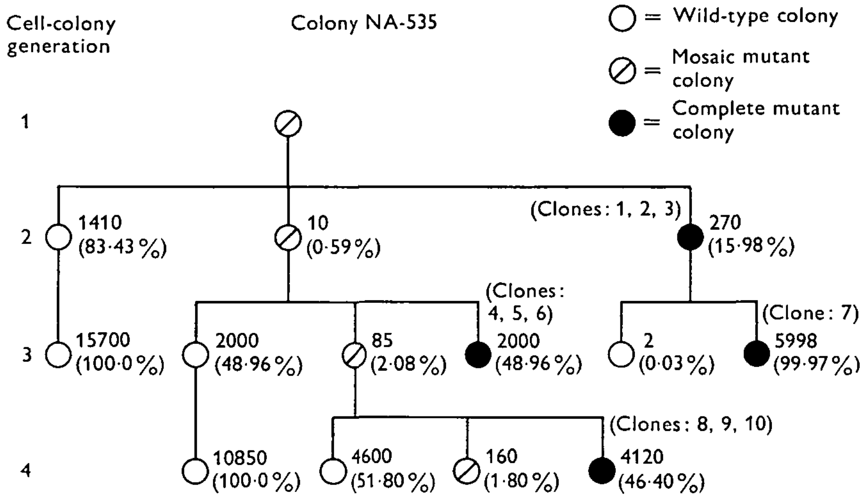

Fig. 2. Pedigree analysis of the NA-535 mosaic colony of *Schizosaccharomyces pombe* obtained after treatment with nitrous acid (NA). For explanation see Fig. 1.

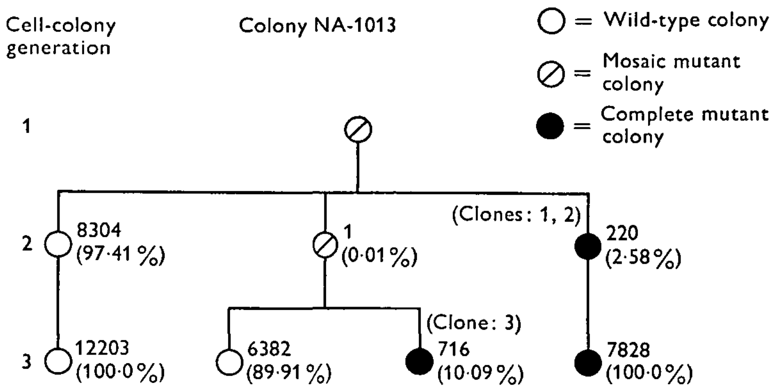

Fig. 3. Pedigree analysis of the NA-1013 mosaic colony of *Schizosaccharomyces pombe* obtained after treatment with nitrous acid (NA). For explanation see Fig. 1.

From the NA-1013 mosaic mutant colony (Fig. 3) clones 1–3 were isolated: they were adenine-dependent at all three temperatures tested and showed an *ad-6* genotype.

From the Na-1019 mosaic clones 1–8 were isolated as shown in Fig. 4. All the clones were adenine-dependent at all three temperatures and showed an *ad–6* genotype.

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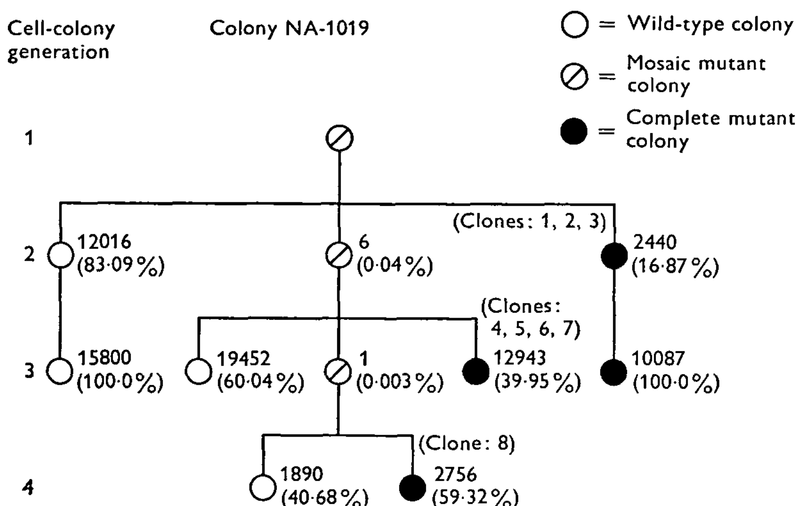

Fig. 4. Pedigree analysis of the NA-1019 mosaic colony of *Schizosaccharomyces pombe* obtained after treatment with nitrous acid (NA). For explanation see Fig. 1.

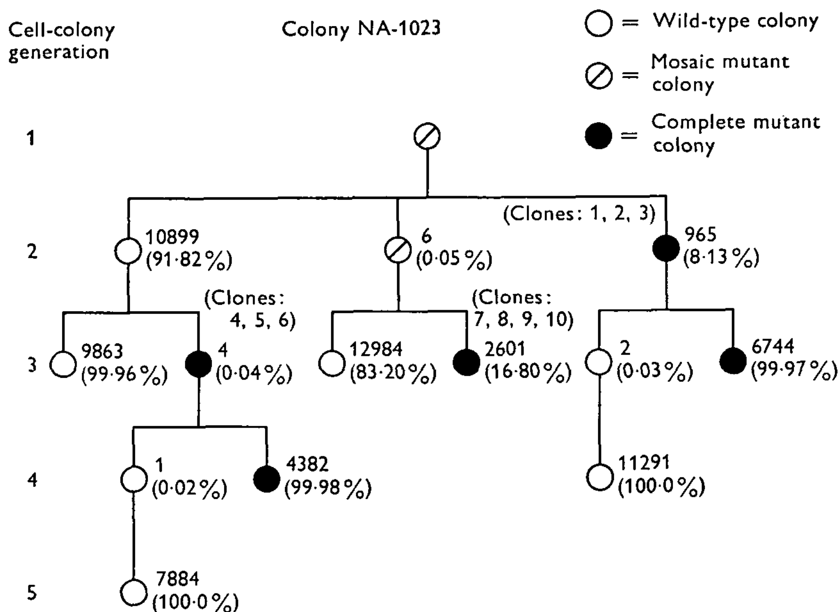

Fig. 5. Pedigree analysis of the NA-1023 mosaic colony of *Schizosaccharomyces pombe* obtained after treatment with nitrous acid (NA). For explanation see Fig. 1.

From the NA-1023 mosaic five cell-colony generations were analysed and clones 1–10 were isolated as shown in Fig. 5. All proved ad_6^- and adenine-dependent at all three temperatures.

No differences among the three buffers tested (pH 3, 4, 5) were found for any of the forty-four isolated subclones in respect to their adenine dependence.

It can be seen that new mosaic mutant colonies arose mainly on replating the

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mosaics found in the previous generations (NA-529, NA-535, NA-1019 and NA-1023). However, in two cases (NA-529 and NA-1023) new mosaics arose from wild-type colonies that had occurred in the 2nd cell-colony generation. No mosaics were obtained from complete mutant colonies.

Table 1. *Recombination among the mutant subclones within each pedigree derived from the four* ad_6^- *mutants (NA-535, NA-1013, NA-1019 and NA-1023) induced by nitrous acid*

Mutant	No. sub-clones	No. crosses	Recombination: No. spores analysed ($\times 10^{-6}$)	Recombination: *ad*+ No.	Recombination: *ad*+ Freq.*	Selfers: No. spores analysed ($\times 10^{-6}$)	Selfers: *ad*+ No.	Selfers: *ad*+ Freq.*
NA-535	10	100	365·9	3	0·008	25·6	0	< 0·039
NA-1013	3	9	19·2	0	< 0·052	21·6	0	< 0·046
NA-1019	8	64	507·3	2	0·004	64·5	1	0·015
NA-1023	10	100	921·2	762	0·827	92·6	71	0·767

* Per 10^6 spores

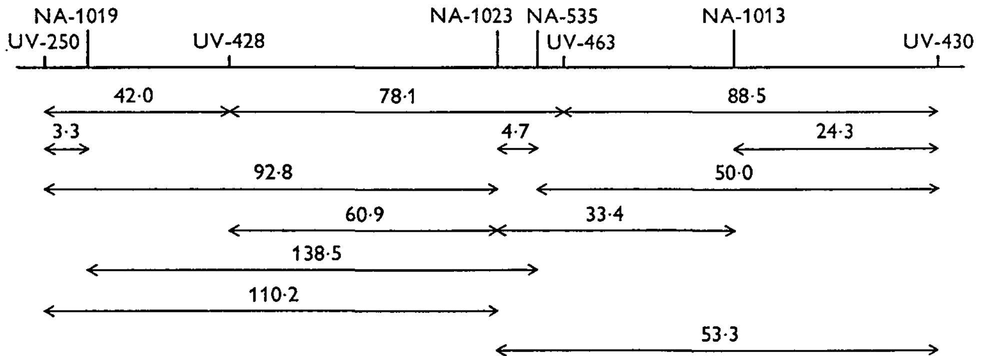

Fig. 6. Linkage relationships of NA-1019, NA-1023, NA-535, and NA-1013 *ad*-6 mutants and UV-250, UV-428, UVI-463, and UV-430 *ad*-6 tester mutants of *Schizosaccharomyces pombe*. Intragenic map distances are presented in terms of prototrophs per 10^6 viable ascospores plated.

Table 1 reports the results of all possible reciprocal crosses among independent mutant clones isolated from each of the four absolutely blocked *ad-6* mutants (NA-535, -1013, -1019 and -1023.) The frequency of *ad*+ colonies in selfing could account for the observed frequency of *ad*+ colonies obtained in crosses among different subclones. This result indicates that the mutation for adenine dependence is at the same site for all the clones deriving from the same original mosaic.

Figure 6 shows the location in the *ad-6* locus of the four mutants analysed: they occupy four different mutational sites within the genetic segment. Each value of recombination frequency is based on the analysis of at least 10^7 ascospores.

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The interallelic complementation patterns of the four mutants are reported in Table 2: different clones of the same mutant always gave the same complementation pattern.

Table 2. *Interallelic complementation patterns of independent clones derived from mosaic colonies of* Schizosaccharomyces pombe

Analysed clones (ad_6^-)	Tester strains						
	UV-566	UV-445	UV-670	UV-271	UV-256	UV-442	UV-588
NA-535/1	+	+	+	−	−	+	+
NA-535/4	+	+	+	−	−	+	+
NA-535/7	+	+	+	−	−	+	+
NA-535/8	+	+	+	−	−	+	+
NA-1013/1	+	+	+	+	+	−	+
NA-1013/3	+	+	+	+	+	−	+
NA-1019/1	−	−	−	+	+	+	+
NA-1019/4	−	−	−	+	+	+	+
NA-1019/8	−	−	−	+	+	+	+
NA-1023/1	+	+	+	−	−	+	+
NA-1023/4	+	+	+	−	−	+	+
NA-1023/7	+	+	+	−	−	+	+

+, Complementation. −, No complementation.

4. DISCUSSION

A genetic instability is produced during the growth of the colonies deriving from cells of *S. pombe* treated with NA; furthermore, in a given cell line, such an instability changes into a fixed mutated state, so that a mutant clone appears in the colony in the form of a mutated sector (mosaic colony). During the growth of the first mosaic colony other unstable clones are produced in which the original instability has been replicated. Replication of the induced instability is demonstrated by the production of new mosaic colonies in the second cell-colony generation. A further replication of instability is also scored in the succeeding cell-colony generations when the cells from newly occurring mosaic colonies are replated. This phenomenon has been analysed for five mosaic mutant colonies induced by nitrous acid at the *ad-6* locus of *S. pombe*. Genetic analyses on a number of independent clones obtained from mosaics scored at succeeding cell-colony generations of each original mosaic have shown that:

(1) All clones had a mutation in the *ad-6* locus.

(2) All independent clones isolated from each original mosaic were mutated at the same site, since no recombinants were recovered in samples of *c.* 10^7 random ascospores obtained from all possible crosses among independent clones isolated from the succeeding cell-colony generations.

(3) Identical complementation patterns were found in diploid cells obtained by crossing independent clones from a given mutant mosaic with seven *ad*-6 tester strains: this is a further indication that the same genetic site has been mutated in all clones for each original mutant.

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These results clearly show that the genetic instability replicates through several cell divisions and produces mutation at a particular codon triplet in the *ad-6* locus. Phenotypic analyses of the adenine requirement at different temperature and pH conditions confirm this finding.

Moreover, the production of instability of the type described is not limited either (i) to a given DNA segment of the *ad-6* locus, since the four mutants studied have been located at different sites within the locus, or (ii) to a given locus, since similar results have been obtained also for mutations induced at the *ad-7* locus (unpublished results).

It seems unlikely that the occurrence of new mosaic mutant colonies in several cell-colony generations with a frequency of 10^{-2}–10^{-5} is due to a spontaneous mutation at the same site, since over-all spontaneous mutants at the *ad-6* and *ad-7* loci have a frequency of 10^{-7} (Loprieno & Bonatti, 1968).

Table 3. *Efficiency of methods of scoring in the analysis of induced genetic instability in* Schizosaccharomyces pombe

Exp. no.	No. of mosaics analysed	Mosaics with instability		No. of colonies evaluated for each mosaic (average)
		No.	%	
1	32	12	37·5	725
2	12	10	83·3	3018

The production of genetic instabilities of the type described in the present paper seems to be common to several other chemical mutagens (Loprieno *et al.* 1965). Nasim (1965) has not observed instability in *S. pombe* when treated with nitrous acid: this may be due to the limited number (300) of colonies scored after replating cells from the original induced mosaics (Nasim, 1967). As shown by the two experiments reported in Table 3, when *c.* 700 colonies were scored for each of thirty-two mosaics, only 37·5 % of them were found to produce mosaics; when *c.* 3000 colonies for each of twelve mosaics were scored, the percentage of unstable mosaics rose to 83·3 %. This result also indicates that the occurrence of genetic instabilities produced by nitrous acid in *S. pombe* may approach 100 %, depending upon the extent of the analysis. Moreover Nasim (1967) demonstrated the occurrence of genetic instability induced by several mutagens. While he established locus specificity for mutant clones from the same mosaic, we have further established site specificity. This result limits the possibilities of interpretation of genetic instability.

Analogous phenomena have been observed in other organisms. In *E. coli* Hill (1963) found instability among spontaneous or u.v.-induced reversions in a tryptophan-requiring strain: she postulated a possible attachment of an episomic element to a suppressor locus. The reversion from auxotrophy to prototrophy could result from the detachment of the episome from the suppressor locus, either spontaneously or after treatment with u.v.-light. An 'attachment–detachment' mechanism of an episomic element for the instability in *S. pombe* seems to be

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unlikely as it would require the assumptions: (1) that several site-specific su^+ active loci are present in the wild type of *S. pombe* and that they are saturated with episomic elements; (2) that nitrous acid (or other mutagens) causes both a mutation in one particular site and detachment from the su^+ locus of the corresponding site-specific episomic element.

In *Aspergillus nidulans* instability has been attributed to aneuploidy (Ball & Roper, 1966), duplication (Bainbridge & Roper, 1966) or translocation (Ball, 1966). Although nitrous acid has been found to produce deletions in Phage T_4 (Tessman, 1962), the main mechanism by which it produces mutations in *S. pombe* (unpublished results), as well as in *Neurospora* (Malling & De Serres, 1967), is base-pair substitutions; therefore chromosomal abnormalities cannot be responsible for all cases of genetic instability observed in *S. pombe*. Moreover, the site specificity demonstrated in the present paper does not allow an interpretation of genetic instability in *S. pombe* in terms of chromosomal abnormalities.

Although the nature of the genetic instability described in the present paper requires further investigation, on the basis of the results presented here it can be excluded that the mechanisms proposed for the occurrence of instability in other organisms are applicable to *S. pombe*, at least for those mutations analysed here.

SUMMARY

A pedigree analysis of several cell-colony generations following a mutagenic treatment with nitrous acid has shown that in *S. pombe* a genetic instability is produced that replicates several times and produces a mutation in independent lines.

It has been shown that the mutants isolated in the progeny of a mosaic colony all contain a genetic alteration that cannot be resolved by genetic analysis and therefore the mutations have occurred at the same genetic site. This finding is confirmed by interallelic complementation and phenotypic analyses.

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