Spontaneous Mutation at the *mtr* Locus of Neurospora: The Spectrum of Mutant Types

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ABSTRACT

We have isolated 135 strains of Neurospora which have mutations at the *mtr* locus resulting from independent spontaneous events. *mtr* is the structural gene for the neutral amino acid permease. The mutants have been characterized by their reversion behavior (both spontaneous and induced) and by hybridization studies of restriction digests of their DNA. About half of the mutants (54%) appear to result from single base-pair substitutions. Thirty-four percent have deletions, including some which extend into neighboring genes. Most of the remaining mutants have insertions. Several of the insertions are tandem duplications of 400–1000 bp and these mutants are unstable, reverting to mtr^+ with a high frequency. When tandem-duplication mutants go through a cross, they are modified: the mutant progeny are fully stable. This modification is probably due to RIP (repeat-induced point mutation). This process has an important bearing on the comparison of germinal to somatic mutation.

THE changing frequencies of alternative gene forms are the basis of evolution and the primary concern of population genetics. In natural populations these changes are governed by four factors: mutation, selection, migration and genetic drift. In experimental situations we can attempt to nullify three of these factors in order to study the fourth. The effects of selection are minimized in a coenocyte like Neurospora: mutant nuclei share gene products with the other nuclei in their vicinity.

We have chosen to study spontaneous mutation at the *mtr* (methyltryptophan resistance) locus in Neurospora. This is the structural gene for the neutral amino acid permease. This gene is especially suitable for our study because it permits selection of both forward and reverse mutants. This is very useful in determining the spectrum of mutant types. The forward mutation system is used to obtain a large sample of independent mutations, and the study of reversion (both spontaneous and induced) helps us to characterize the different mutant types (base substitutions, frame-shifts, deletions and insertions).

Forward mutants at *mtr* are selected on growth medium supplemented with an inhibitory analog of a neutral amino acid (methyltryptophan or fluorophenylalanine). Reversions are selected from an *mtr* strain which is auxotrophic for a neutral amino acid. Growth on medium supplemented with that amino acid can occur only in those cells which have recovered the uptake system by mutation. The *mtr* gene has been cloned, so probes are available to examine the molecular properties of the mutants.

MATERIALS AND METHODS

Strains: *mtr* mutants were isolated from conidia of two strains, both of the genotype *col-4 trp-2* . The phenotype of *col-4* is colonial (nonspreading) vegetative growth, and the phenotype of *trp-2* is a nutritional requirement for tryptophan or anthranilic acid. The first strain (CTa) was mating type *a* and derived its genetic background from Oak Ridge wild type. The second strain (ACTA) was mating type *A* and was the product of a cross of CTa to a wild-type strain from Africa called Adiopodoumé (here called Adio) which has been shown by KINSEY and HELBER (1989) to carry a transposable element.

Media: All media were made from Vogel's minimal (DAVIS and DE SERRES 1970) variously supplemented. Plates contained sorbose medium, for which the carbohydrates were 1.5% sorbose and 0.1% dextrose. Carbohydrate for media in flasks or tubes was 2% sucrose. Supplements were: anthranilic acid (Ant), L-tryptophan (Trp), L-arginine (Arg), *p*-fluorophenylalanine (Fpa), pyridoxine (Pdx) and pantothenate (Pan). The concentrations of Ant, Trp and Arg were normally 50 mg/liter. Fpa was 15 mg/liter and Pdx and Pan were 5 mg/liter.

The *trp-2* strains can grow on medium supplemented with either Trp or Ant. The latter does not depend upon the neutral amino acid permease for uptake. Thus *trp-2* strains can grow on Ant whether they are mtr^+ or *mtr*. The selective medium for *mtr* mutants was supplemented with Ant + Fpa. The counter-selective medium (for mtr^+ revertants) was called Trp + Arg; it contained 0.2 of the normal tryptophan supplement (thus 10 mg/liter) and twice the normal amount of arginine (thus 100 mg/liter). On this medium the cell can only take up tryptophan if the neutral amino acid permease is functioning. Tryptophan can also be taken up by the general amino acid permease, but on this medium that system is blocked by arginine.

Isolation of mutants: The procedure was designed to insure that each mutant resulted from a separate mutation. About 200 conidia of CTa or ACTA were spread on a plate of Ant medium and incubated overnight at 33°. Individual colonies that had each arisen from a single cell were cut out

and transferred to tubes of solid Ant medium. For each of the four experiments (two with each strain), 15–20 single-cell colonies were isolated and grown in separate tubes.

The tube cultures were grown two days at 33° followed by 3–5 days at 22°. Conidia were harvested by adding 5 ml sterile water to each tube and shaking and mixing thoroughly before filtering the suspension through glass wool to remove clumps. A sample was diluted and counted in a hemocytometer, and 10^7 cells were added to 45 ml of melted Ant + Fpa at 50° and poured into two plates. These plates were incubated 3 days at 33° before being scored for resistant colonies (putative *mtr* mutants). From each tube culture (pair of plates), a single large colony was grown in a tube of Ant medium to include in the spontaneous mutation study.

Mutation at *mtr* in a heterokaryon: A separate experiment involved spontaneous *mtr* mutants which arose in a heterokaryon. The first component strain of the heterokaryon was *cot-1* (colonial growth at high temperature) *trp-2 ylo-1* (yellow conidia). The second component was *pdx-1* (pyridoxine-requiring) *mtr col-4 arg-2* (arginine-requiring) *R* (round ascospores). *pdx-1 mtr col-4 arg-2* form a cluster of linked genes in that order, spanning a total length of about three map units. Both components of the heterokaryon were mating type *A*.

Even though the second component was *mtr*, the heterokaryon was sensitive to fluorophenylalanine because *mtr* is recessive. Thus Fpa + Pdx + Arg provided a selective medium for heterokaryotic cells with mutation at *mtr* in the first component. This experimental design permitted the detection and recovery of *mtr* mutants that resulted from deletions extending into essential genes (DE SERRES and OSTERBIND 1962; STADLER, MACLEOD and LOO 1987). The deletion mutants had a recessive lethal phenotype and were detected by the failure of the heterokaryon to produce any homokaryotic conidia of the genotype *cot trp ylo mtr*; these could be easily recognized by their distinctive *cot* morphology on plates (of Ant + Fpa) incubated at 33°. Mutants with deletions large enough to extend to any of the linked markers which were segregating in the heterokaryon (*pdx-1*, *col-4*, *arg-2*) were also recognized by the expression of these recessive traits in the *mtr* heterokaryons. Those mutants that did not have recessive lethal damage (and were viable as *cot trp ylo mtr* homokaryons) were put through the same genetic tests as described for the mutants in CTa and ACTA.

The molecular analysis of the *mtr* mutants resulting from large deletions (those causing recessive lethal damage) posed a special problem. We wished to learn whether these mutants had lost all DNA homologous to the *mtr* probe. However, these strains could only grow as heterokaryons, so the extracted DNA included the *mtr* region from the other component. Therefore we constructed a heterokaryon in which one component carried the *mtr* region from Mauriceville-1c A, a strain which shows many restriction fragment length polymorphs (RFLPs) in comparisons to standard laboratory strains (METZENBERG *et al.* 1985). We found that the *Cla*I digest of Mauriceville yields an *mtr* band that is absent in our standard strains. We constructed a strain carrying *cot-1*, *trp-2*, *ylo-1* and markers flanking the *mtr* locus: *col-4* and *pdx-1*. This was crossed to a strain carrying linkage group IV from Mauriceville (kindly provided by R. L. METZENBERG), and *cot trp ylo* progeny were isolated that were col^+ pdx^+. One of these was used as the first component of our heterokaryon after the Mauriceville origin of its mtr^+ gene was confirmed by *Cla*I digestion.

Analysis of mutants: The putative mutants were tested on plates of sorbose medium of the two counterselective types: Ant + Fpa for *mtr* and Trp + Arg for mtr^+. The original isolates were picked from crowded plates (5×10^6 conidia), so that many of them were mixtures of mutant and nonmutant material and showed growth on both media. These were purified by streaking on Ant + Fpa, incubating overnight and picking an isolated colony which arose from a single cell. These macroconidia frequently contain more than one haploid nucleus, but, since *mtr* mutants are recessive, this procedure should be sufficient to isolate a pure-mutant culture. This was confirmed by repeating the growth test. The majority of the mutants were stable and grew well on Ant + Fpa, but not at all on Trp + Arg. These were examined further for induced reversion.

Induced reversion tests: Conidia were isolated from fresh tube cultures into water suspensions and counted. Conidia (5×10^6) were added to 2.5 ml of melted 1% agar at 50° and poured immediately over a plate of Trp + Arg. The overlayer in some cases also contained 10 mg of nitrosoguanidine (NG) or 70 mg of ICR-170. An overlayer with no mutagen was used for the control plate and for the UV test. For the latter, the conidia were treated with 800 J of acute UV (at 100 J/min) before being added to the overlayer mix. (This dose of UV kills about 50% of the conidia.) Control platings were made of the same overlayer mixtures on minimal sorbose plates to check for trp^+ revertants (because these were also expected to grow on Trp + Arg). The plates were incubated 2–3 days at 33°. The production of 10–200 mtr^+ colonies on a plate of treated cells was considered to be evidence of induced reversion.

Genetic analysis: *col-4* and *pdx-1* (pyridoxine requirement) flank the *mtr* locus and are about 3 map units apart. Putative *mtr* mutants were crossed to *pdx-1 trp-2*. The progeny were scored for resistance on Ant + Fpa + Pdx medium and for the linkage of this trait to *col-4* and *pdx-1*.

Molecular analysis: Neurospora DNA was prepared from lysed spheroplasts or mycelium ground with sand by the method of ZOLAN and PUKKILA (1986). Restriction enzymes were from BRL or Boehringer Mannheim and were used according to the manufacturers' instructions. Southern transfers were made to nitrocellulose (S & S) or Hybond-N (Amersham). Hybridization was to *mtr* plasmid pCVN 2.9 (STUART, KOO and VOLLMER 1988) nick translated with ^{32}P (NEN) or biotin-II-dUTP (BRL). Biotinylated probes were detected with streptavidin-alkaline phosphatase using the Blugene system (BRL).

RESULTS

A random sample of spontaneous mutations at *mtr* was obtained by selecting a single mutant from each separate culture. Two sets of mutant isolations were done with each of two strains, *col-4 trp-2 a* (CTa) and *Adio col-4 trp-2 A* (ACTA). A total of 68 separate cultures were plated, but eight failed to yield any *mtr* mutants. Thus there were 60 mutants for genetic and molecular studies.

The 60 independent mutants were purified by vegetative reisolation and were characterized by the growth tests on Ant + Fpa and on Trp + Arg shown in Table 1. These tests involved streaks of conidial suspensions. A few of the mutants continued to grow in both counterselective media even though they were repeatedly reisolated from single-cell colonies. These were named "ambiguous" mutants and are believed to be intermediate alleles producing a reduced or

TABLE 1

Classification of *mtr* mutants by growth tests

Strain	~300 on Ant	~300 on Ant + Fpa (*mtr* grows)	~300 on Trp + Arg (*mtr*$^+$ grows)	~30,000 on Trp + Arg
trp mtr$^+$	~300	0	~300	Confluent
trp mtr (stable)	~300	~300	0	0
trp mtr (highly unstable)	~300	~300	0	2–200
trp mtr (ambiguous)	~300	~300	~300	Confluent

Conidia were suspended in water at ~30,000 per drop, and a single drop was streaked on sorbose medium supplemented with Trp and Arg. The suspension was diluted 100-fold, and a single drop was streaked on each of the three media. The streaked plates were incubated 24 hr at 33° before the colonies were counted.

altered permease activity. Six of the mutants produced a few (2–200) colonies on Trp + Arg in streaks containing about 30,000 conidia, and this result was repeatable when the strains were reisolated from single-cell colonies on Ant + Fpa. These were called "highly unstable" mutants. One mutant strain produced spontaneous revertants at frequencies 20–100 times lower. It was called "slightly unstable." Only the stable mutants could be examined further for induced reversion by plating on Trp + Arg after treating with mutagens. Induced reversion tests were performed on all 44 of the stable mutants with three mutagens: UV, NG and ICR-170.

The *mtr* gene has been cloned by STUART, KOO and VOLLMER (1988), so that a labeled probe is available for hybridization studies of restriction digests of mutant *mtr* genes. DNA was prepared from the mutant strains, digested with *Xho*I, run on agarose gels and hybridized with the *mtr* probe (Figure 1). Some of the mutants were also studied in digests from other restriction endonucleases: *Msp*I, *Hpa*II, *Sau*3AI, *Mbo*I. The results of these analyses and the reversion tests are shown in Table 2.

Seventeen of the mutants revert after treatment with either UV or NG. They show no changes in the *Xho*I restriction digests and are presumed to be base-substitution mutants. Eleven mutants revert only after UV treatment. They are believed to be base-substitution mutants, but it is possible that they include one or more frame-shift mutants (see below).

Fourteen of the mutants do not revert after mutagen treatment. Of these, five show detectable deletions, and some of the others may have smaller deletions. The remaining mutants include seven unstable and eight ambiguous. The ambiguous mutants grow on both counterselective media and are presumed to have an altered or reduced level of permease activity. They have normal restriction digests and are probably base-substitution missense mutants.

The highly unstable mutants revert to *mtr*$^+$ with frequencies about 100 times the forward mutation frequency. They have interesting molecular properties. Four of the six mutants show all three normal bands in the *Xho*I digests plus a new small band of 500–1000 base pairs (Figure 1). The same kind of change occurred when one of these *mtr* mutants was digested with *Sau*3AI and with *Mbo*I (Figure 2). This is the pattern expected for a tandem duplication of the size of the new band if the duplicated segment includes one of the internal cut sites. RAMBOSEK and KINSEY (1984) determined the nucleotide sequence of an unstable mutant in the *am* gene in Neurospora and showed that it contained a tandem duplication. The same basis was found for an unstable *cyc-1* mutant in Saccharomyces (DAS, CONSAUL and SHERMAN 1988). The *Xho*I digests of the other two highly unstable mutants have only three bands: two in the normal positions and the third increased by about 400 bp. We propose that these are also tandem duplications, but that the duplicated segments do not include *Xho*I cut sites.

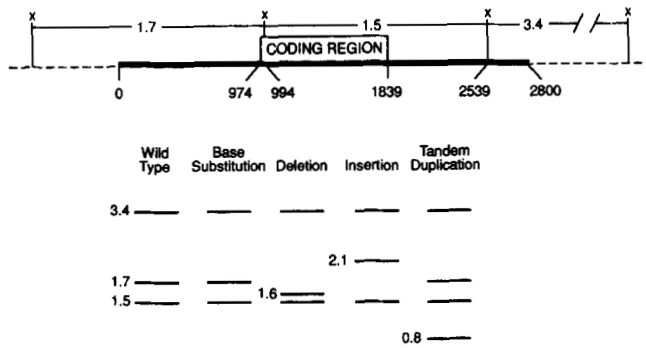

FIGURE 1.—Molecular characterization of mutant *mtr* genes by *Xho*I digestion. X = *Xho*I site. The heavy bar represents the 2.8-kb cloned fragment which includes the *mtr* gene (STUART, KOO and VOLLMER 1988). The proposed coding region of KOO and STUART (1991) is shown. It is based on their examination of the total sequence for ORFs. The lower part of the figure represents the hybridization of *Xho*I restriction digests of wild type and several classes of *mtr* mutants. The deletion mutant has been sequenced and shown to be deleted for bp 429 to 521. The insertion mutant has been sequenced and found to contain a tandem duplication of bp 240 through 623. The mutant labeled "tandem duplication" has not yet been sequenced.

SELKER *et al.* (1987) discovered that when a cross is made of a Neurospora transformant which contains a duplication of the transformed gene, this region is subject to repeat-induced point mutation ("RIPing"): methylation and concerted base-substitution mutation (C to T transitions; SELKER 1990). When we cross our highly unstable mutants, it appears that they are RIPed. Most of the *mtr* mutant progeny are *stable*, and hybridization of restriction digests shows that *Mbo* I sites have been lost and that the DNA has been methylated (see Figure 2).

The slightly unstable mutant reverts about 100-fold less frequently than the highly unstable mutants. It has an insertion of about 30 bp. This is difficult to detect in the *Xho*I digest, but is seen clearly in the *Mbo*I digest by the separation of two small bands of

TABLE 2

Classification of 60 *mtr* mutants isolated from simple strains

Class of *mtr* mutant	Parent Strain		Restriction pattern[a]	Possible molecular type
	col-4 trp-2	Adio *col-4 trp-2*		
Stable				
Induced Reversion				
UV and NG	8	9	Normal	Base substitution
UV only	6	5	Normal	Base substitution
NG only	1	0	Normal	Base substitution
ICR and UV	1	0	Normal	Frame-shift
No reversion	9	5	Deletion (5/14)	Deletion
			Normal (9/14)	Small deletion
Highly unstable	5	1	Insertion	Tandem duplication
Slightly unstable	1	0	Insertion	Insertion
Ambiguous	3	5	Normal	Base substitution
Other	0	1	Methylated	Unknown

All of the stable mutants were screened for reversion after treatments with UV, NG and ICR-170.

[a] *Xho*I digests of the mutants were hybridized with the *mtr* probe. The stable mutants with no induced reversion and the unstable mutants were also digested with some or all of the following restriction enzymes: *Msp*I, *Hpa*I, *Sau*3AI, *Mbo*I, *Apa*I, *Kpn*I.

nearly equal size. We have seen no evidence of RIP when this mutant was crossed. The *mtr* progeny were all slightly unstable, like the parent.

The difference between the two original strains used in our mutation experiment is that the Adio strain is known to carry a 7-kb transposable element (KINSEY and HELBER 1989). We have seen no evidence of genetic instability in this strain, however. We can estimate the forward mutation rate at *mtr* in these experiments, and it appears to be about 1.5×10^{-7} per division in CTa and slightly *lower* in the Adio strain (D. STADLER and H. MACLEOD, unpublished results). Furthermore, none of the 26 mutants isolated from the Adio strain contain the 7-kb insert.

ICR-170 has been shown to revert frame-shift mutants in Neurospora and several other fungi. Only one of our mutants responded to this agent. In contrast, 29 mutants responded to UV and/or NG treatment. Frame-shift mutants are also reverted by UV, and it has been proposed that ICR-170 can only revert frame-shift deletions and not frame-shift insertions (BURNS *et al.* 1986). It is possible that there are some frame-shifts among the mutants in Tables 2 and 3 that reverted only after UV treatment.

All of the mutants discussed above are believed to be at the *mtr* locus. Twenty-two of them have been crossed, including members of all classes, and resistance to Fpa remained coupled with *col-4* in over 95% of the progeny of every cross. *col-4* is about 2 map units from *mtr* .

One mutant did not fit into any of the classes already described. Hybridization studies showed that the DNA in the *mtr* region of this strain was methylated. The mutant could not be isolated free of a heterokaryon with a "helper" nucleus, so it was presumed to carry recessive lethal damage. The mutant did not come through a cross, so we cannot be certain that the change detected at *mtr* is responsible for the Fpa-resistant phenotype.

A second experiment on the spectrum of spontaneous mutation at *mtr* was carried out in a heterokaryon (two genetically different haploid strains sharing a common cytoplasm). One component was mtr^+ while the second was *mtr* . The heterokaryotic cells were sensitive to Fpa. (*mtr* mutants are recessive.) By plating on medium supplemented with Fpa, we selected heterokaryotic cells in which the first component had mutated to *mtr*. The advantage of this system is that it detects and reveals all *mtr* mutants, even deletions which extend into essential genes flanking the *mtr* locus. Such mutants have a recessive lethal phenotype, but can be harbored in a heterokaryon. Of 75 mutants isolated in this system, 9 (12%) were deletions of this type. The frequency is very similar to that observed by DE SERRES (1988) in an experiment of the same type. He scored spontaneous mutation at the *ad-3* locus of Neurospora in a heterokaryon and reported that 25 of 172 mutants (14%) were multilocus deletions. Six of the *mtr* deletions extended far enough to reveal one or more of the flanking markers: *pdx-1* on the left and *col-4* and *arg-2* on the right. Flanking markers on both sides were deleted in three of these mutants.

The molecular analysis of the deletion mutants was limited to the 0.6-kb *Cla*I band unique to the Mauriceville strain, because the DNA could only be obtained from the heterokaryon (see MATERIALS AND METHODS). Three of the mutants with deletions extending to flanking markers were tested and all had lost the 0.6-kb band. Three of the mutants had deletions which did not extend to flanking markers but did include essential genes (as revealed by their reces-

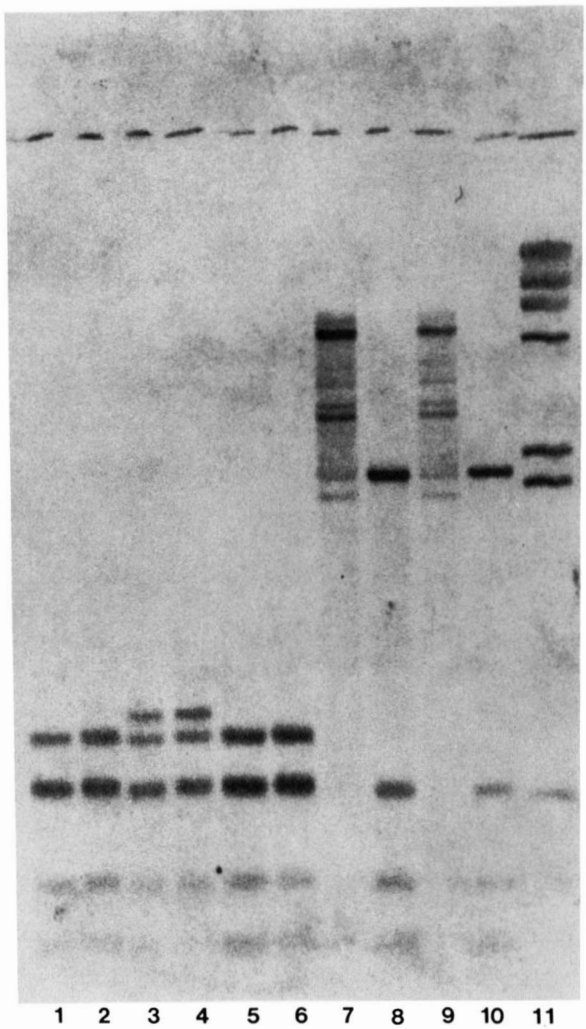

FIGURE 2.—Restriction digest analysis of a highly unstable *mtr* mutant. The first lane of each pair contains DNA digested with *Sau*3AI, the second with *Mbo*I. These endonucleases are isoschizomers which cut the sequence GATC. *Mbo*I cuts even when the C is methylated, while *Sau*3AI does not. Lanes 1, 2: wild type (*mtr*$^+$) control; lanes 3, 4: *mtr* mutant SR24; lanes 5, 6: spontaneous revertant of SR24; lanes 7, 8: *mtr* from the cross progeny of SR24; lanes 9, 10: second DNA preparation from the same progeny strain; lane 11: marker (lambda digested with *Hin*dIII; band sizes in kb: 23.1, 9.4, 6.7, 4.4, 2.3, 2.0, 0.56). The loss of *Mbo*I sites in lanes 8 and 10 indicates the base substitutions characteristic of RIPing; the comparison of these digests to the corresponding *Sau*3AI digests (lanes 7 and 9) shows methylation.

sive lethal phenotype). *Cla*I digests from two of these were examined. The 0.6-kb band was missing from one and smaller than normal in the other.

The remaining 66 mutants from the heterokaryon experiment would be expected to correspond to those of the first experiment. The stable mutants were tested for reversion after treatment with UV and with ICR-170. Restriction digests of the DNA of some of the mutants were examined. The results and the tentative classifications are shown in Table 3.

The spectra of mutants from the two experiments are similar for the two major classes: UV-revertible (base pair substitutions) and nonrevertible (deletions). However, highly unstable mutants were completely absent in the heterokaryon experiment, while they made up 10% of the total mutants from the simple strains. It would be interesting to know whether this is a real difference based on a sequence difference in the *mtr*$^+$ genes of the parent strains. The strain used in the heterokaryon experiment, Mauriceville, is known to show many RFLPs in comparison to the standard laboratory strains (METZENBERG *et al.* 1985).

Two of the *mtr* mutants have now been sequenced and, in both cases, the results confirm the predictions from restriction digest analysis (see Figure 1). One is a highly unstable mutant which had been deemed a 400-bp insertion by the increase in size of one *Xho*I band; it turns out to have a tandem duplication of 383 bp. The second is a nonrevertible mutant which had been predicted to have a 100-bp deletion on the basis of the reduced size of one *Xho*I band; the sequence shows a 92-bp deletion. Both of the sequenced *mtr* mutants are located several hundred base pairs upstream of the proposed *mtr* open reading frame (ORF) of KOO and STUART (1991). However, those authors point out that there is a second ORF of 174 bp in this region, and they suggest that it may play a role in *mtr* function.

DISCUSSION

Evolution results from the interplay of mutation and selection. Much has been said about the role of selection, but mutation has been largely taken for granted. Population biologists seem to have concluded that the patterns of selection are complex enough to engage their full attention, so it has been convenient to assume that mutation is simple: the infrequent occurrence of single base substitutions, randomly distributed in time. This assumption is implicit in the use of the "molecular clock" method for determining patterns of phylogeny.

Our results do not fit very well to the assumptions of the molecular clock. Nearly half the mutants involve more extensive changes than single base substitutions. In the heterokaryon experiment, 12% of the *mtr* mutants were large deletions causing recessive lethal damage. Summing the results of Tables 2 and 3 to account for the remaining 88%, we find: 54% base substitutions, 22% intragenic deletions and 4% tandem duplications. The remaining 8% include equal numbers of small insertion mutants, frame-shift mutants and mutants of unknown nature.

Spectra of spontaneous mutations in other microorganisms have shown a wide range of results. SCHAAPER, DANFORTH and GLICKMAN (1986) found that the majority of mutations in the *lacI* gene of *Escherichia coli* were not base substitutions, even when they excluded the two-thirds of total mutants which were

TABLE 3

Classification of 66 *mtr* mutants isolated from a heterokaryon

Class of *mtr* mutant	Number	Restriction pattern[a]	Possible molecular type
Stable			
Induced reversion (UV)	38	3 normal 35 not tested	Base substitution
Induced reversion (ICR and UV)	3	Not tested	Frame-shift
No reversion	17	Deletion (5/17)	Deletion
		Normal (12/17)	Small deletion
Slightly unstable	3	Insertion	Insertion
Ambiguous	2	Normal	Base substitution
Other	3	Methylated	Unknown

All of the stable mutants were screened for reversions after treatments with UV and ICR-170.
[a] *Xho*I and *Msp*I digests of all the tested mutants were hybridized with the *mtr* probe.

frame shifts at a single hot-spot. On the other hand, KUNZ *et al.* (1989) reported that over 80% of mutations at *SUP4* in Saccharomyces were base substitutions, and DE JONG, GROSOVSKY and GLICKMAN (1988) found 27 base substitutions among 30 spontaneous mutants at the APRT locus in hamster cells.

The source of variation for evolution is *germinal* mutation. Can we assume that the spectrum of germinal mutants will be the same as that of somatic mutants? There are important differences in the ways they arise. Germinal mutations are measured per organism generation and may have arisen at any point in that process. This means we are concerned with the total mutation for several rounds of mitosis and one round of meiosis. There may be unique mutational processes in meiosis. Gene conversion and unequal crossing over are known to give rare nonparental genetic types, and these may be scored as mutations. It is also possible that meiosis (or some stage of the sexual cycle) screens the new mutants which have arisen in the preceding mitoses and can modify or eliminate them.

We have observed one example of sexual cycle screening of new vegetative mutants in this study. This is the change of unstable *mtr* mutants to stable mutants. These unstable mutants appear to result from spontaneous tandem duplications. Preliminary analysis indicates that these duplications were subjected to RIP when they went through a cross. It has been shown that RIPing involves multiple base-pair substitutions within the duplicated segment (SELKER 1990). Thus a spontaneous tandem duplication followed by a cross would produce a germinal mutation involving complex changes: a duplicated segment and multiple changes of sequence.

If such complex mutational events were frequent, they would pose a serious problem for our understanding of evolutionary change. We do not know the frequency of tandem duplications in the genome at large. In the *mtr* gene they appear to be quite frequent: 6 out of 60 mutants in our first experiment. It is possible that several of these duplications have a common start site which is a hotspot for such events. Other spontaneous mutation surveys in which tandem duplication mutants were identified (RAMBOSEK and KINSEY 1984; DAS, CONSAUL and SHERMAN 1988) have not shown a high frequency of this class of mutants.

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