Spontaneous Mutation During the Sexual Cycle of *Neurospora crassa*

Michael K. Watters and David R. Stadler

Department of Genetics, University of Washington, Seattle, Washington 98195

Manuscript received September 15, 1994
Accepted for publication September 22, 1994

ABSTRACT

The DNA sequences of 42 spontaneous mutations of the *mtr* gene in *Neurospora crassa* have been determined. The mutants were selected among sexual spores to represent mutations arising in the sexual cycle. Three sexual-cycle-specific mutational classes are described: hotspot mutants, spontaneous repeat-induced point mutation (RIPs) and mutations occurring during a mutagenic phase of the sexual cycle. Together, these three sexual-cycle–specific mutational classes account for 50% of the mutations in the sexual-cycle mutational spectrum. One third of all mutations occurred at one of two mutational hotspots that predominantly produced tandem duplications of varying lengths with short repeats at their endpoints. Neither of the two hotspots are present in the vegetative spectrum, suggesting that sexual-cycle–specific mutational pathways are responsible for their presence in the spectrum. One mutant was observed that appeared to have been RIPed precociously. The usual prerequisite for RIP, a duplication of the affected region, was not present in the parent stocks and was not detected in this mutant. Finally, there is a phase early in the premeiotic sexual cycle that is overrepresented in the generation of mutations. This "peak" appears to represent a phase during which the mutation rate rises significantly. This phase produces a disproportionally high fraction of frame shift mutations (3/6). In divisions subsequent to this, the mutation rate appears to be constant.

SPONTANEOUS mutation has long been a popular area of study. This long-standing interest stems from the insight it may give into the very heart of genetics, the mechanisms responsible for faithfully replicating the genome for the next generation while allowing rare mutations that permit a species to evolve. These studies illuminate the engine of evolution, the mutational process, by telling us what forms those changes take, in what proportions and at what frequencies. All three are vital to understanding the relationships between the genomes of different species.

The majority of studies of spontaneous mutation have concerned themselves only with vegetative cells. This unfortunately has left the sexual cycle and meiosis largely ignored in regard to their effects on spontaneous mutation. Because evolution is driven by germinal mutations (including both mutations occurring during the sexual cycle as well as vegetative mutants incorporated into germinal cells), it is important to know if the vegetative mutation studies are representative of germinal mutation as a whole.

Previous studies of spontaneous mutation in meiosis have suggested that differences do exist. The first report that meiosis might have special mutagenic affects was in *Saccharomyces cerevisiae* (MAGNI and VON BORSTEL 1962). These authors observed that certain auxotrophic alleles appeared to revert to prototrophy at much higher rates in meiosis than in mitosis. MAGNI later proposed that the "Meiotic Effect" resulted from a rare unequal exchange of DNA during meiotic recombination that resulted in frameshift mutations. This phenomenon was absolutely dependent on the presence of a homologous copy of the auxotroph in both parents such that the mutants reverted when selfed but not when crossed to stocks deleted for the gene in question (MAGNI 1963, 1964). Similar "selfers" were described in several systems, including Salmonella (DEMEREC 1962), Ascobolus (PASZEWSKY and SURZYCKI 1964), maize (GAVAZZI and COLELLA 1973), Schizophyllum (KOLTIN *et al.* 1975) and Neurospora (BAUSUM and WAGNER 1965).

Evidence for a meiotic effect in forward mutation was less convincing . Two studies of the *CAN1* gene of *S. cerevisiae* gave conflicting results. One found evidence for increased mutation rates in meiosis (MAGNI 1964), whereas the other did not (WHELAN *et al.* 1979). Studies in barley and maize (LINDGREN 1975) revealed evidence for increased rates of forward mutation in meiosis as well as increased sensitivity to mutagens during meiosis. A study of forward mutation in *Schizosaccharomyces pombe* (FRIIS *et al.* 1971) showed no evidence for an increase in the rate of mutation during meiosis. However, it did show a strong effect on the spectrum of mutations generated. These authors observed increases in the proportion not only of frameshift mutants but also of other mutants that were neither base substitutions nor frameshifts and presumably included insertions and deletions.

Corresponding author: Michael K. Watters, Department of Molecular and Medical Genetics, Oregon Health Sciences University, 3181 SW Sam Jackson Park Rd., Portland, OR 97201.
E-mail: wattersm@ohsu.edu

Neurospora has a well-described example of a sexual-cycle–specific mutational event. Duplications sent through a cross are subject to repeat-induced point mutation (RIP) (SELKER *et al.* 1987; CAMBARERI *et al.* 1989; SELKER 1990). In RIP, duplicate sequences are detected and altered during the sexual cycle. Both copies are inactivated at high frequency. Affected sequences are typically heavily methylated as well as being subjected to multiple base substitutions. DNA sequencing of RIPed regions revealed that changes were exclusively GC→AT mutations. Changes were biased toward sites where adenine was immediately 3′ of the affected cytosine. A similar effect, MIP, which methylates but does not mutate duplications, has been described in Ascobolus (RHOUNIM *et al.* 1992). These examples serve to demonstrate the potential of the sexual cycle for generating specific effects on spontaneous mutation.

Here, we report studies of sexual cycle mutation at *mtr* (methyltryptophan resistance), the structural gene for the neutral amino acid permease in Neurospora. It is especially useful because it permits selection of both forward and reverse mutations. The ability to select rare mtr^+ spores in a mutant population is useful in two steps of our analysis: reversion testing to assign tentative mutation types and deletion mapping of mutants to assist in sequencing. In this paper we report the results of sequencing a spectrum of spontaneous mutations occurring during the sexual cycle and discuss their relationship to the spectrum of vegetative mutants.

MATERIALS AND METHODS

Strains: *mtr* mutants were isolated from spores of a cross between two strains with the genotypes: *trp-2 pdx-1 A* and *trp-2 col-4 a*. The phenotype of *trp-2* is a nutritional requirement for tryptophan or anthranilic acid. *pdx-1* requires pyridoxine and *col-4* is a morphological marker that results in colonial (nonspreading) vegetative growth. The *pdx-1* and *col-4* loci flank the *mtr* locus (3 m.u. proximal and 1 m.u. distal to *mtr*, respectively) and were used to determine which parental chromosome was present in the mutants and also to monitor recombination in the region. Both parent strains derived their genetic background from Oak Ridge wild-type as did the following strains, used for deletion mapping: 1564 (*trp-2 pdx-1 MTR1564*), 59 (*trp-2 col-4 MTR59*), 1X (*trp-2 col-4 MTR1X*) and 30 (*trp-2 col-4 MTR30*).

Media: Vegetative growth media were made from Vogel's minimal (DAVIS and DESERRES 1970) with various supplements. Supplements were anthranilic acid (Anth), L-tryptophan (Trp), L-arginine (Arg), *p*-fluorophenylalanine (Fpa) and pyridoxine (Pdx). The concentrations of Anth, Trp and Arg were normally 50 mg/liter. Fpa was 15 mg/liter and Pdx was 5 mg/liter. Plates contained sorbose medium, for which carbohydrates were 1.5% sorbose and 0.1% dextrose. Carbohydrate for media in tubes was 2% sucrose. All crosses were performed in glass plates with ~20 ml crossing medium (WESTERGAARD and MITCHELL 1947) without sugar. A single round 9-cm Whatman #1 filter paper was placed on top of the gelled medium before inoculation to serve as the carbon source.

The *trp-2* strains can grow on medium supplemented with either Trp or Anth. The latter does not depend upon the neutral amino acid permease for uptake. Thus, *trp-2* strains can grow on Anth whether they are mtr^+ or *mtr*. The selective medium for *mtr* mutants (M^-S) was supplemented with Fpa, Anth and Pdx. On this medium, wild-type cells die because they take up the Fpa via the neutral amino acid permease (*mtr* gene product), whereas *mtr* mutants survive by virtue of their permease being defective. The counterselective medium (for mtr^+) was called M^+S. In addition to Pdx, it contained 0.2 of the normal tryptophan supplement (10 mg/liter) and twice the normal arginine supplement (100 mg/liter). On this medium the cell can only take up the 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 *mtr* mutants: Multiple individual cultures of the two parent strains were grown from isolated colonies on Anth + Pdx medium. These cultures were used to start individual cross plates. After the formation of protoperithecia, the cross plates were fertilized with similar individual cultures.

Crossing plates were inoculated with the maternal parent and incubated at 25° for 1 week before being fertilized. Most crosses were performed with the *col-4* line as the maternal parent. After fertilization, the inverted cross plates were incubated an additional 3 or 4 weeks at 25°C. Ascospores were harvested by washing the lid of the cross plate with 10 ml of sterile water. To this suspension was added 50 μl of 5% NaOCl to eliminate conidia (as described by KINSEY *et al.* 1980). The suspension was then centrifuged and the pelleted spores resuspended in 1 ml of sterile water. This suspension was then added to 2.5 ml of 1% agar and heat shocked at 60° for 45 min to break dormancy. After heat shock, the mixture was overlaid on M^-S medium. Spore recoveries ranged from 10^5 to 4×10^6 spores per cross plate (average was 5×10^5). Potential *mtr* mutants were scored and harvested after incubating 2 days at 33°. All potential mutants were twice reisolated from streaks on M^-S medium before retesting for *mtr* phenotype by growth tests on the two counterselective media.

All *mtr* mutants recovered were included for study. Mutants of *mtr* are recessive; therefore, growth under selective conditions requires that they must contain only *mtr* nuclei. This only happens when the mutational event results in a homoduplex by the end of the postmeiotic division. Clusters of one presumably result from mutational events that occur during the postmeiotic division. One can also imagine that size one clusters would result from events earlier in Meiosis proper if the associated heteroduplex was not "corrected" until the postmeiotic division. Events that occur (or reside in heteroduplex DNA) after this division would lead to heterokaryotic spores that would not grow on M^-S and thus would not have been detected. It is also possible that mutants originating in vegetative divisions might be incorporated into a perithecium and be collected in spores. We would expect that any such vegetative mutants would give rise to large clusters of mutants because half the contents of the affected perithecium would be mutant. Studies of single isolated perithecia (data not shown) suggest that a single perithecium yields about 500 spores under our cross and collection conditions. Thus, a vegetative mutant incorporated into a perithicium should yield a cluster of ~250 mutant spores. Two of our 52 mutants occurred in clusters near that size (119 and 220). Because, however, these cannot be distinguished from events occurring in the very earliest stage of the sexual cycle, they were retained and included for study.

Given the low probability that any given cross plate would yield mutants (only 52 out of 1500 cross plates produced mutants), it seems likely that all mutant spores arising from

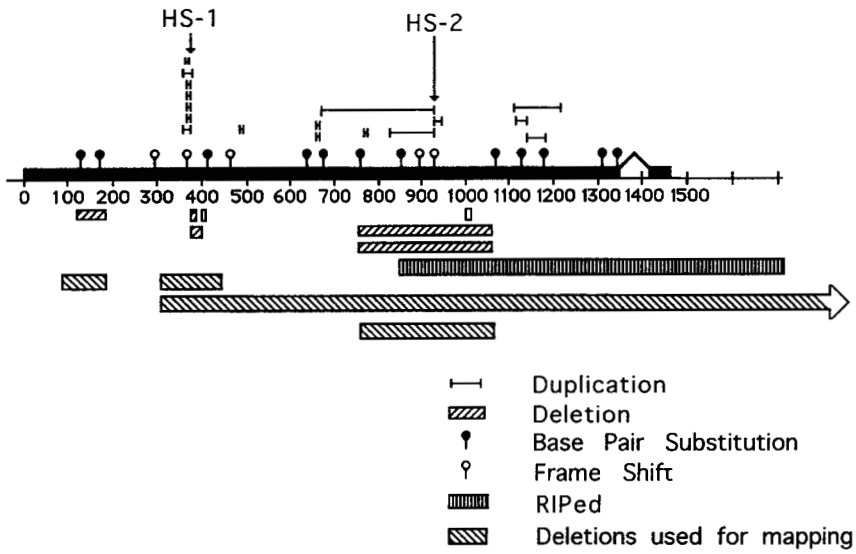

FIGURE 1.—Schematic representation of 17 duplications, seven deletions, 12 base substitutions, five frameshifts and one RIPed mutant among sexual cycle spontaneous mutants. The box immediately above the line represents the open reading frame (ORF) with one small intron. Bars above the line denote the endpoints of duplications. ▨, deletions; ▩, range of C → T transitions in RIPed mutant pA24; ●, base substitutions; ○, frameshifts; ▧, deletion mutants used to map the spectrum of sexual cycle mutants. The 3′ end of Δ1564 extends an undetermined distance past the end of the ORF. Also noted on the sequence are the two hotspots (HS-1 and HS-2) largely distinguished by duplication mutants.

a single cross plate were siblings, descended from a single mutational event. This hypothesis was tested by marker testing several individual spore colonies isolated from the same cross plate (*i.e.*, potential sibling mutants). For nearly every plate tested, all of the potential siblings were of the same genotype, having only one of the two parental combinations of markers flanking *mtr* (either *pdx-1 col-4*$^+$ or *pdx-1*$^+$ *col-4*). The only exceptions were rare recombinants within a cluster (data not shown). Therefore, all the mutants arising from a single cross plate must have descended from a single mutational event. Thus, the number of mutant spores gathered from a single cross plate, or its "cluster size," potentially provides information regarding the time during the sexual cycle at which the mutation occurred (late mutant = small cluster). This will be discussed further below.

Deletion mapping: Crosses to a set of four *mtr* deletion mutations were used to localize *mtr* mutants (RAMBOSEK and KINSEY 1983). The *mtr* deletion in Δ1X was isolated after X-ray treatment, Δ1564 after UV treatment and the deletions in Δ30 and Δ59 were spontaneous. The deletion endpoints of Δ30, Δ1X and Δ59 have been determined by sequence analysis (see Figure 1). They represent deletions of 93, 160 and 312 bp, respectively. The deletion endpoints of Δ1564 have not been sequenced, but the 5′ endpoint has been localized to within 40 bp by Southern analysis.

The deletion strains served as the maternal component in all crosses except those involving Δ1564, which was used as the paternal component. Crosses were performed and harvested as described above. The spores were plated on M$^+$S medium to score recombinants.

Molecular analysis: Neurospora DNA was prepared from mycelium ground with sand by the method of ZOLAN and PUKKILA (1986). Restriction enzymes from BRL or Boehringer Mannheim were used according to manufacturers' instructions. Southern transfers were made to Qiabrane (Quiagen). Hybridization was to polymerase chain reaction (PCR)-amplified *mtr* DNA labeled with biotin-II-dUTP (BRL). Biotinylated probes were detected with streptavidin-alkaline phosphatase using the Blugene system (BRL).

Various portions of the *mtr* region were amplified by PCR using a variety of 20-mer oligonucleotides. The program used in all amplifications consisted of one 10-min cycle at 94°, followed by 25 cycles of 1 min at 95°, 2 min at 55° and 3 min at 72°. A final cycle of 10 min at 72° ended the program.

The amplified DNA was run on 2% agarose gels either uncut or digested with restriction enzymes to check for band length changes. Using this method, insertions/deletions as small as 15 bp were easily detected.

Amplified DNA to be sequenced was first treated with T7 gene 6 exonuclease using the method of U.S. Biochemical. Resulting single-stranded DNA was sequenced using the chain-termination method (SANGER *et al.* 1977), using the Sequenase kit (U.S. Biochemical), according to the manufacturer's specifications.

RESULTS

A random sample of spontaneous mutations occurring during the sexual cycle was obtained by selecting *mtr* mutants on M$^-$S medium among spores from a cross between the two strains: *trp-2 pdx-1 A* and *trp-2 col-4 a*. A total of 1500 cross plates were examined, of which 52 yielded *mtr* mutants. Each cross plate yielded an average of 5×10^5 spores. On most of those 52 plates, more than one mutant colony was observed. The distribution of these "clusters" of mutations is shown in Table 1. The flanking marker combinations revealed that paternal and maternal nuclei were almost equally represented among the mutants (data not shown).

The 52 independent mutants (one for each cross plate yielding *mtr* mutants) were purified by vegetative reisolation and characterized by deletion mapping, restriction digests and sequencing. Deletion mapping and restriction digests were used to localize the mutations to assist in sequencing. Of the 38 mutations that had been localized by either deletion mapping or restriction digests, 36 were sequenced. In addition, six of the non-localized mutations were sequenced for a total of 42 sequenced mutations.

The results of sequencing the mutations are given in Table 2. The physical distribution of the mutations is shown in Figure 1. Immediately obvious from the distri-

TABLE 1

Distribution of cluster sizes

Cluster sizes	Total recovered	Sequenced	Hotspot	Nonhotspot
1	27	21	9	12
2	9	7	2	5
3	1	1	—	1
4	5	4	2	2
6	1	1	—	1
9	1	1	—	1
13	1	1	1	—
19	2	2	—	2
21	1	1	—	1
42	1	1	—	1
53	1	1	—	1
119	1	1	—	1
220	1	—	—	—

bution of mutations is the presence of two mutational hotspots (HS-1 and HS-2), each of which has several (10 and 4, respectively) events whose endpoints are contained within a narrow region of the gene.

Hotspot 1 (HS-1, Figure 2) is a 20-bp region that contains at least one endpoint of 10 independent mutations (7 duplications, 2 deletions and 1 frameshift). All seven of the duplications are associated with short tandem repeats (2–4 bp) at their endpoints. Neither of the deletions have repeats at their endpoints.

Hotspot 2 (HS-2, Figure 3) is an 8-bp region containing one endpoint of four independent mutations (3 duplications and 1 frameshift). As with HS-1, the three duplications of HS-2 are associated with short tandem repeats (2–8 bp) at their endpoints.

Mutant pA24 shows signs of RIP. The sequence of the affected region of pA24 is given in Figure 4. This mutant has a total of 48 C→T transitions in a 1-kb region that covers a portion of the open reading frame and the intron and extends into the 3′ untranslated region of the gene (Figure 1). Attempts to uncover a duplication of *mtr* in mutant pA24 via Southern analysis and PCR amplification were both unsuccessful (data not shown).

DISCUSSION

In this paper we describe the DNA sequence changes of 42 spontaneous mutations of the *N. crassa mtr* gene that occurred during the sexual cycle. We undertook this study to determine any differences in spontaneous mutation between the sexual cycle and vegetative growth. We have recovered representatives of five different classes of events (base substitution, frameshift, duplication, deletion and RIP). A variety of mutational mechanisms are presumed to be represented.

Sexual-cycle hotspots: The two sexual-cycle mutational hotspots were distinguished by having unusually high concentrations of mutational events in a confined region of the gene. They differ from other described hotspots, such as the *Escherichia coli Lac I* hotspot (SCHAAPER *et al.* 1986), which generates large numbers of identical (or complementary) mutations. At the *Lac I* hotspot, a triple tandem repeat of a four-base sequence tends to gain or lose one copy of the repeat at a high frequency, accounting for 67% of observed spontaneous mutations in *Lac I* (SCHAAPER *et al.* 1986). The two sexual-cycle hotspots observed here produce a wider array of mutations.

Of the 14 mutations located within the sexual cycle hotspots, 11 distinct changes were observed. Both hotspots predominantly produce tandem duplication mutations (70%). This contrasts with the spectrum of nonhotspot sexual-cycle mutation that has only 25% duplications. The hotspot mutational spectrum contrasts even more sharply with the spectrum of vegetative *mtr* mutations, which contains only 3% (1/31) duplications (DILLON and STADLER 1994). The largest duplication detected in the sexual cycle spectrum is M58 (242 bp) in hotspot 2. Notable is the fact that this duplication exited the sexual cycle without being affected by the RIP process. Although there are several reasons this might have occurred (duplication occurred after the

TABLE 2

Spectrum of sexual cycle spontaneous *mtr* mutations by class

Class	Sexual-cycle mutant total	Hotspot	Peak	Non–sexual-cycle specific	Vegetative control[a]
Base sub.	12 (29)	—	1 (17)	11 (52)	15 (48)
Frameshifts	5 (12)	2 (14)	3 (50)	—	4 (13)
Deletions	7 (17)	2 (14)	1 (17)	4 (19)	11 (35)
Duplications	17 (40)	10 (71)	1 (17)	6 (29)	1 (3)
RIP	1 (2)	—	—	—	—
Totals	42	14	6	21	31

Values in parentheses are percents.
[a] DILLON and STADLER (1994).

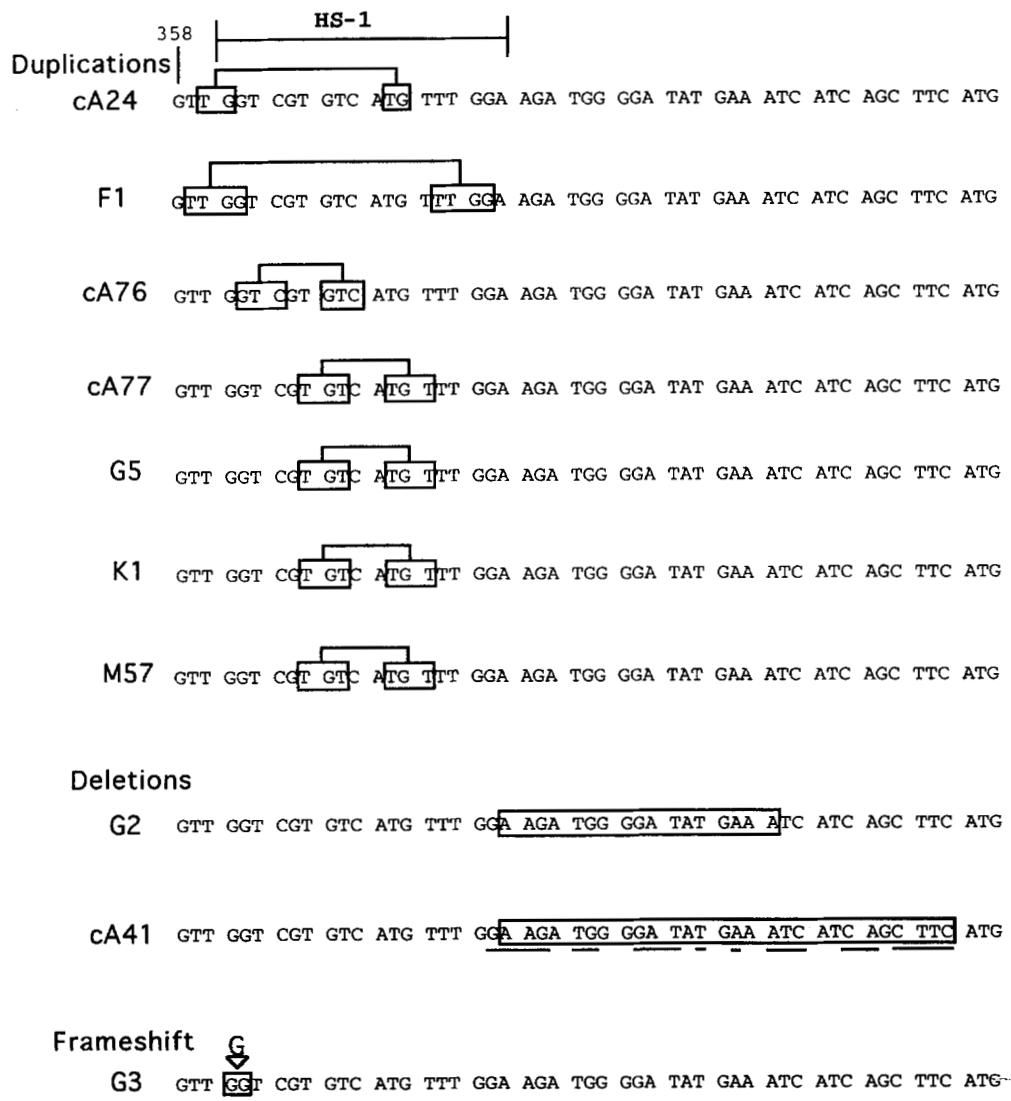

FIGURE 2.—Diagram of mutations in hotspot 1. The wild-type sequence is listed for each mutant with the mutation noted on the sequence. For duplication mutants, boxed regions represent endpoint repeats potentially accounting for their occurrence. For deletions, the boxed regions represent those bases absent in the mutant. The extent of HS-1 is marked by the bar at the top of the figure. A proposed DNA misalignment associated with hotspot 1 is underlined on mutant cA41. This palindrome is potentially capable of directing the formation of deletion mutant cA41. Pairing of the bases in this palindrome could also stabilize nearby slipped DNA structures leading to the hotspot for duplications and deletions.

window of activity for RIP, duplications not immediately subject to RIP or require some activating event early in sexual cycle, and so on), later tests on this mutant (data not shown) suggest that it is too short to be detected by the RIP mechanism.

The hotspot duplications were all flanked by short (2–8 bp) tandem repeats at their endpoints, consistent with their formation by slippage during replication (STREISINGER *et al.* 1966). Hotspot 1 (HS-1) has an adjacent palindrome that may contribute to the region's high mutability. Noted in Figure 2, the sequence 378–405 is capable of folding into an imperfect palindrome that might account for the deletion in cA41 and might also contribute to short duplications and deletions in the area by stabilizing slipped templates during replication. However, tandem and inverted repeats at HS-1 and HS-2 cannot totally account for these regions' specific mutability because these regions are not hotspots for mutation during vegetative growth (DILLON and STADLER 1994). Although 6 of the 31 vegetative mutants were localized to the palindrome immediately adjacent to HS-1, only 1 (the endpoint of a deletion) is localized within HS-1. None of the mutants in the vegetative spectrum map to HS-2.

RIPed mutant pA24: The affected region in pA24 is shown in Figure 4. A total of 48 GC → AT transitions have been sequenced in pA24 over a 1-kb region covering the 3′ end of the gene and 3′ untranslated re-

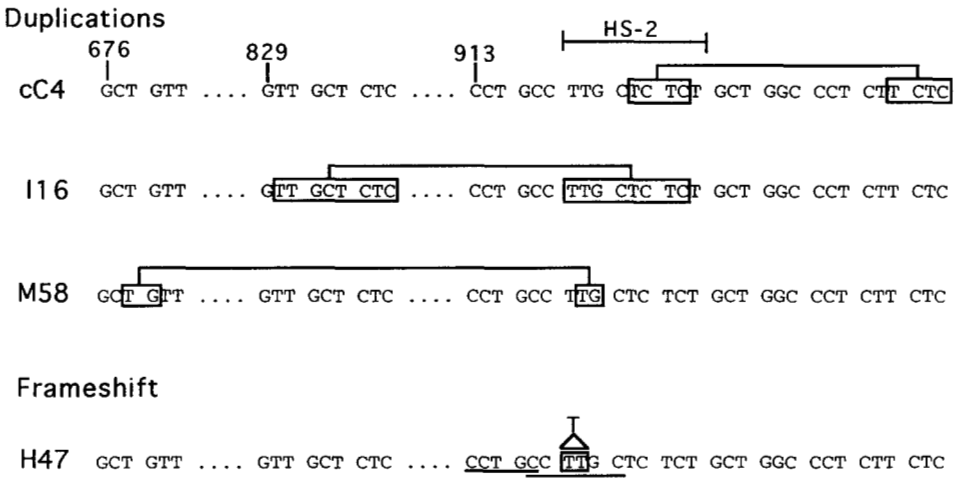

FIGURE 3.—Diagram of mutations in hotspot 2. Sequence notations are the same as for Figure 2. The underlined bases on mutant H47 denote a five base imperfect repeat that could have served to template the mutation.

gions. RIP is characterized by multiple GC → AT mutations, so we suspected that pA24 may have been subjected to RIP. Unlike previously reported RIP sequences, all of the 48 GC → AT transitions in pA24 were C → T on the sense strand, as opposed to the mix of G → A and C → T that have been previously reported for RIPed regions. This leads us to conclude that pA24 was subject to only a single round of RIP.

```
796                                826
ATG CAC ACC CCC TCC GAC TAC AAG AAG TCC ATC GTT GCT CTC GGC TTG ATT GAA ATC TTC

856                                886
ATC TAC ACC GTT ACT GGT GGC GTC GTT TAC GCT TTC GTC GGC CCC GAG GTC CAG TCT CCT

916                                946
GCC TTG CTC TCT GCT GGC CCT CTT CTC GCC AAG GTT GCT TTC GGC ATT GCC CTC CCC GTC

976                                1006
ATC TTC ATC TCT GGC AGT ATC AAC ACT GTT GTC GTC AGC AGG TAT CTG ATT GAG CGC ATC

1036                               1066
TGG CCC AAC AAC GTC ATT CGC TAT GTC AAC ACC CCA GCG GGT TGG ATG GTT TGG CTT GGT

1096                               1126
TTT GAC TTT GGC ATT ACC CTC ATT GCC TGG GTT ATT GCT GAG GCC ATC CCT TTC TTC TCT

1156                               1186
GAT CTG TTG GCC ATC TGC TCG GCT CTC TTC ATT TCC GGT TTT AGC TTC TAT TTC CGT GCC

1216                               1246
TTG ATG TAT TTC AAG ATC ACC AGG AAC GAT GCC AAG AGC CAG GGC AAG AAG TAC TTC TTG

1276                               1306
GAT GCC CTC AAC ATG CTC TGC TTC GTC ATC GGC ATG GGC ATT CTT GGT ATT GGT ACC TAC

1336
GCC GCT ATT CAG GAC (ATTGTAAGTTTGGCCCGCTTTTCTGTTTACTCTTTGCACACAAATGCTAACT
                     intron
1403
TGCTTCTCAG) ATG GAC CGT TAC GAC CAT GGC AAG GTT TCG AAG CCT TAT AGC TGT GCT CCC

1464
TTG GCT TAA CAGGCCCAACGCACGCTTATGATCCTGTTGTTTTTTTTTGGATGATTTAATTAAAGTTG
      stop
1532
CGCAGTGATTGACGTCTGTCTTCACCCGCGATTGCCCCTTTGTATACCCCCTCAGACTTGCCGGCCTGG

1602
GGAAATGTTTTGAGTATTTCTATTTTCGGAGTTTCAGGATTTGGCACAAAGCAAACCAGCGCGGGTTGAAA

1673
CCGTGGTGTGGTCGCGGTGCGCTGCTGCATTGGTAGTGCTTGTTCCAGGTTTTTGTTTGGTGGTTTGGATG

1744
CGTGCACCACTTTTTTTTTTAACGTTTTATTGCATGCATGTATTATATGGGAAAGTCATGGGACATGGCAA

1815
CTATACGAACCGACGCAAAGATAGGATGGGATGGATGATGGATGGACGTACGATCCAACGC
```

FIGURE 4.—Sequence of the affected region of mutant pA24 that shows signs of RIP. The wild-type sequence is listed with the affected bases underlined. Changes are confined to an ~1-kb region of the gene and are exclusively C → T transitions on the coding strand.

Mutant pA24 is interesting because it marks the first observance of a spontaneous RIP event arising from wild-type strains. It is also unique in that neither the cross parents nor the mutant carries traces of a duplication that may have served to initiate the event. Given the absence of a duplication in either the parents or mutant cluster pA24, we believe the most likely triggering event for RIP in pA24 was the occurrence in the sexual cycle of a transient duplication. An unlinked duplication could have triggered RIP in the perithecium, which gave rise to mutant cluster pA24, only to be later lost due to random segregation. Another possibility is that a free duplication unincorporated into the genome might serve to trigger RIP only to be lost in later divisions. Alternately, one can speculate that pA24 was created by a RIP-like mechanism in the absence of any duplication. The RIP sites of pA24 conform to previous observations (CAMBARERI *et al.* 1989) that CpA sites are targeted in preference to other dinucleotides.

The changes in pA24 are exclusively C → Ts on the coding strand (see Figure 4). If all 48 changes occurred as independent events, they should be a mix of C → T and its symmetrical product, G → A, on a given strand. Because that is not the case in this mutant, it suggests one of two models for the RIP process. The first model is that RIP occurs after a single initiating interaction that serves to orient the process to a single strand (see Figure 5). After this initiating event, the RIP mechanism could progress in a processive manner, altering sites as it translated down the DNA. This model would predict that after a single round of RIP, only one type of base substitution (C → T or G → A) would be observed on a single strand. The second mechanism suggested is one that generates C → T changes randomly (see Figure 5) along both strands. If the resulting heteroduplex is not repaired before replication, then the resulting

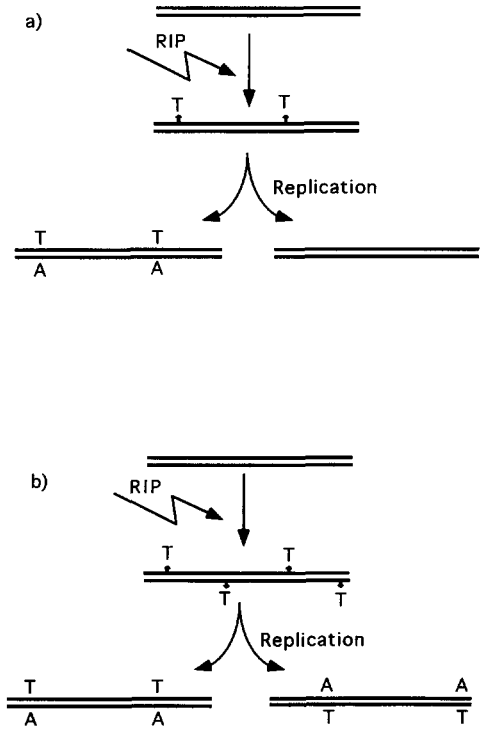

FIGURE 5.—(a) RIP model 1: strand specific. Under this model, RIP is a single strand processive event that orients itself randomly at each round of RIP. This model would produce RIPed strands that showed only C → T (or all G → A) changes on a strand after one round of RIP. (b) RIP model 2: randomly distributed independent events. Under this model, RIP events are randomly distributed along both strands but are not repaired before replication. As with model 1, this model predicts the formation of strands with only one type of change on a strand. However, two different patterns are generated by a single event. Only the altered bases of a strand are shown above.

sequences, after replication, would be populated exclusively by C → T changes on the old strands and G → A on the newly synthesized strands. Both models would produce a mixture of C → T and G → A changes on a strand after multiple rounds of RIP.

The models have predicted differences when one looks at the total expected products of a single RIP event. Under the first model, all the descendants of a RIP event will have the same RIP pattern (*i.e.*, distribution and type of changes). The second model, however, would predict that two completely dissimilar RIP patterns would be generated by a single round of RIP (see Figure 5). An examination of the sibling mutants in cluster pA24 in combination with a more detailed examination of RIP patterns in other lines should help discriminate between these two models.

Time of mutation: In an attempt to determine the time of mutation for the mutants in our spectrum, we examined the number of sibling mutant spores (cluster size) produced by a cross plate. A cluster of a given size n could have originated in one of three ways: (1) two or more independent events giving mutants totaling n, (2) a single event that produced n mutants and (3) a single event that produced $n + x$ mutants, only n of which were recovered.

The low probability of a given cross plate yielding *any* mutants (52 mutations detected out of 1500 cross plates tested) suggests that it is highly unlikely that two events would have arisen coincidentally on the same plate, providing strong evidence against case 1 above. To further test for coincident mutation, sibling mutants (all the mutants arising from a given cross plate) from all clusters were compared for their markers flanking *mtr* that were segregating in the cross. If a cluster consisted of a mixture of two events, then there should be only a 50% chance that both events would occur in the same parental background. In every cluster, only one parental combination of flanking markers was present. The only clusters that showed mixed combinations of flanking markers were those in which rare siblings were recombinant. We therefore conclude that the observed clusters represent only single events.

The question of the recovery rates of the mutants in a cluster is more complex to address. Because we observe cluster sizes other than simple powers of two (1, 2, 4, 8 and so on), we know we are not recovering 100% of the mutants in every cluster. What we can do is look at the smallest clusters greater than one (2s and 4s) and make use of the special properties of these small clusters to examine the rates of recovery from a single ascus. There is some evidence that recovery rates from these small clusters are quite good. For clusters of size 2, there are two possible origins: a true cluster of size 2 or a true cluster size of 4 or more, only two of which were recovered.

For first case, the two mutants could only have emerged from the sexual cycle as sister spores and should therefore be identical for all markers, including the mating type locus that is unlinked to *mtr*. For the latter case, because *mtr* is unlinked to mating type, there is only a 50% chance that both spores would carry the same allele for mating type.

A total of nine clusters of size 2 were recovered. In all nine cases, both sibling spores carried the same allele for mating type and the same combination of flanking markers. In addition, in one cluster (K2) both siblings were recombinant for the flanking markers *pdx-1* and *col-4* (total map distance ~ 4 m.u.). It is highly unlikely that these recombinants could have arisen independently. A much more likely explanation is that the observed clusters of size 2 arose as true size 2 clusters and that the whole cluster was recovered.

A similar analysis was carried out for clusters of size 4 with similar results. Results from size 2 and 4 clusters are consistant with their originating as clusters of two or four that were fully recovered. If we assume the results from these small clusters can be extended to large

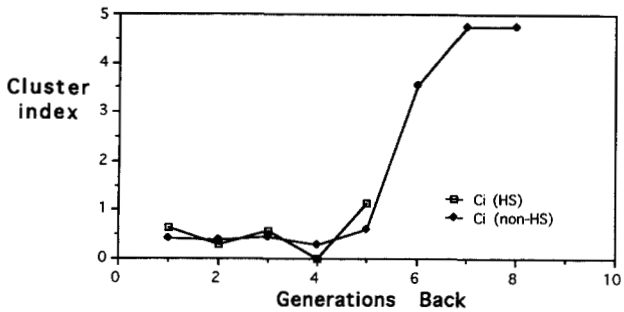

FIGURE 6.—Graph of the cluster index (Ci) for hotspot and nonhotspot mutation in the sexual cycle. Under the assumption that the mutation rate is constant per replication, Ci is a constant (1/2). This holds roughly true for both hotspot mutants and most nonhotspot mutants. However, the early generations of the sexual cycle are overrepresented in the mutational spectrum. This suggests that the mutation rate is higher during this phase of the sexual cycle than during later phases.

clusters, we can make predictions regarding the distribution of cluster sizes among all mutations.

Mutations occurring in earlier divisions should give rise to a larger cluster of mutants than mutations occurring later. The size of clusters should increase in powers of two (1, 2, 4, 8 and so on) depending on how early the event occurred [*i.e.*, cluster size (Cs) = 2^{x-1}, where x is the number of nuclear generations between the mutational event and scoring of that mutant, including the generation in which the mutation occurs]. If the probability of mutation remains constant per nucleus per generation, then the number of events from successivly earlier cell generations will be proportional to the series 1/2, 1/4, 1/8 and so on [*i.e.*, cluster fraction (Cf) = $(1/2)^x$, with x defined as above]. Therefore, 50% of all mutational events should occur in the last division and produce clusters of size 1, 25% of mutational events producing clusters of size 2, 12.5% producing clusters of size 4 and so forth. In addition, an index (Ci) defined as the size of a cluster multiplied by the proportion of events of that size should be a constant if the above assumptions hold true (*i.e.*, Ci = (Cs)(Cf) = 1/2). If Ci deviates from a constant, that would indicate that one of our assumptions (constant mutation rate or the geometric increase in cluster size with time of event) fails at that point. For the purposes of our analysis, a cluster is assumed to have originated from the smallest natural cluster size (see above) that could have given rise to it, that is, an observed cluster of three is counted as a size 4 cluster.

Examining the observed distribution of cluster sizes for mutations in the sexual cycle we see the following. For mutations associated with the two hotspots, the observed cluster size distribution is very close to that predicted under the assumption of constant rate (Figure 6).

For mutations not associated with the two hotspots, however (Figure 6), a different trend is seen. Although Ci remains constant for later divisions (similar to HS mutants), it deviates strongly from a constant for the largest clusters (which represent early divisions). Under the hypothesis of constant mutation rate, Ci should remain a constant over all cluster sizes. Although this is true for mutations occurring during later divisions (both HS and non-HS), the nonhotspot mutations show a peak in mutation among the earliest observed events. One way we can address the significance of the peak is to calculate the probability of finding 6 of the 27 nonhotspot mutants occurring that early. From the above discussion, we can see that the probability that any *given* mutant occurs during the "peak" phase (cluster size ≥ 32) is 1/32. Thus, the probability of observing six (or more) peak mutants in our sample is 1.79×10^{-4}, considerably below a 1% significance level. This allows us to reject the hypothesis that a given mutant has a 1/32 chance of occurring in the early divisions and thus reject the hypothesis that mutation rates are constant through the sexual cycle. This suggests that an early phase of the sexual cycle is unusually mutagenic, producing mutations at a higher rate than during later phases of the sexual cycle. For mutations occurring after this peak, Ci is nearly constant, suggesting that our assumptions hold approximately true for the later divisions.

The spectrum of these peak mutations is given in Table 2. Examples of all mutational types (base substitution, frameshift, duplication and deletion) are present among the peak mutants. However, all three of the nonhotspot frameshifts occurred as peak mutations. Because frameshifts are uncommon in the spectrum (11% of the nonhotspot mutants), it is highly improbable (P = 1.9%) that three of the six peak mutants would be frameshifts.

It is apparent that during the sexual cycle of Neurospora, there is a phase, occurring early, that is highly mutagenic. The mechanisms of mutation during this phase must be altered as well because of the observance that frameshifts are disproportionally represented in the peak mutant spectrum. One possibility might be that one or more repair pathways are shut down (or that lesions are shunted to alternate pathways) during this phase. The result would be what amounted to a temporary "mutator" phenotype during this phase.

If we remove the three defined sexual-cycle–specific mutational classes (hotspot, RIP and peak mutants) from the spectrum (Table 2), we are left with a spectrum that we must conclude is different [chi-square analysis of the two spectra yields p(spectra same) < 5%] from the vegetative spectrum obtained for *mtr* in Neurospora. The sexual-cycle spectrum remains biased toward duplications. Perhaps with a larger collection of mutants, additional duplication hotspots might be

observed. Alternatively, sexual cycle mutation might simply be biased toward duplications in general.

If we examine Neurospora for the Meiotic Effect as it was described by MAGNI and VON BORSTEL (1962), we find none. The mutants that have the strongest potential for originating in meiosis (clusters of size 1 and 2) show no propensity for frameshifts nor do they occur more frequently than expected, judging from the distribution of cluster sizes. The findings, therefore, do not support the existence of the Meiotic Effect in Neurospora *per se*. However, if we shift our attention to the peak (described above), we find that it shows some of the characteristics associated with the Meiotic Effect. Namely, it shows an increase in the rate of mutation and a disproportionate amount of frameshift mutants, as was seen both by MAGNI (1963, 1964) and FRIIS *et al.* (1971). However, the differences between the peak mutants and the Meiotic Effect are clear. Most obviously, the peak mutations do not occur in meiosis but in the early stages of the sexual cycle. Second, although the Meiotic Effect is absolutely dependent on the presence of a homologous copy of the gene (MAGNI 1963), the peak mutations occur before karyogamy (thus taking place in the absence of a homologue within the affected nucleus).

In recent years there have been two reports of unusual mutational activities during the sexual cycle of Neurospora. SELKER *et al.* (1987) described RIP in the sexual cycle. BUTLER and METZENBERG (1989) observed that in Neurospora, the nucleolus organizer region was subject to a high rate of size changes during the sexual cycle. We have now added to these the observation of sexual-cycle–specific mutational hotspots and have highlighted a phase occurring early in the sexual cycle that has an increased rate of mutation and altered mutational spectrum. It is becoming apparent that in Neurospora at least, the sexual cycle is characterized by discrete mutational processes and that, as a result, germinal mutations may be quite different from somatic mutations.

We are grateful to W. D. STUART and K. KOO for providing strains and plasmids. We thank H. MACLEOD and D. DILLON for helpful comments and suggestions. This work was supported by a grant from the National Science Foundation.

LITERATURE CITED

BAUSUM, H. T., and R. P. WAGNER, 1965 "Selfing" and other forms of aberrant recombination in isoleucine-valine mutants of Neurospora. Genetics **51:** 815–830.

BUTLER, D. K., and R. L. METZENBERG, 1989 Premeiotic change of nucleolus organizer size in Neurospora. Genetics **122:** 783–791.

CAMBARERI, E. B., B. C. JENSEN, E. SCHABTACH and E. U. SELKER, 1989 Repeat induced G-C to A-T mutations in Neurospora. Science **244:** 1571–1575.

DAVIS, R. H., and F. J. DESERRES, 1970 Genetic and microbiological research techniques for Neurospora crassa. Methods Enzymol. **17:** 79–143.

DEMEREC, M., 1962 "Selfers"—attributed to unequal crossovers in Salmonella. Proc. Natl. Acad. Sci. USA **48:** 1696–1704.

DILLON, D., and D. STADLER, 1994 Spontaneous mutation in *mtr* locus in Neurospora: the molecular spectrum in wild-type and mutator strain. Genetics **138**: 61–74.

FRIIS, J., F. FLURY and U. LEUPOLD, 1971 Characterization of spontaneous mutations of mitotic and meiotic origin in the *ad-1* locus of *Schizosaccharomyces pombe*. Mutat. Res. **11:** 373–390.

GAVAZZI, G., and C. COLELLA, 1973 Different rates of spontaneous reversion of an unstable R allele (R2nc) during meiosis and mitotic division of the gametophyte. Mutat. Res. **19:** 269–271.

KINSEY, J. A., J. R. S. FINCHAM, M. A. M. SIDDIG and M. KEIGHREN, 1980 New mutational variants of Neurospora NADP-specific glutamate dehydrogense. Genetics **95:** 305–316.

KOHLER, S. W., G. S. PROVOST, A. FIECK, P. L. KRETZ, W. D. BULLOCK *et al.*, 1991 Spectra of spontaneous and mutagen-induced mutations in the *lacI* gene in transgenic mice. Proc. Natl. Acad. Sci. USA **88:** 7958–7962.

KOLTIN, Y., J. STAMBERG and R. RONEN, 1975 Meiosis as a source of spontaneous mutations in *Schizophylum commune*. Mutat. Res. **27:** 319–325.

LINDGREN, D., 1975 Sensitivity of premeiotic and meiotic stages to spontaneous and induced mutations in barley and maize. Hereditas **79:** 227–238.

MAGNI, G. E., 1963 The origin of spontaneous mutations during meiosis. Proc. Natl. Acad. Sci. USA **50:** 975–980.

MAGNI, G. E., 1964 Origin and nature of spontaneous mutations in meiotic organisms. J. Cell. Comp. Physiol. **64** (Suppl. I): 165–171.

MAGNI, G. E., and R. C. VON BORSTEL, 1962 Different rates of spontaneous mutation during mitosis and meiosis in yeast. Genetics **47:** 1097–1108.

PASZEWSKY, A., and S. SURZYCKI, 1964 "Selfers" and high mutation rate during meiosis in *Ascobolus immersus*. Nature **204:** 809.

RAMBOSEK, J. A., and J. A. KINSEY, 1983 Fine structure mapping of the AM (GDH) locus of Neurospora. Genetics **105:** 293–307.

RHOUNIM, L., J. L. ROSSIGNOL and G. FAUGERON, 1992 Epimutation of repeated genes in *Ascobolus immersus*. EMBO J. **11:** 4451–4457.

SANGER, F., S. NIKLER and A. R. COULSON, 1977 DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:** 5463–5467.

SCHAAPER, R., B. DANFORTH and B. GLICKMAN, 1986 Mechanisms of spontaneous mutagenesis: an analysis of the spectrum of spontaneous mutation in the *Escherichia coli lacI* gene. J. Mol. Biol. **189:** 273–284.

SELKER, E. U., 1990 Premeiotic instability of repeated sequences in *Neurospora crassa*. Annu. Rev. Genet. **24:** 579–613.

SELKER, E. U., E. B. CAMBARERI, B. C. JENSEN and K. R. HAACK, 1987 Rearrangement of duplicated DNA in specialized cells of Neurospora. Cell **51:** 741–752.

STREISINGER, G., Y. OKADA, J. EMRICH, J. NEWTON, A. TSUGITA *et al.*, 1966 Frameshift mutations and the genetic code. Cold Spring Harbor Symp. Quant. Biol. **31:** 77–84.

WESTERGAARD, M., and H. K. MITCHELL, 1947 Neurospora V. A synthetic medium favoring sexual reproduction. Am. J. Botany **34:** 573–577.

WHELAN, W. L., E. GOCKE and T. R. MANNEY, 1979 The *can1* locus of *Saccharomyces cerevisiae*: fine-structure analysis and forward mutation rates. Genetics **91:** 35–51.

ZOLAN, M. E., and P. J. PUKKILA, 1986 Inheritance of DNA methylation in *Coprinus cinereus*. Mol. Cell. Biol. **6:** 195–200.

Communicating editor: R. H. DAVIS