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Spontaneous and Induced *rho* Mutants of *Saccharomyces cerevisiae*: Patterns of Loss of Mitochondrial Genetic Markers

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The deletion which leads to spontaneous *rho* mutants occurs preferentially at a unique region covering genes *oxi3*, *pho1*/O_{II}, and *mit175*. The frequency of loss of genetic markers in this region was significantly higher than in other regions as determined with a 15- marker system. When various mutagenic treatments were applied, this specific pattern of deletion was also observed, but it was dramatically amplified. This suggests that the basic mechanism of *rho* production is the same in yeast mitochondrial genomes in both spontaneous and induced mutants.

The cytoplasmic "petite mutation" (*rho* mutation) in the yeast *Saccharomyces cerevisiae* results from extensive deletions of wild-type (*rho*$^+$) mitochondrial DNA (mtDNA) sequences (4, 11). Many chemical and physical agents are known to induce efficiently this class of mutation.

The loss of mtDNA sequences has been studied in primary clones of *rho* mutants induced by different treatments: ethidium bromide (2, 12, 13), photoaddition of psoralen derivatives (12, 14), UV irradiation (M. Heude and E. Moustacchi, Genetics, in press), mutation of nuclear genes (24), and phosphate starvation (26). The structure of the deletions can be determined genetically by using the numerous mitochondrial genetic markers now available (9), including mutations which confer drug resistance (17, 27) and *mit* mutations, which are specific defects in mitochondrial genes (1, 3, 10, 15, 20, 23, 25, 28). Biochemical analysis, with restriction enzymes, of mtDNA has also been performed (6, 16, 18, 22). These deletion studies have allowed the localization of some 30 mitochondrial genes on the circular genetic map of mtDNA.

Most studies on the mtDNA of *rho* mutants have been carried out with mutants induced by mutagens, especially ethidium bromide, because of its unusually high mutagenic efficiency. The *rho* mutation can also occur spontaneously, and it has been often questioned whether the mechanism of deletion and the structure of *rho* mtDNA produced are the same in induced and spontaneous mutants (7, 19; R. Goursot, Third cycle thesis, University of Paris, Paris VII, 1978).

In the case of induced *rho* mutants, detailed studies of deletions, using many genetic markers (11-13, 24; Heude and Moustacchi, in press), have shown that the different segments of mtDNA are not deleted at random. The unique region carrying genes *oxi3* and *oli2* appears to be preferentially deleted, irrespective of the nature of the mutagen. Such a specific pattern of deletion may be related to nonrandom distribution of the targets for mutagens in the mtDNA. Alternatively, but without excluding the latter hypothesis, it may result from some essential features of replication or recombination, or both, of yeast mtDNA.

In our study, we examined the fate of 15 mitochondrial genetic markers in independent spontaneous *rho* mutants to determine whether any specific pattern of deletion could be observed as in induced *rho* mutants.

MATERIALS AND METHODS

Strains. Spontaneous *rho* mutants were isolated from three strains.

Two isomitochondrial respiratory-sufficient (*rho*$^+$) strains, MH41-7B (**a** *ade2 his1*) and MH41-7B/O11 (**a** *ade2 his1 betO11*), differed only by one nuclear gene, *betO11* (11), which confers resistance to ethidium bromide. These strains carry four drug resistance mitochondrial markers, *C321*^{r}, *E514*^{r}, *O145*^{r}, and *P454*^{r}, which confer resistance to chloramphenicol, erythromycin, oligomycin (locus O_I), and paromomycin respectively.

The other *rho*$^+$ strain, MH32-6D (*α ade2 his1*), carries the resistance markers *C321*^{r}, *E221*^{r}, *O144*^{r} (locus O_{II}), and *P454*^{r}.

A list of *mit* tester strains is given in Table 1.

Use of these various strains permitted examination of 15 different genetic markers.

Media. Complete glucose medium, complete glycerol medium, drug-containing media, and minimal medium were as described by Fukuhara et al. (12).

Isolation of independent spontaneous *rho* clones. In each experiment, *rho*$^+$ strains were first grown to stationary phase in complete glycerol liquid medium. Cells were then plated onto complete glucose agar. After 5 days of growth at 30°C, 400 red clones (*ade2*) characteristic of the *rho*$^+$ genotype were trans-

TABLE 1. *Tester strains used*

Marker tested	Tester strain	Nuclear genotype
C321^r, E514^r, O145^r, P454^r	IL125-10C/1	α
C321^r, E221^r, O144^r, P454^r	1073	a *leu3*
asp/mit170	FF1210-6C/170	α *ura*
asp/mit170	170-6D	a *ura*
tsm8	SM50-11C (Schweyen)	α *ura*
ts982	FF1210-6C/982	α *ura*
oxi1/mit201	FF1210-6C/201	α *ura*
oxi2	M9-3 (Tzagoloff)	α
oxi2/mit231	231-1B	a *ura*
oxi3/mit3771	771-3A/3771 (Schweyen)	α *op1 ade1*
oxi3	M12-193/H4A	a *ade2 his1*
pho1/C2-3	IL125-10C/C2-3	α *ura*
mit175	FF1210-6C/175	α *ura*
cob1/mit226	FF1210-6C/226	α *ura*
cob2	M9-228 (Tzagoloff)	α
cs990	cs990-01B	a *ura*
ts983	FF1210-6C/983	α *ura*

ferred individually into 400 liquid complete glucose tubes and allowed to grow to stationary phase for five to six generations. Each culture was then plated, after suitable dilutions, onto a complete glucose medium plate. Among the red *rho*$^+$ colonies, some white colonies (presumptive *rho* clones) were seen. Only one white colony was picked per plate. The frequency of white colonies in each culture fluctuated between 0 and 5%. The picked colonies were aligned on complete glucose medium plates and grown to full spots.

Further replica-plating onto glycerol medium plates allowed elimination of *rho*$^+$ clones which had been erroneously picked. In each experiment, 300 to 350 clones among the 400 picked were found to be true *rho* clones. The remote possibility of selecting nuclear "petites" (*pet*) or *mit* deletion mutants could be discarded since the frequency of such mutants is known to be on order of 10^{-4} to 10^{-6} within the respiratory-deficient population.

Genetic analysis. The detection of drug resistance markers in *rho* clones was performed as described by Deutsch et al. (8) and that of *mit*$^+$ alleles was performed as described by Fukuhara et al. (12). Only the method for crossing differed. Each matrix plate carrying the spots of primary clones was replica-plated either onto minimal medium, for the crosses in which prototrophic selection of diploids after mating with a tester strain was possible, or onto complete glucose medium for the other crosses. On each plate, a drop of one tester strain at density of about 10^7 cells per ml was seeded onto each imprint. The occurrence of mating was carefully checked after growth. Diploids were then replica-plated onto selective media.

RESULTS

Loss frequencies of individual markers. The frequency at which individual markers in the population of *rho* clones were lost is shown in Table 2. The different markers are presented according to their order on the circular genetic map of mtDNA. The same *mit* tester strains were used in the analysis of the clones derived from strains MH41-7B and MH41-7B/O11. Similar results were obtained with the two strains. Consequently, the data of these two experiments were pooled, and the resulting mean values are given in Table 2. The percent losses fluctuated from the lowest value of 47.3%, corresponding to *cob1*, to the highest value of 69.6%, corresponding to *mit175*. As for strain MH32-6D of the opposite mating type, although a smaller number of markers was studied, similar results were observed for those in common with the two other strains.

Genotypic classes of deletions. A complete analysis of the genotypes of the different *rho* clones was performed (Fig. 1). The majority of the *rho* clones exhibited single deletion patterns. Among the 597 *rho* clones isolated from MH41-7B and MH41-7B/O11, in which the genotype was completely determined, only 2.5% retained the 15 markers studied, 6.2% lost all of the markers, and 7.4% were multiple deletions. In the case of MH32-6D, in which eight markers were examined, these values were 2.3, 12.7, and 1.7%, respectively, for a total of 299 clones. Large variations were observed in the frequency of each genotypic category. Ninety-three different genotypes, interpreted as single deletions, were encountered among the 210 possibilities for a 15-marker system. The segment *oxi3 -pho1 -mit175*, alone or in association with other markers, was included in numerous types of deletions. From the different associations of marker losses the gene order was determined to be the same as that already published (12, 24).

Disjunction in each interval and genetic map. In Table 3, the disjunction frequencies between each pair of adjacent markers are reported for strains MH41-7B and MH41-7B/O11. In each case, the orientation of sequence deletion to the left or to the right is compared with that expected from the different loss values of each marker found in Table 2. A strong asymmetry is observed for some segments, such as *oxi3-pho1*, *pho1-mit175*, *mit175-cob2*, and *cob2-cob1*.

The genetic map was deduced from these

TABLE 2. *Percent loss of individual mitochondrial markers in independent spontaneous rho mutants*

Strain	Loss (%) of marker:						
	E	*C*	*mit170*	*tsm8*	*ts982*	*oxi1*	*oxi2*
MH41-7B	53.4 ± 5.5	52.8 ± 5.5	53.8 ± 5.5	50.9 ± 5.5	49.5 ± 5.5	42.5 ± 5.5	51.5 ± 5.5
MH41-7B/O11	56.6 ± 5.7	56.8 ± 5.7	57.8 ± 5.6	56.5 ± 5.7	57.1 ± 5.6	58.1 ± 5.6	51.1 ± 5.7
	(54.9 ± 4.0)[b]	(54.7 ± 4.0)	(55.7 ± 3.9)	(53.6 ± 4.0)	(53.2 ± 4.0)	(50.2 ± 4.0)	(51.3 ± 4.0)
MH32-6D	59.8 ± 5.2	60.1 ± 5.2	60.5 ± 5.2				61.5 ± 5.2

[a] The two numbers are the lowest and highest numbers of *rho* clones examined; the sample size depended on the marker studied.

[b] Numbers in parentheses are means. The number of *rho* clones from each strain studied were pooled, and the ponderate mean percentages of loss are given.

data, using distance coefficients relating the separation frequencies to the loss frequencies as proposed by Schweyen et al. (24). This map, with the distances between the markers, is presented in Fig. 2, curve A.

Correlation between loss frequency and position on the map. The loss frequency of each marker has been plotted as a function of its map position on Fig. 3, curve A. A progressive decline appears on each side of the preferentially lost region, *oxi3-pho1-mit175*. Although a higher marker density on each side should be necessary, this progressive decline does not seem to be symmetric, as already observed by Schweyen et al. (24). A preferential direction of loss appears on the *oxi3* side as compared with the *mit175* side.

Comparison of spontaneous *rho* with induced *rho* clones. Data from Fukuhara et al. (12) concerning 3-carbethoxypsoralen- or ethidium bromide-induced *rho* mutants have been plotted on Fig. 3, curve B. These data, when compared with the pattern of spontaneous *rho* mutants, clearly show that the sensitive region is the same in both cases, but the regions flanking the sensitive region are lost much less often in induced than in spontaneous *rho* mutants. For example, only 27% of the *rho* clones lost the *E-C* markers after mutagenesis, whereas twice as many spontaneous *rho* clones lost these markers. The same ratio was observed for the O_1 and *ts983* markers. For intermediate markers, this ratio was less pronounced. This points to a special sensitivity of the *oxi3-pho1-mit175* region to mutagenic agents, the loss of markers outside of this region being mostly associated with loss of the region, and declining progressively with the distance.

Figure 3, curve C, represents Schweyen et al. data on marker losses in *rho* clones produced in the course of growth of *tsp25* at 35°C (24). In this case, a striking parallel can be observed with the curve obtained with spontaneous *rho* clones. This suggests that in *tsp25* mutants the events leading to *rho* formation are analogous to those arising spontaneously. Moreover, this highlights the fact that the extent of the difference between the markers lost least and most often is independent of the extent of *rho* production.

Another interesting feature concerning deletion genetic mapping should be noted. The map derived from the analysis of spontaneous *rho* mutants does not greatly differ from that obtained for induced *rho* mutants (Fig. 2). The major differences between the three maps lie in the distance between *oxi2* and *P* and between *ts983* and *E-C* or O_1 and *E-C*.

DISCUSSION

The mechanism by which the *rho* mutation is generated both spontaneously and after mutagenic treatments is not known. In an attempt to follow the early steps by which deletions of mtDNA sequences are produced, we examined the fate of markers in spontaneous, independent *rho* mutants. Our previous analysis of mutagen-induced *rho* mutants has clearly shown that one region of the mitochondrial genome is deleted highly preferentially (12; Heude and Moustacchi, in press).

Several features of spontaneous *rho* mutants are similar to those of induced ones. (i) The different markers are lost as large deletions, the majority of *rho* mutations corresponding to single deletions (Fig. 1). (ii) There is a non-homogeneous distribution of the deletions along the mitochondrial genome, the region carrying the three contiguous markers (*oxi3, pho1/*O_{IL}, and *mit175*) being preferentially lost (Table 2; Fig. 3, curve A). Statistical analysis of the data (standard deviation, X^2 homogeneity tests, comparison of intra- and intergroup variances) shows that this group of adjacent markers has a significantly different frequency of loss as compared with the other markers. (iii) The genetic map, deduced from the analysis of spontaneous *rho* mutants is essentially similar to those derived from the study of induced *rho* mutants (Fig. 2), since the same gene order is found. However,

TABLE 2—*continued*

Loss (%) of marker:									No. of *rho* clones[a]
P	*oxi3*	O_{II}/*pho1*	*mit175*	*cob2*	*cob1*	O_I	*cs990*	*ts983*	
54.4 ± 5.5	60.0 ± 5.5	57.4 ± 5.5	65.6 ± 5.3	50.0 ± 5.6	45.2 ± 5.5	47.7 ± 5.5		45.7 ± 5.5	315–327
59.1 ± 5.6	68.8 ± 5.3	63.3 ± 5.5	73.7 ± 5.0	47.2 ± 5.7	49.5 ± 6.3	50.6 ± 5.7		50.5 ± 5.9	293–308
(56.7 ± 3.9)	(64.4 ± 3.8)	(60.3 ± 3.9)	(69.6 ± 3.7)	(48.6 ± 4.0)	(47.3 ± 4.0)	(49.1 ± 4.0)		(48.0 ± 4.0)	619–635
69.4 ± 4.9	72.3 ± 4.7	68.6 ± 4.9					55.9 ± 5.7		304–357

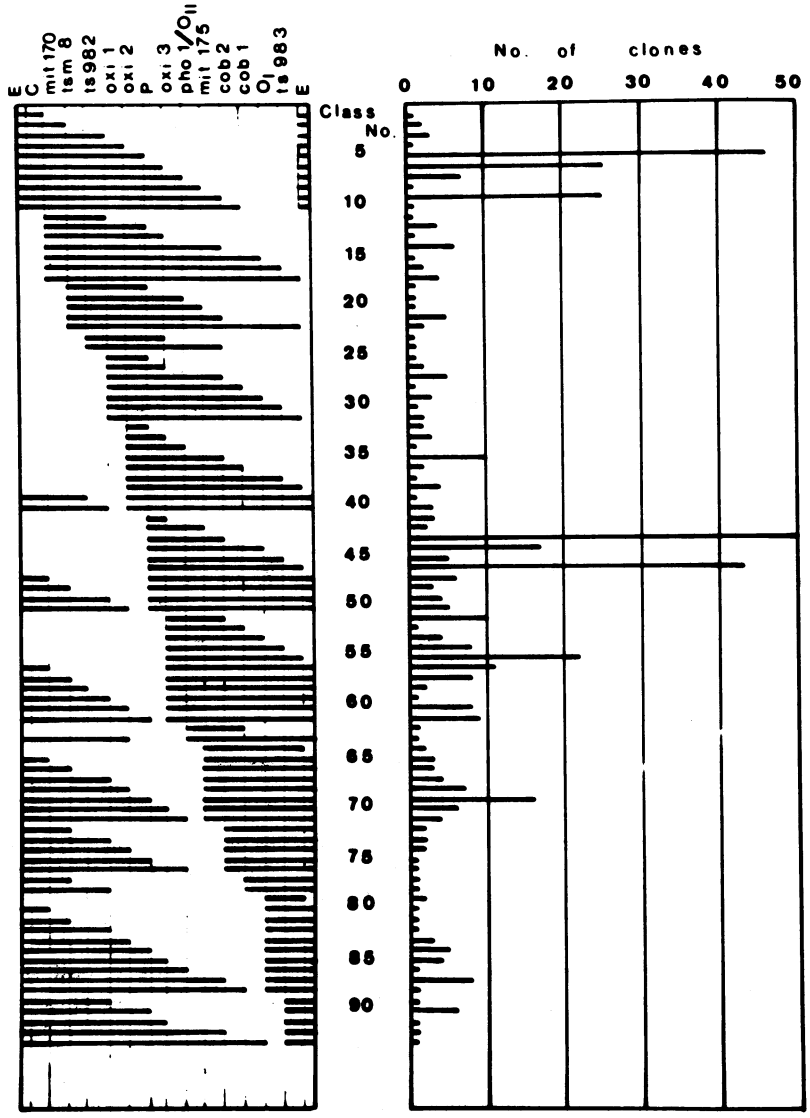

FIG. 1. *Various mitochondrial genotypes found in spontaneous rho mutants (left) and number of clones found for each class (right). (Left panel) The mtDNA sequences lost in the rho mutants (heavy line) were determined by the loss of the genetic markers indicated on top of the diagram. A total of 501 clones with a single-deletion genotype were found.*

the difference in the estimated distances between genes is quite large in some regions. It is probably too early to decide whether the distances determined by gene disjunction should specifically change according to the way in which the *rho* clones are produced. We note also

TABLE 3. *Frequency of disjunction of mitochondrial genetic markers*

Pair of adjacent markers	No. of clones disjuncted	Orientation of sequence deletion (no. of clones)		Calculated orientation observed (%)	
		To the left	To the right	Left	Right
C-mit170	41	22	19	54 (49)[a]	46 (51)
mit170-tsm8	30	20	10	67 (52)	33 (48)
tsm8-ts982	5	3	2	60 (50)	40 (50)
ts982-oxi1	37	22	15	59 (53)	41 (47)
oxi1-oxi2	54	27	27	50 (49)	50 (51)
oxi2-P	231	91	140	39 (45)	61 (55)
P-oxi3	132	48	84	36 (42)	64 (58)
oxi3-pho1	14	13	1	93 (54)	7 (46)
pho1-mit175	47	4	43	9 (40)	91 (60)
mit175-cob2	129	121	8	94 (71)	6 (29)
cob2-cob1	7	5	2	71 (51)	29 (49)
cob1-O_I	54	27	27	50 (48)	50 (52)
O_I-ts983	28	17	11	61 (51)	39 (49)
ts983-E	192	81	111	42 (43)	58 (57)
E-C	1	0	1	(50)	(50)

[a] Numbers in parentheses are the expected values.

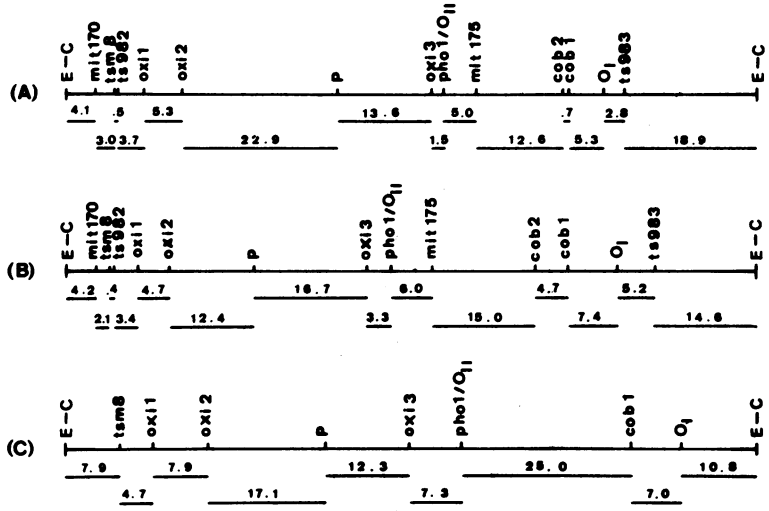

FIG. 2. *Genetic map. Distances between markers are expressed as percentages of the total genome length. For convenience, the circular map is represented by a linear arrangement of genes. The distances of map (A) are those estimated from our analysis of spontaneous rho mutants. For comparison, the data from Fukuhara et al. (12) have been reported on map (B), and those from Schweyen et al. (24) have been reported on map (C).*

that the positions of genetic markers as shown on the available physical maps (6, 16, 18) are somewhat different from those estimated genetically.

It may be asked whether the high frequencies of loss of the *oxi3*, *pho1/O_{II}*, and *mit175* markers are related to their primary sequences. Indeed, this region is located in the quadrant of the physical map which is characterized by the presence of long deoxyadenylate-deoxythymidylate clusters (21). However, unless the inclusion of this region in the whole *rho*$^+$ genome is taken into consideration, such a hypothesis is difficult to reconcile with the fact that these same genes, when included in a *rho* mtDNA molecule, can become highly stable with or without a mutagenic treatment (data not shown). Another explanation of high loss frequencies in this region could be related to the properties of the *mit* tester strains. *mit* mutations in the *oxi3* region being often relatively large deletions (5), the use of such tester strains would correspond to an artificial extension of the length of the target, leading to the observation of high loss frequen-

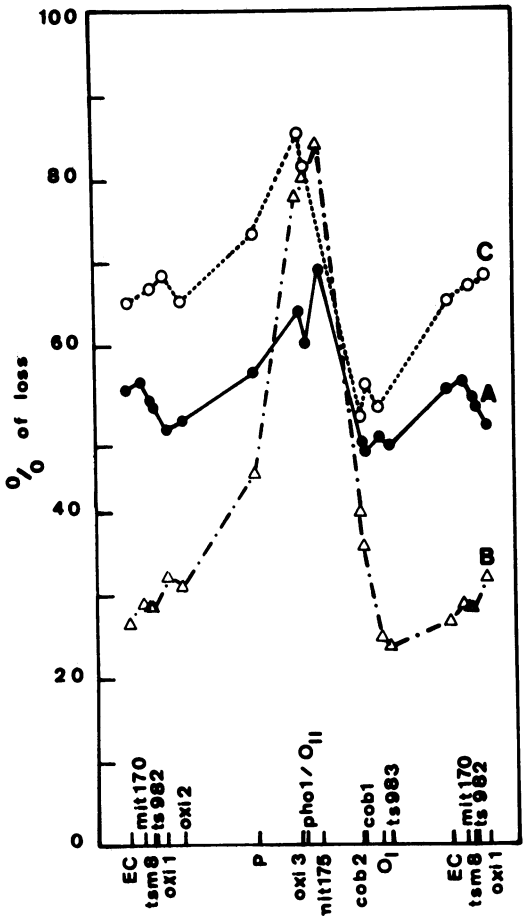

FIG. 3. *Frequency of loss of the different markers as a function of their location on our genetic map. Data from spontaneous rho mutants are reported on curve (A)* (●). *For comparison, the data of Fukuhara et al. (12), concerning ethidium bromide or 3-carbethoxypsoralen plus 365-nm mutagenesis, are reported on curve (B)* (△). *Those of Schweyen et al. (24), concerning rho induction by high-temperature growth of tsp25 mutants, are reported on curve (C)* (○).

cies of such markers. To avoid such an artefact, we selected tester strains corresponding to *mit* point mutations. Consequently, it seems likely that the preferential loss of the *oxi3*, *pho1/*O_{II}, and *mit175* markers resulted from some properties in the organization or the function of the whole *rho*$^+$ mtDNA molecule that may be related to replication or recombination or both. It is interesting that the sensitive region, beside its richness in deoxyadenylate-deoxythymidylate clusters, is particular in the sense that various yeast strains appear to differ in the presence or absence of large sequence inserts in this region of the mitochondrial genome (22).

Although our data show a good overall agreement with those obtained for the *rho* clones derived from high-temperature growth of *tsp25* mutants (Fig. 3, curves A and C), some differences appear when comparing spontaneous *rho* clones with those selected after mutagenic treatment (either UV irradiation [Heude and Moustacchi, in press], or photoaddition of 3-carbethoxypsoralen, or ethidium bromide treatment) (Fig. 3, curves A and B). The major difference lies in the relative sensitivity of the *oxi3-pho1/*O_{II}*-mit175* region as compared with that of other regions. The preferential loss of this region is more pronounced after mutagenesis than in spontaneous *rho* mutants. The magnitude of the difference between the markers lost more often and those lost less often depends upon the mutagenic treatment applied (Fig. 3; Heude and Moustacchi, in press). The larger the difference, the less often are the markers furthest away from the sensitive region lost. This may reflect the exceptional sensitivity of this region which leads to a high proportion of deletions extending away from this region associated with a decreasing probability of reaching one given marker as a function of the distance.

When one considers the percent loss of each marker as a function of its map location, the regions to the left of the *oxi3-pho1/*O_{II}*-mit175* region are more often lost than those located to the right, as already observed for *rho* clones derived from *tsp25* (24). Although estimations of distances lack precision, deletion mapping of *rho* mutants of diverse origins and physical mapping lead to similar distribution of the markers.

In conclusion, deletions occur preferentially in a unique region covering genes *oxi3*, *pho1/*O_{II}, and *mit175*. The loss frequencies of genetic markers in this region are significantly higher than those in other regions. This specific pattern of deletion is amplified when various mutagenic treatments are applied. This observation may suggest that the basic mechanism of *rho* induction is the same in spontaneous and induced mutagenesis, the mutagenic treatments amplifying the loss of the region specifically deleted. This would explain why some types of *rho* mutants, e.g., those carrying only the *oxi3* region, are very rare among mutagen-induced mutants, whereas such mutants can be more easily found in spontaneous *rho* clones. An interesting approach to the properties underlying the preferential loss would be the analysis of marker losses in various well-defined *rho* mutants.

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