Spontaneous Mutation at the *mtr* Locus in Neurospora: The Molecular Spectrum in Wild-Type and a Mutator Strain

Davin Dillon[1] **and David Stadler**

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

Manuscript received March 31, 1994
Accepted for publication May 27, 1994

ABSTRACT

Sequence analysis of 34 *mtr* mutations has yielded the first molecular spectrum of spontaneous mutants in *Neurospora crassa*. The great majority of the mutations are base substitutions (48%) or deletions (35%). In addition, sequence analysis of the entire *mtr* region, including the 1472-base pair open reading frame and 1205 base pairs of flanking DNA, was performed in both the Oak Ridge and Mauriceville strains of Neurospora, which are known to be divergent at the DNA level. Sixteen sequence differences between these two strains have been found in the *mtr* region, with 13 of these in DNA flanking the open reading frame. The differences consisted of base substitutions and small frameshifts at monotonic runs. This set of sequence differences has allowed a comparison of mutations in unselected DNA to those mutations that produce a phenotypic signal. We have isolated a mutator strain (*mut-1*) of Neurospora in which the spontaneous mutation rate at various loci is as much as 80-fold higher than in the non-mutator (wild type). Twenty-one *mtr* mutations in the mutator background have been sequenced and compared to the non-mutator spectrum, revealing a striking increase in −1 frameshift mutations. These frameshifts occur exclusively within or adjacent to monotonic runs and can be explained by small slippage events during DNA replication. This argues for a role of the *mut-1* gene in this process.

THE spectrum of spontaneous mutation at the sequence level has been determined in several different organisms at various loci, including extensive work involving the *lacI* gene in *Escherichia coli* (SCHAAPER *et al.* 1986; SCHAAPER and DUNN 1991) and the *SUP4-0* tRNA gene in *Saccharomyces cerevisiae* (GIROUX *et al.* 1988) This level of analysis has been facilitated by the existence of stable plasmids in these organisms. In *Neurospora crassa*, stable episomes have not been found, making this type of analysis less feasible until recent advances in polymerase chain reaction (PCR) amplification.

We report here the sequence analysis of 34 mutations at the *mtr* locus, the structural gene for the neutral amino acid permease (PALL 1969). The spectrum of spontaneous mutational types at the DNA level in *N. crassa* was desired for several reasons. One objective was the determination of the "baseline" spectrum of spontaneous mutation at the *mtr* locus, to be used for comparison to the spectra generated by mutant strains with altered mutational specificity. We have isolated one such "mutator" strain, and this comparison of mutation spectra at *mtr* has given us some insight into the role of the mutator gene in DNA repair. It is also of interest to compare the baseline (somatic) spectrum to the spectrum of germinal mutations (M. WATTERS and D. STADLER, unpublished). A second objective was to study possible mechanisms involved in the formation of various mutational types, including deletions and insertions.

The *mtr* locus was chosen as a mutational target for two reasons. First, there existed a direct selection for both forward mutations and revertants, facilitating the collection and initial characterization of the mutants (STADLER *et al.* 1991). Second, the gene had previously been cloned and sequenced (STUART *et al.* 1988; KOO and STUART 1991) which allowed a more detailed molecular study of the mutants.

The optimal mutational target is one that will both allow and report the occurrence of all mutational events, resulting in an unbiased spectrum. The *mtr* gene is likely to fall short of this expectation, as is the case of most if not all mutational targets. Large deletions that include essential genes will not be recovered in a haploid system. For this reason we have used a heterokaryon (two haploid strains sharing a common cytoplasm), allowing the recovery of this mutational class (STADLER *et al.* 1991). Another source of bias is the undetected base substitutions that are silent at the amino acid level or occur in an insensitive region of the protein. To test the effects of bias on our mutational target, another approach was employed in which bias should be reduced, if not eliminated. This approach utilized a different strain of *N. crassa* known as Mauriceville. This strain is known to diverge at the sequence level from Oak Ridge, the usual lab strain (METZENBERG *et al.* 1985). We reasoned that the sequence comparison of these two strains for the regions flanking the *mtr* open reading frame (ORF) would yield a set of differences representing a spectrum

[1] Present address: Infectious Disease Research Institute, 41 Dravus Street, Suite 75, Seattle, Washington 98109.

of mutation that formed under little selective influence. This would then allow the comparison of this spectrum to the *mtr* spectrum, which depended upon display of variant phenotypes.

We also report the development of a system to detect mutator strains and the isolation and characterization of one such strain, including the molecular spectrum of *mtr* mutations produced. The isolation and characterization of a number of these strains in prokaryotes (TREFFERS *et al.* 1954; LIBERFARB and BRYSON 1970; DEGNEN and COX 1974; NGHIEM *et al.* 1988; MICHAELS *et al.* 1990) has led to a detailed understanding of systems involved in controlling or limiting spontaneous mutation. The fact that many of these mutator strains have no secondary phenotype, such as mutagen sensitivity, demonstrates the necessity of detecting these genes by screening directly for mutator activity.

Recently, similar approaches in eukaryotic systems have been successful. Progress in *S. cerevisiae* includes the characterization of the mutator effect in radiation-sensitive strains *rad1*, *rad18* and *rad52* (KUNZ *et al.* 1989, 1990, 1991), and the isolation and characterization of PMS1 (post-meiotic segregation) (WILLIAMSON *et al.* 1985; W. KRAMER *et al.* 1989) and two MSH (*mutS* homolog) genes (REENAN and KOLODNER 1992a,b), presumptive components of a post-replicative mismatch repair system. Recent progress in mammalian systems includes the molecular characterization of the mutator phenotype in Chinese hamster ovary cells with imbalances in deoxyribonucleoside triphosphate pools (PHEAR and MEUTH 1989), and in Werner syndrome, an autosomal-recessive human disorder (FUKUCHI *et al.* 1989).

MATERIALS AND METHODS

Strains: Two heterokaryons were used for *mtr* mutant isolation. One component in both heterokaryons was *pdx-1* (requires pyridoxin), *col-4* (colonial morphology), *mtr* (resistant to *p*-fluorophenylalanine), *arg-2* (requires arginine), *pan-2* (requires pantothenate) *A*. The second component in each heterokaryon had the following markers in common: *cot-1* (colonial at elevated temperature), *ylo-1* (yellow conidia), *trp-2* (requires tryptophan or anthranilate) *A*. They differed, however, in strain background with one being fully Oak Ridge (O), while the other was primarily Oak Ridge yet was Mauriceville (M) for linkage group IVR, including the region containing the *mtr* gene (STADLER *et al.* 1991). The Mauriceville strain is known to contain numerous sequence differences from Oak Ridge based on restriction fragment length polymorphism data (METZENBERG *et al.* 1985).

The following strains were used for deletion mapping: OR30 (*cys-15 mtr30 col-4 trp-2 pan-2 ylo-1 a*), OR1X (*mtr1X col-4 trp-2 ylo-1 a*), OR59 (*mtr59 col-4 trp-2 ylo-1 a*), OR1564 (*pdx-1 mtr1564 trp-2 a*).

Media: Media were made from Vogel's minimal (DAVIS and DESERRES 1970) with various supplements. Media in plates contained 1.5% sorbose and 0.1% dextrose; media in tubes contained 2% sucrose. Crossing medium (WESTERGAARD and MITCHELL 1947) was used with a Whatman No. 1 filter substituting as the carbon source. Supplements used were pyridoxin (Pdx), arginine (Arg), *p*-fluorophenylalanine (Fpa), cysteine (Cys), anthranilic acid (Ant), tryptophan (Trp), pantothenate (Pan), and adenine (Ade). Concentrations of Arg, Cys, Ant, Trp, and Ade were 50 mg/liter unless otherwise noted. Fpa concentration was 30 mg/liter in *mtr* mutant isolation and 15 mg/liter for all other uses. Concentrations of Pdx and Pan were 5 mg/liter.

Supplementation of the *trp-2* mutants was accomplished by the use of either Trp or Ant. The uptake of Ant is independent of the *mtr* (neutral amino acid permease) system. *trp-2* strains that carry either the *mtr* or the mtr^+ allele can grow on media with Ant. Trp is taken up by both the neutral and general amino acid uptake systems, but by using media supplemented with one-fifth the usual amount of Trp (10 mg/liter) and twice the usual amount of Arg (100 mg/liter) the general uptake system can be blocked, resulting in the dependence of Trp uptake solely on the *mtr* uptake system. This ratio, referred to as Trp + Arg, was used when testing for the mtr^+ phenotype in reversion tests and deletion mapping.

Isolation of *mtr* mutants: Individual cultures of the two heterokaryons were grown from isolated colonies on Pdx + Arg medium. Conidial suspensions in water were counted and 2×10^7 conidia were plated in medium with Pdx + Arg + Fpa. Because the medium lacked Trp and Pan, only the heterokaryotic conidia could grow. In addition, the presence of the inhibitor Fpa prevented growth of all conidia with a functional mtr^+ gene, due to the recessive behavior of the *mtr* mutation. Only those heterokaryons that had an *mtr* mutation in the *cot-1* component were able to grow. One resistant colony from each plate was picked and grown in medium with Pdx + Arg. All mutants were purified by re-streaking twice on medium with Pdx + Arg + Fpa. The *cot-1 mtr trp-2 ylo-1 A* homokaryons were isolated by streaking conidial suspensions on medium with Fpa + Ant at elevated temperature (33°), and picking colonies showing the recessive *cot-1* morphology (restricted radial growth).

Spontaneous *mtr* mutants in the mutator strain were isolated from a simple haploid, homokaryotic culture. Individual cultures of the *mut-1 trp-2 ylo-1 A* strain were grown up from isolated colonies on medium with Ant. Conidial suspensions were counted and 4×10^6 plated in medium with Fpa and Ant. One resistant colony from each culture was selected and purified by re-streaking twice on medium with Fpa and Ant.

Induced reversion tests: Conidial suspensions in water were made from fresh cultures and counted. Conidia (5×10^6) were plated in 2.5 ml of melted 1% agar (50°) over medium with Trp + Arg. Mutagen exposure was either 800 J UV (100 J/min for 8 min) before plating, or the addition of 70 μl 6-chloro-9-[3-([2-chloroethyl]-ethylamino)propylamino]-2-methoxyacridine (commonly known as ICR 170) at a concentration of 1 mg/ml to the agar overlay. Plates were scored for colony number after 2 days. Samples were judged unstable if they showed more than five colonies on the control plate (no mutagen). Samples were judged revertible by UV or ICR 170 if they showed more than five colonies on their respective plates over background. The non-revertible *trp-2* allele (75001) was present in these strains, so *trp-2* reversion was not a complicating factor.

Deletion mapping: A set of four *mtr* deletion strains were used to localize *mtr* mutations. The *mtr* mutation in deletion strain OR1X was isolated after X-ray treatment, in deletion strain OR1564 after UV treatment, and the *mtr* mutations in deletion strains OR30 and OR59 were spontaneous. The deletion endpoints of the *mtr* mutations *mtr 30*, *mtr 1X*, and *mtr 59* have been sequenced and specify deletions of lengths 93, 160, and 312 base pairs (bp), respectively. The deletion endpoints of deletion *mtr1564* have not been sequenced but the

5′ endpoint has been localized to within 40 bp by Southern analysis. The four deletion strains all contain the same non-revertible *trp-2* allele as in the *mtr* mutants isolated, so *trp-2* recombination or reversion was not a complicating factor in deletion mapping crosses or reversion tests. Crosses were made with the deletion strains as the maternal component except those involving OR1564, which was used as the paternal component. Crosses were done in inverted glass Petri dishes with a 9-cm Whatman No. 1 filter as a carbon source. Plates were incubated at 25°, for 4–6 weeks. General recoveries ranged from 5×10^5 to 5×10^6 viable ascospores. Ascospores were harvested as described by KINSEY *et al.* (1980) and plated in 2.5 ml melted 1% agar, overlaid on medium with Trp + Arg (Pan, Cys and Pdx added in appropriate crosses). Viability was tested after one day by counting germinated ascospores. Colonies were scored after 2 days. All mutants were crossed to the set of deletion strains except the unstable and ambiguous mutants, since they spontaneously produced colonies on the counter-selective media, and could not be reliably mapped.

Amber suppression: Suppression of the amber mutation was accomplished by crossing the strain *col-4 ssu ad-2 trp-2*^{s} (*s* = suppressible allele) *a* to the strain *mtrM2 ad-2 trp-2 A*. The suppressor (*ssu*) is presumed to be an allele of amber suppressor *ssu-1* (BURNS *et al.* 1984), based on suppression behavior (STADLER *et al.* 1987) and mapping information (D. STADLER, unpublished). The *ad-2* allele present in both parents was suppressible, so the segregation pattern of the suppressor in the progeny was signalled by adenine independence. The *trp-2* allele in the second parent was not suppressible. The morphological mutation *col-4* is tightly linked to *mtr*, so wild-type morphology signalled the presence of *mtr* in the progeny. Isolates that contained the non-suppressible *trp-2* and *mtr* genes (non-colonial), with or without the suppressor, were tested for their ability to grow on the counter-selective medium (one-fifth Trp and twice the normal Arg and Ade), indicating restoration of *mtr*$^+$ activity.

Molecular analysis of *mtr* mutants: DNA was prepared from lysed spheroplasts by the method of ZOLAN and PUKKILA (1986). Restriction enzymes were from BRL or Boehringer Mannheim and used according to manufacturer's specifications. Southern transfers were made to nitrocellulose (Schleicher & Schuell). DNA probes were labeled by nick translation with ^{32}P using the *mtr* subclone pCVN2.9 (STUART *et al.* 1988).

Various portions of the *mtr* region were amplified by PCR amplification using a variety of 20-mer oligonucleotides. The program used in all PCR amplifications consisted of one 10-min cycle at 94°, followed by 25 cycles consisting of one minute at 95°, 2 min at 55°, and 3 min at 72°. One final cycle of 10 min at 72° was included. Amplified DNA was treated with T7 gene 6 exonuclease using the method of U.S. Biochemical Corp. Resulting single-stranded DNA was sequenced using the chain-termination method (SANGER *et al.* 1977) using the Sequenase kit (U.S. Biochemical Corp.), according to the manufacturer's specifications. For the regions including the three discrepancies from the published sequence (KOO and STUART 1991), both strands were sequenced, and dITP was used to clarify ambiguous regions.

Mutator screen: The genotype of the strain used in the genetic screen to detect mutations was *sn* (snowflake) *cr* (crisp) *trp-2* (requires tryptophan) *ad-2* (requires adenine) *al-2* (albino conidia) *cyh-1* (cycloheximide resistant) *A*. The double mutant *sn cr* confers a morphology amenable to replica plating, with shortened aerial hyphae and a colonial (non-spreading) margin.

Mutagenesis involved either X-ray exposure (108 rad/sec for 2–3 min–approximately 50% lethality) or *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (NG). Mutagenesis by NG was performed by dissolving NG in 0.2 M Na-acetate buffer, pH 5.4, at a concentration of 1 mg/ml. A conidial suspension in water (2×10^6 conidia/ml) was made and a portion added to an equal volume of NG solution. The suspension was incubated in a 30° shaker bath, and aliquots were withdrawn at 10, 30, and 50 min. All aliquots were filtered using Whatman 540 filter paper and rinsed three times with 0.1 M Na-acetate buffer, pH 5.4. Conidia were recounted after being recovered by vortexing in a vial with 2 ml water. Mutagenized conidia were plated at a density of 100 cells/plate on medium supplemented with 2% sucrose + Ant + Ade (non-selective), grown three to 5 days at 33°, and transferred to room temperature to conidiate. Colonies were then replica plated to selective media to distinguish three different kinds of mutants.

The loci used to monitor mutation levels were *mtr*, *mep* and *trp-2*. The *mtr* gene encodes the permease responsible for the uptake of neutral amino acids, and forward mutations in this gene can be readily revealed by demanding resistance to an amino acid analog such as Fpa. Mutations at the *mep* loci (PENDYALA *et al.* 1979) confer resistance to 6-methylpurine (MeP). The last signal monitored was reversion of *trp-2*^{s}, allowing growth on medium lacking tryptophan.

Master-plate colonies that showed growth on at least two of these selective plates were considered mutator suspects and were retested for increased frequency of mutation at the *mtr* locus. Conidial suspensions were counted and 5×10^6 conidia plated in medium with Fpa + Ant + Ade. Strains that gave threefold or greater increase over wild type were retested using three to five subisolates.

Calculation of mutator effect of *mut-1*: The strains used to calculate mutation rates were *pdx-1* (requires pyridoxin) *trp-2 A*, with and without *mut-1*. In both strains the revertible *trp-2*^{s} allele was present. The two mutational events monitored were forward mutation at *mtr* and *trp-2* reversion. For each experiment nine to 11 separate isolates were grown up in test tubes or flasks. All conidia were harvested, filtered and counted, and suspensions were plated in medium with Fpa + Pdx + Ant to score *mtr* colonies and medium with Pdx to score *trp-2* reversion. Plates were incubated at 33° for 3 days. Conidial viability was tested by plating on nonselective medium. Median frequency of mutants/conidium was calculated, correcting for viability. For the measurement of mutation to dominant *trp*$^+$, these values were converted to mutants/nucleus by dividing by 2.2, the average number of nuclei/conidium. For the measurement of mutation to recessive *mtr*, these values were multiplied by a correction factor of five, because only the one-fifth of the conidia that are uninucleate are expected to express the recessive mutation. In both the *mtr* and *trp-2* calculation the final population size (N_2) was estimated by multiplying the average number of recovered conidia per culture by 2.2 nuclei per conidium, and multiplying by four, based on the estimate that only one-fourth of the nuclei are present in the conidia, the remainder present in the unrecovered filamentous tissue. The mutation rates were calculated using the method of mutant accumulation described by DRAKE (1991).

Mapping of *mut-1*: Linkage of *mut-1* to *cot-1* (on linkage group IVR) was initially discovered during outcrossing of the strain. Further localization was accomplished by crossing the mutator to the following multiply marked strains: (i) *pyr-3* (requires uridine) *cot-1* (colonial morphology), *al-2 A*, (ii) *ad-6* (requires adenine) *cot-1 al-2 A*, (iii) *trp-4* (requires tryptophan) *pan-1* (requires pantothenate) *a*, (iv) *his-5* (requires histidine) *pyr-3 A*. All of these loci except *al-2* and mating type (*A*, *a*) are on linkage group IVR. Progeny with parental and recombinant orientation of linked markers were scored for the presence of *mut-1* by testing for mutator activity on the *mtr* locus. Mutator activity was determined by plating

suspensions of 5×10^6 conidia on medium with Fpa, with appropriate supplements. Generally, non-mutator strains give zero to five colonies in this test, while strains containing *mut-1* give 40–200 colonies.

Mutagen sensitivity and mutability: The two strains used in mutagen sensitivity and mutability tests were *cot-1 trp-2 ylo-1* (yellow conidia) *A* and *trp-2 ylo-1 mut-1 A*. The non-mutator strain contained the temperature-sensitive morphological marker *cot-1*, allowing separate survival counts of the two strains in experiments using mixed cultures (which assured equal exposure of the two strains in the UV and X-ray mutagenesis). Conidia from fresh cultures of each strain were suspended, filtered and counted. Mixtures were exposed to UV (100 J/min) or X-ray (108 rad/sec) with aliquots removed at various time points. Suspensions were plated on medium with Fpa + Ant to measure *mtr* mutation, and dilutions plated on Ant to measure viablity. Plates were scored after 1–3 days at 33°. Sensitivity to methyl methanesulfonate (MMS) was measured qualitatively by testing drops of the conidial suspensions on plates with 0.15% MMS + Ant.

Dominance test of *mut-1*: Progeny from the cross *col-4 ad-2 a* to *trp-2 ylo-1 mut-1 A* were isolated and tested for markers and for heterokaryon compatibility. Progeny of the following genotypes were used in the dominance studies: *col-4 trp-2 A*, *trp-2 ylo-1 mut-1 A*, *ad-2 A* and *ad-2 mut-1 A*. Heterokaryons constituted of strains with different auxotrophic markers were detected by demanding growth in the absence of both supplements. Nuclear ratios were calculated by determining frequencies of homokaryons, counting nuclear numbers in stained conidia, and applying appropriate calculations (STADLER and CRANE 1979). The mean *mtr* mutant frequency was determined using five isolates for each strain. Plating was done as described above.

RESULTS

Isolation of *mtr* mutants: Seventy-five independent *mtr* mutants (from 117 independent cultures) were isolated from two heterokaryons in which the first component was mutant at the *mtr* locus and flanked by two nutritional markers, *pdx-1* and *arg-2*. The second component in each of the two heterokaryons contained the same markers (*cot-1 trp-2 ylo A*) in different backgrounds, one being entirely Oak Ridge (O), while the other was partly Mauriceville (M), including the *mtr* region (see MATERIALS AND METHODS). Mutations at the *mtr* gene in the second components were readily isolated by demanding resistance to the amino acid analog, Fpa. Heterokaryons without a mutation at the *mtr* gene in the second component were sensitive to Fpa, due to the recessive nature of the *mtr* mutants. Pyridoxin and arginine were also present in the selection medium, so that large deletions that included these genes could be recovered.

Viability of the homokaryons carrying the new *mtr* mutations was tested by using the recessive temperature-sensitive *cot-1* mutation present in the second component, which causes restricted radial growth at the restrictive temperature. Sixty-six of the isolates produced this colony type, indicating homokaryon viability, and were used for subsequent analysis. The other nine *mtr* mutants resulted from large deletions extending into essential genes in the flanking regions. This was demonstrated at the molecular level in five of the strains in the Mauriceville background by the loss of a Mauriceville specific restriction fragment in the *mtr* region (STADLER *et al.* 1991).

Reversion analysis of *mtr* mutants: Reversion tests of the 66 homokaryon-viable mutants were performed using UV and ICR 170, a frameshift mutagen. Tentative mutational classification was based on the results of the reversion tests. Mutants that were reverted solely by UV were classified as carrying base substitution mutations. Those that were reverted by ICR 170 were classified as carrying frameshifts. Mutants not revertible by either mutagen were classified as deletions. Mutants exhibiting instability (spontaneous revertant frequencies of 10^{-4} to 10^{-6}) were classified as tandem duplications.

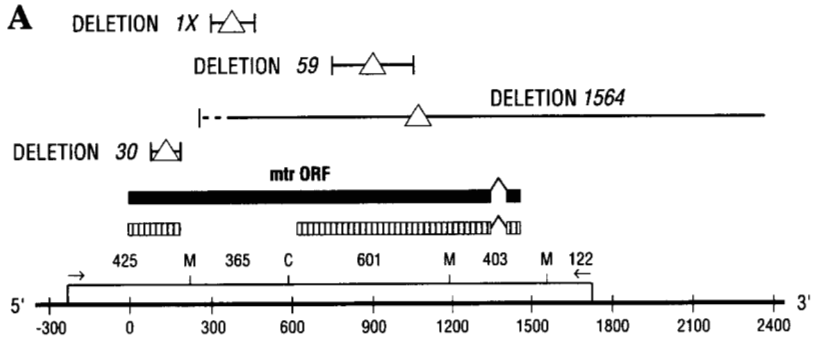
Ambiguous mutants were able to grow on both selective media (Fpa + Ant, Trp + Arg). Although reversion tests were not possible with these mutants, they are presumed to be base substitution mutations that only partially impair the permease. Sequence analysis of two ambiguous mutants in another strain was consistent with this presumption; both carried base substitutions resulting in a missense change.

Three mutants exhibited extreme instability (cultures contained up to 80% revertants). The *mtr* region of these strains showed apparent hypermethylation based on finding methylation ladders when genomic DNA was cut with methylation-sensitive restriction enzymes and probed with the subclone containing the *mtr* gene (data not shown). Neither the *mtr* phenotype nor methylation were retained through a cross. The basis of this is not understood.

Deletion mapping of *mtr* mutants: To facilitate sequence analysis of the *mtr* mutants, the collection of mutants was crossed to strains carrying a set of well characterized deletions (Figure 1A) to further localize the mutants. A cross that yields no mtr^+ recombinants indicates that the lesion in the unmapped mutant is within (or very near) the region covered by the deletion. Fifty-five of the 58 mutations tested were successfully localized.

The efficacy of the deletion mapping can be judged by the results. Sequence analysis based on map location was attempted on 35 *mtr* mutant strains. In 31 mutant strains a mutation was observed within the deletion or within 50 bp of the deletion endpoints. In four of the mutant strains, complete analysis of the region within or near (within 50 bp) the deletion revealed no changes. The deletion mapping accuracy is therefore 89% (31/35).

Sequencing of *mtr* mutants: The 31 sequenced mutations were located in two regions, those mapping to the 5′ portion of the gene, including the *mtr-30* deletion and the *mtr-1X* deletion, and those mapping to the region containing the *mtr-59* deletion (Figure 1). The



B

Number of *mtr* mutants that map to the different regions.

Strain	*1X*	*30*	*1564*	*59*	5' of *1X*
mut-1$^+$	17	3	17	15	3
mut-1	0	1	16	7	4

FIGURE 1.—Deletion mapping of the *mtr* mutants. (A) Location of the four deletions and the proposed open reading frames. The two smaller open reading frames (vertical lines) have been previously proposed by KOO and STUART (1991). The newly proposed larger single open reading frame is denoted by the solid bar. (B) Distribution of *mtr* mutants in the *mut-1* and non-mutator backgrounds. Mutants in the *1564* category are not within *1X* or *59*. Mutants in the 5′ of *1X* and *1564* category are not within *30*. The location and size of the amplified DNA fragment used in PCR digests is shown. Included are the cut sites for the restriction enzymes *Cla*I and *Msp*I and the size fragments produced (in base pairs).

endpoints of these deletions have all been sequenced and the deletions are of lengths 93, 160 and 312 bp, respectively. A spectrum of the 31 mutants segregated by class is shown in Table 1. This represents a random sample of *mtr* mutants based solely on location determined by deletion mapping. Base substitutions and deletions accounted for 26 of the 31 mutants. Four of the remaining five mutants were frameshifts (frameshifts were defined as mutations involving the loss or gain of 1 or 2 bp).

In addition, the three mutations believed to be tandem duplications based on reversion test results were also sequenced. Their location was determined by size differences of PCR amplified fragments within the *mtr* ORF. All *mtr* mutations were sequenced directly from PCR-amplified DNA (see MATERIALS AND METHODS).

A breakdown of the different mutants is shown in Table 2. The position given for all sequenced mutations is based on the distance from the predicted translational start site (see APPENDIX). The reversion behavior of the mutants is also included. Two areas may be hypermutable for base substitutions (383–385, 464–466) and there is one deletion hotspot (217–232), producing three identical mutants. The sequence at the endpoints of all 11 deletions contain significant direct repeats as do two of the three tandem duplications.

Mauriceville *vs.* Oak Ridge: The entire *mtr* regions of the Oak Ridge and Mauriceville strains were sequenced including both 5′ and 3′ regions, a total of 2677 bp. These strains are known to be divergent at the sequence level (METZENBERG *et al.* 1985). Sequence analysis of the region yielded 16 sequence differences between the two strains (Table 3). Only three of these differences were within the 1472-bp *mtr* ORF, and all were silent third position changes at the amino acid level. The remaining 13 differences were located in the 1205 bp of 5′- and 3′-flanking DNA. The higher frequency of divergence

TABLE 1

Molecular spectrum of *mtr* mutants

Mutational class	Number	Totals
A. Spectrum of *mtr* mutants in wild-type background		
Base substitutions		15 (48)[a]
Transitions	9	
Transversions	5	
Complex	1	
Deletions		11 (35)
Frameshifts		4 (13)
(+1)	1	
(+2)	2	
(−2)	1	
Insertions		1 (3)
B. Spectrum of *mtr* mutants in *mut-1* background		
Base substitutions		6 (29)
Transitions	6	
Transversions	0	
Complex	0	
Deletions		1 (5)
Frameshifts		13 (62)
(+1)	1	
(+2)	0	
(−1)	12	
(−2)	0	
Insertions		1 (5)
Tandem duplication	1	
Non-tandem insertions	0	

[a] Numbers in parentheses are percentages.

observed in the flanking regions compared to the ORF is significant ($P < 0.005$). The spectrum of differences in the flanking regions consists of base substitutions (9/13), frameshifts (3/13) and a small insertion/deletion (1/13). The categories of insertions and deletions cannot be separated since the founder strain is not known.

Isolation of mutator strains: The screen developed to isolate mutator strains was based on increased frequency of spontaneous mutation at two distinct loci. This approach has been successfully employed elsewhere (DEGNEN and COX 1974) to reduce the number of false positive signals.

TABLE 2

Sequence of mtr mutations in wild-type background

Mutant	Position	Change	Number	Reversion
A. Base substitutions				
M-54	158, 160	TCG → CCT	1	None
M-25	313	G → A	1	UV
M-2, 20	383	G → A	2	UV
M-29	384	G → A	1	UV
O-39	385	G → T	1	UV
M-26	464	T → G	1	None
M-34, O-23	466	T → C	2	UV, none
M-5	755	C → T	1	UV
M-58	762	C → A	1	None
M-21	824	C → A	1	UV
M-19	824	C → T	1	UV
M-30	988	G → C	1	UV
M-39	1038	G → A	1	UV

Mutant	Position	Size (bp)	Repeated bases[a]	Number	Reversion
B. Deletions					
M-10, 36, 53	217–232	16	TCGCTACCC	3	None
M-41	283–301	19	CTCA	1	None
O-32	366–410	45	TGT	1	UV
O-36	407–410	4	TGTT	1	UV
M-12	454–468	15	GGCACCATCA	1	None
M-17	666–684	19	TGGTCT(C/T)GCTG	1	UV, ICR 170
O-27	979–1146	168	TTC(A/T)TCTCTG	1	UV
M-32	1002–1004	3	TGT	1	None
M-15	1082–1091	10	TGGTTT(T/G)G	1	UV

Mutant	Position	Mutational type	Sequence[b]	Repeat[c]	Reversion
C. Frameshifts and insertions					
M-38	265–266	−2-bp frameshift	TCTCTCTG		None
M-71	383–386	+2-bp frameshift	TGGGGGGA		ICR 170
M-18	531–548	18-bp, tandem duplication		CATTC	Unstable
M-43	702–703	+2-bp frameshift	AGGGGA		ICR-170
M-35	876–877	3-bp insert	GGCTGCGTC		UV
O-10	1056–1057	+1-bp frameshift	CGCTTAT		UV
M-52	1157–1224	68-bp tandem duplication		None	Unstable
M-37	1290–1352	63-bp tandem duplication		ACAT	Unstable

[a] Sequence of the direct repeats at the endpoints of the deletions.
[b] Underlined sequence represents added or deleted base pairs.
[c] Sequence of the direct repeats at the endpoints of the insertions.

The basic strategy involved the use of a *sn, cr* (snowflake, crisp) strain, suitable for replica plating. A conidial suspension was mutagenized with either NG or X-ray and plated nonselectively at concentrations that allowed the formation of isolated colonies. After these colonies conidiated, they were replica-plated to test for the appearance of *mtr* mutants and for either *mep* or trp^+ mutants.

Isolates that showed growth on both selective media were then retested for increased mutation at the *mtr* locus. The *mtr* mutant frequency was calculated for the mutator candidates, and an arbitrary level of mutation (threefold increase over wild type) was used to designate strains as mutator mutants. Of the 96 original suspect mutators, five showed at least a threefold effect at *mtr* upon retesting. Of the five, two had reasonably strong mutator effects at *mtr* (a minimum 10-fold increase over wild type). One of these remaining two mutator strains exhibited variable growth, with changing morphologies and a tendency to become aconidial, making the strain difficult to study. Because it was not possible to separate this characteristic from the mutator phenotype, further analysis was confined to the remaining mutator strain, isolated by X-ray mutagenesis. The locus responsible for the mutator activity was designated *mut-1*.

Quantifying the mutator effect of *mut-1*: Two systems were used to study the effect of *mut-1* on mutation frequency. These were forward mutation at the *mtr* locus and reversion of the $trp\text{-}2^s$ allele. The results of these tests (Table 4) indicate *mut-1* caused an 80-fold increase in the mutation rate (a 140-fold increase in mutant frequency) at the *mtr* locus and a 10-fold increase in the rate of reversion of the $trp\text{-}2^s$ mutation (a 14-fold increase in mutant frequency).

Mapping of *mut-1*: Initial mapping crosses indicated linkage (approximately 11 m.u.) to cot-1 (colonial at restrictive temperature) on the right arm of chromosome *IV*. Successive crosses demonstrated that *mut-1* was

TABLE 3

Sequence differences between Oak Ridge and Mauriceville

Position	Mutational class	Oak Ridge[a]	Mauriceville[a]
−70	Base substitution	CCAAA	CCCAA
−10	Base substitution	TTTAC	TTCAC
57	Base substitution	CCAGA	CCGGA
222	Base substitution	CTTCC	CTACC
273	Base substitution	GTCGG	GTTGG
1714–1718	Frameshift	TTTTT	TTTT
1730–1732	Frameshift	GTTTG	GTTTTG
1756–1765	Frameshift	TTTTTTTTTT	TTTTTTTT
1985	Base substitution	AGAGA	AGGGA
2059	Base substitution	AACAT	AATAT
2085	Base substitution	AACAC	AAAAC
2123	Base substitution	TAATTT	TATTTT
2131	Base substitution	ATCCT	ATTCT
2136	Base substitution	TGACT	TGAAT
2138	Base substitution	TCCCA	TTCCA
2151–2155	Insertion/Deletion	GCTTTTTTTTTTTTTTTTT	GTTTTTTTTTTTT

[a] Sequence difference is underlined.

TABLE 4

Quantitative mutator effect of *mut-1*

Strain background	Mutation rates and mutant frequencies	
	Forward mutation at *mtr* locus	Reversion of *trp-2*
trp-2, pdx-1, mut-1$^+$	2.5×10^{-7} (2.8×10^{-7})	1.1×10^{-9} (4.7×10^{-9}) [a]
trp-2, pdx-1, mut-1	2.0×10^{-5} (3.9×10^{-5})	1.1×10^{-8} (6.4×10^{-8})

Median mutant frequencies (number of mutants/viable conidium) are shown in parentheses and were derived from 9–11 isolates, plating 0.5–1 $\times 10^7$ for *mtr* mutation and 1–2 $\times 10^8$ for *trp-2* reversion. These values were used to calculate the mutation rates (refer to MATERIALS AND METHODS).

[a] Mutant frequency calculated using the mean number of revertants.

tightly linked (either proximal or distal) to *trp-4* (<3 m.u.).

Testing of *mut-1* for mutagen sensitivity and mutability: Strains containing *mut-1* were tested for sensitivity to UV, X-ray and MMS. Both *mut-1* and the non-mutator control strains contained similar markers, but only the non-mutator strain contained *cot-1*. This allowed surviving colonies to be distinguished and counted after the strains were mixed together and subjected to UV and X-ray mutagenesis. No difference in sensitivity to UV, X-ray or MMS was observed (data not shown).

These strains were also used to test for induced mutation. The mutagens tested were UV and X-ray, and forward mutation at the *mtr* locus was monitored. No difference in mutability by either mutagen was observed between the *mut-1* strain and non-mutator strain; both produced similar numbers of induced mutants (data not shown).

Dominance test of *mut-1*: Several heterokaryons were constructed with 1:1 nuclear ratios in which neither, one or both components contained *mut-1*. These were tested for mutator activity at the *mtr* locus. Heterokaryons with the *mut-1* mutation in one of the two components gave similar *mtr* mutant frequencies to the non-mutator strain. Only strains in which both components contained the *mut-1* mutation gave significantly elevated frequencies of *mtr* mutants (approximately 100-fold increase in mutant frequency), arguing that the mutator activity of *mut-1* is recessive (data not shown).

Spectrum of *mtr* mutants in a *mut-1* strain: An informative characteristic of a mutator strain is the type(s) of mutation produced by the strain; the altered spectrum of mutation can be used to infer the types of mutational change normally corrected by the wild-type allele of the mutator gene. For our spectrum, the *mtr* locus was used as the mutational target.

A collection of 41 independent *mtr* mutants (from platings of 46 independent cultures) was isolated in the strain *mut-1 trp-2 ylo-1 A*. Mutants were selected by plating in medium with Fpa + Ant. Reversion tests were performed on all *mtr* mutants, using UV and ICR 170.

Eleven of the 41 mutants were scored as unstable. The instability could be due to the instability of the mutation itself or due to the effects of the mutator. To discriminate between these two possibilities, the 11 unstable *mtr* mutants were separated from the mutator (see MATERIALS AND METHODS) and the reversion tests repeated. Of the eleven unstable mutants, only two remained unstable in the absence of *mut-1* and remained classified as tandem

TABLE 5

Sequence of *mtr* mutations in the mut-1 background

Position	Change	Number	Sequence[a]	Reversion
A. Base substitutions				
165	G → A	1	CTG*G* AAACGT	None
466	T → C	1	ATG*T* GGGGCA	None
557	C → T	1	TCCTC*C* CAGTT	UV
1125	G → A	1	CTG*G* GTTATTGCTGAG	UV
1166	C → T	1	TGTTGG*C* CATCT	Ambiguous
1328	G → A	1	TATTG*G* TACCTA	Ambiguous

Position	Mutational type	Number	Sequence[b]	Repeat[c]	Reversion
B. Frameshifts, insertions and deletions					
190–192	3-bp deletion	1	GTCGTCCTCATCGTC		UV
666–681	16-bp tandem duplication	1		TGGT	Unstable
803–807	−C	2	ACCCCCT		ICR 170
803–807	+C	1	ACCCCCT		UV
851	−T	1	GAAATC		UV
1067–1070	−C	6	ACCCCA		ICR 170
1194–1197	−T	3	GTTTTA		None

[a] Wild-type sequence shown with base affected in italics. Sequences that could be involved in a slippage-mispairing mechanism are underlined.
[b] Wild-type sequence shown with base(s) affected underlined.
[c] Sequence of the direct repeats at the endpoints of the insertion.

duplications. The other nine were reclassified depending on their behavior in the absence of the *mut-1* allele.

Twenty-eight of the *mtr* mutations in the *mut-1* background were successfully localized by deletion mapping (Figure 1). The distribution of these mutations is compared to the distribution of *mtr* mutations in the non-mutator strain (Figure 1). A significant difference was observed, with a much greater fraction of mutants in the non-mutator background mapping to deletion *1X*.

Molecular analysis of *mtr* mutants from *mut-1*: PCR amplification of the *mtr* region was successfully performed on 39 of the 41 *mtr* mutants. The remaining two mutants were not tested due to inability to make a DNA preparation for one strain and failure of PCR to amplify the *mtr* region in the other. This procedure consisted of PCR amplification of the *mtr* region for each of these mutants and the subsequent digestion by a combination of *Cla*I and *Msp*I (Figure 1). The digested DNA was visualized by staining agarose gels with ethidium bromide. Only two of the 39 mutants revealed alterations, one containing a small insertion and the other a small deletion in the 601-bp fragment. The mutant with the small insertion was one of the two deemed unstable in reversion tests and on later sequencing proved to be a 16-bp tandem duplication. The mutant with the small deletion was scored as ICR-revertible and was not sequenced.

Sequencing was attempted on an unbiased subset of the mutants (30/41). Deletion mapping information on these mutants was used, when available, to direct sequencing efforts. In 21 of the 30 mutants a sequence change was found. The position given for each mutant is based on the distance from the putative translational start site. The sequences of 6 base substitutions were determined, all of which were transitions. Three of them can be templated by mispairing with neighboring sequences during DNA replication (Table 5A). Thirteen frameshifts–defined here as the loss or gain of 1 or 2 bp–were sequenced, 12 of which were −1 frameshifts (Table 5B). The frameshifts occurred at only four sites within the *mtr* gene, with one extreme hotspot accounting for six of the mutations. Twelve of the 13 frameshifts occurred within monotonic runs of at least four G:C or A:T residues, with the remaining frameshift occurring adjacent to a run of three A:T residues.

A direct comparison of the sequenced *mtr* spectrum in the *mut-1* background to that sequenced in the non-mutator strain can be made. This comparison shows that frameshift mutations are dramatically increased in the mutator strain (56% *vs.* 13%). The majority of the remaining mutations in the mutator background were base substitutions (34%), all of which were transitions.

DISCUSSION

We have presented the sequence analysis of 34 *mtr* mutants in the wild-type strain, sequence analysis of 21 *mtr* mutants in the *mut-1* mutator strain, and the sequence differences between the Oak Ridge and Mauriceville strains for the *mtr* region. With this information we can begin to address several interesting questions.

Mechanisms: The molecular spectrum of the spontaneous mutants has provided some mechanistic clues. Most apparent is the presence of monotonic runs at the sites of frameshifts (Tables 2C and 5B), and the presence of direct repeats of variable length at the endpoints of 11 of 12 deletions (Tables 2B and 5B) and three of the four tandem duplications (Tables 2C and 5B). These data are consistent with the DNA synthesis-slippage model first proposed by STREISINGER *et al.* (1966). In this

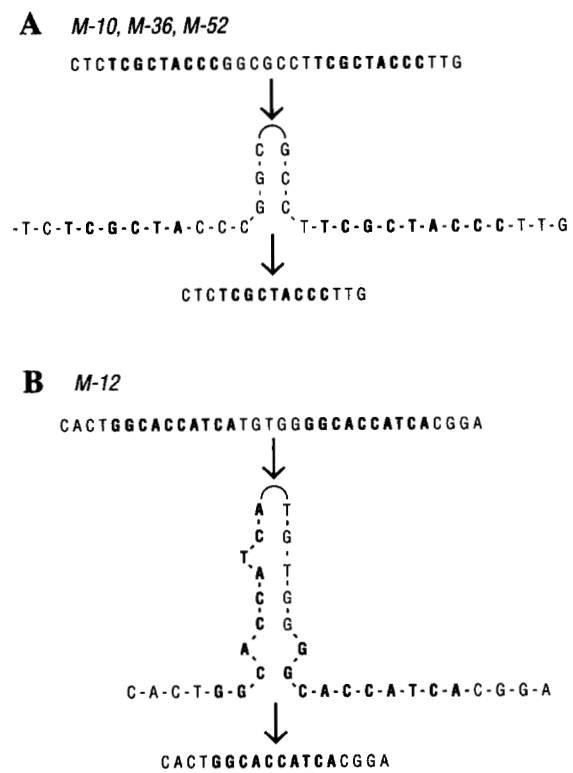


FIGURE 2.—Potential secondary structure of DNA during replication. Direct repeats are shown in boldface. Inverted repeats can act to reduce distance between repeats (A) or to stabilize the slippage intermediate (B).

model the occurrence of frameshift mutations was explained by the formation of slippage intermediates at monotonic runs during DNA replication. This model was expanded to account for the formation of deletions and tandem duplications, where direct repeats at endpoints could stabilize slippage intermediates (ALBERTINI *et al.* 1982). In addition, the presence of inverted repeats has been observed within or adjacent to deletions (SCHAAPER *et al.* 1986), which could reduce the distance separating the direct repeats or assist in the stabilization of the slippage intermediate. The presence of direct repeats in nearly all of our deletion and tandem duplication mutants is consistent with slippage during DNA synthesis. In addition, 2 of the 10 different deletion mutants recovered, including the deletion hotspot recovered three times, contained inverted repeats (Figure 2). The inverted repeat in Figure 2A could potentially act to reduce the distance between the 9-bp direct repeats, while the inverted repeat in Figure 2B could play a role in stabilization of the slippage intermediate. Interestingly, the deletion hotspot shown in Figure 2A was recovered in all three cases in the Mauriceville strain, where a sequence difference (relative to the Oak Ridge strain) creates a 9-bp direct repeat at the endpoints, rather than an 8/9 imperfect direct repeat present in the Oak Ridge strain.

The presence of direct repeats at the endpoints of all 11 deletion mutants in the non-mutator strain contrasts with the observation in the *lacI* spectrum (SCHAAPER *et al.* 1986; SCHAAPER and DUNN 1991) where a substantial fraction of the deletions (7/22 and 20/71, respectively) have no significant direct repeats at the endpoints. This may reflect an additional mechanism of deletion formation present in *E. coli* that is lacking in *N. crassa*.

The characterization of the mutator strain offers some clues toward the mechanism of mutation and the role of the *mut-1* gene product. The mutator strain was shown to have wild-type sensitivity to UV, X-ray and MMS. In addition, wild-type induced mutability was observed. These results argue that the *mut-1* gene product is not involved in a general repair system responsible for the repair of both spontaneous and induced DNA damage.

Instead, the spectrum of *mtr* mutants produced in the *mut-1* background strongly suggest a role for *mut-1* in maintaining accurate DNA replication, specifically in the prevention or correction of DNA slippage. The sequenced spectrum of 21 *mtr* mutations is dominated by frameshifts (13/21), all of which are part of or adjacent to monotonic runs. All of these could have formed via the slippage of a single base pair within the monotonic run. The vast majority of the frameshifts were −1 frameshifts (12/13). Six base substitution mutations were found, all of which were transitions. Three of these could be templated by slippage events involving nearby sequences (Table 4). The remaining two mutations, a small deletion and a small tandem duplication, both contained repeated sequences in the region that could have mediated a slippage event.

Collectively, 18 of the 21 *mtr* mutations could have been the result of a small slippage event. Some of the *mtr* mutants in the non-mutator spectrum can also be explained by slippage events, although the majority of these involve deletion and duplication formation involving relatively larger slippages. Therefore, a possible role for the *mut-1* gene product is the prevention or correction of these smaller slippage events. Likely candidates are subunits of the DNA polymerase or a component of a post-replicative mismatch correction system. Indeed, the phenotype of *mut-1* most closely resembles the methyl-directed mismatch repair mutants *mutH*, *mutL* and *mutS* in *E. coli*. These mutations are recessive, cause an increase of approximately 250-fold in *lacI*$^-$ mutant frequency, and yield a mutational spectrum at *lacI* dominated by transitions (75%) and −1 frameshifts (25%) (SCHAAPER and DUNN 1987). Moreover, the vast majority of these −1 frameshifts (116/122) occurred at monotonic runs of two or more, with an extreme hotspot at a monotonic run of 5 A:T bp. The spectrum of mutation observed in these mutants is strikingly similar to that observed in the *mut-1* background. The only significant difference is the proportion of frameshifts to base pair transitions, which could be explained by a difference in

the sensitivity of the mutational targets to base substitution mutations.

The spectrum of detected mutation at *lacI* was limited to i^{-d} mutants, which are not formed by +1 frameshifts (SCHAAPER and DUNN 1987). The contribution of this type of mutation at *lacI* in the mismatch repair mutant spectrum is not known. No such selection bias is present in the *mtr* spectrum.

The loss of the mismatch repair system in *mutH*, *mutL* and *mutS* strains is believed to yield mutational spectra that better reflect the errors produced by the polymerase. If the *mut-1* gene product is a component of a similar mismatch repair system, it argues that the polymerases in these different systems have extreme similarity in their ability to accurately replicate DNA, with similar limitations of fidelity.

The sequence of the PMS1 gene and MSH genes in *S. cerevisiae* (W. KRAMER *et al.* 1989; REENAN and KOLODNER 1992a) have shown homology to *mutL* and *mutS* respectively in *E. coli*, arguing for similar mismatch repair systems in eukaryotes. The *pms1* mutant phenotype includes mutator activity, and this gene has been shown to be necessary for repair of mismatches (B. KRAMER *et al.* 1989). A strong mutator effect (85-fold increase in the spontaneous mutation rate to canavanine resistance) was also observed for an *msh-2* mutant, along with an increase in post-meiotic segregation, consistent with a loss of mismatch repair activity (REENAN and KOLODNER 1992b). A spectrum of mutation in the *pms1* and *msh-2* backgrounds is not yet available for comparative purposes.

The frameshifts recovered in the *mut-1* background all involve monotonic runs, occurring predominantly at runs of 4 or 5 bp (12/13). Both C:G and A:T base pairs appear susceptible. However, the *mtr* ORF contains 10 monotonic runs of four or more, while mutations are found at only three sites. No mutations were recovered at the longest monotonic run, a single run of 6 bp located at the extreme 5′ end of the gene. However, the translational start site of the *mtr* gene has not been unambiguously determined, and it is possible that this monotonic run is slightly upstream of the open reading frame, and frameshifts at this site might not produce a mutant phenotype. The single run of 5 bp is a mutational hotspot with three frameshifts occurring here including the only +1 frameshift. This is consistent with the idea that longer monotonic runs are more mutable in the *mut-1* background. Also consistent with this idea is that only one of the frameshifts occurs at a monotonic run of less than four despite the frequent occurrence of 3-bp runs (49) in the *mtr* ORF. Less clear is why the remaining nine frameshifts within monotonic runs of four occur at only two sites. Clearly these are mutational hotspots in the *mut-1* background, but a comparison of these to the other seven runs of four shows no obvious differences in the neighboring nucleotides, such as additional monotonic runs that might further stabilize a slippage event. Nor is there any indication of the presence of significant inverted repeats (that might also stabilize a slippage event) in the immediate flanking sequences (10 bp). This does not rule out the possibility of more complex inverted repeats involving sequences farther away.

Deletion mapping revealed differences in *mtr* mutant distribution between the mutator and non-mutator backgrounds. Specifically, 17 mutants in the non-mutator strain mapped to the *1X* deletion, while none of the mutants in the mutator strain mapped to this interval. There are three moderate mutational hot spots within this region in the non-mutator background. This region does not appear to contain a frameshift hotspot in the mutator background, despite the existence of two monotonic runs of 4 bp.

Another interesting question involving the frameshift mutations is why twelve of the 13 are −1 frameshifts. This bias could arise in at least two ways. First, if the *mut-1* gene product were involved in some type of replication-associated mismatch repair system, then this would argue that the bias toward the −1 frameshifts was due to the action of the polymerase. In a DNA slippage model, this would mean that the slippage occurred predominantly in one direction, in which the newly synthesized strand slipped forward relative to the template strand.

A second possibility is that the *mut-1* gene product is a part of the polymerase and in the mutant form it alters either the frequency or spectra of errors. Assuming the presence of a functional mismatch repair system, the spectrum of mutation being observed could be quite different from that which is occurring; the spectrum observed representing the classes of mutants not normally repaired by the mismatch repair system or the result of overloading the repair system.

Oak Ridge *vs.* Mauriceville sequence: Determination of the true mutational spectrum is an important goal, with impact on areas ranging from evolution to human genetic disease. One difficulty present in virtually all spectrum studies is the problem of detection bias. By using a specific phenotype as a mutational signal, these studies inevitably bias the mutational spectrum one observes. For our spectrum, we select for the loss of function of a specific protein, biasing our spectrum toward those mutants capable of reducing or eliminating the function of the protein. Deletions, duplications, and frameshifts usually produce profound effects at the amino acid level, allowing the recovery of the majority of these mutants when they occur. Base substitution mutations, however, will not be recovered with such regularity. Approximately 24% of possible base substitution mutations are silent at the amino acid level and will certainly be missed. In addition, many base substitutions

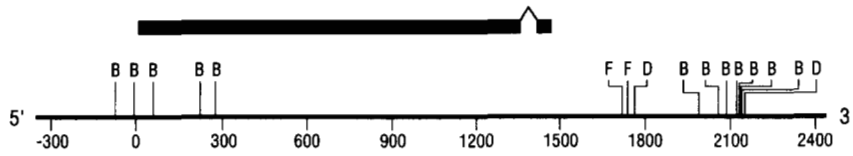


FIGURE 3.—Distribution of sequence differences between Oak Ridge and Mauriceville (B, base substitution; F, frameshift; D, insertion/deletion).

result in neutral changes or occur in regions of the protein less sensitive to alteration, increasing the bias against the recovery of these mutations.

One method of eliminating this bias is to use the base substitution mutants that cause chain termination to estimate the total number of base substitution mutants (DRAKE 1991). This is accomplished by multiplying the number of these mutants by 64/3, the correction factor representing the sixty four possible codon combinations divided by the three chain terminating codons. Five of the base substitution mutants in our spectrum fell in this category, yielding $5 \times (64/3) = 107$ base substitution mutants. This contrasts sharply with the 15 base substitution mutants we recovered. The most critical assumption in using this method is that the recovered chain terminating mutations occur at frequencies identical to the mutations not recovered. If instead, these represent mutational hotspots, then the number of base substitution mutants will be exaggerated. The fact that four of our five chain terminating mutants occur within a 3-bp region involving a monotonic run of four C:G residues argues that it may be a mutational hotspot and the use of this method to eliminate bias is inappropriate.

Another method to limit this type of bias is to utilize a system not dependent on a functional protein. To determine the "selection" bias present in our system, the entire *mtr* region, including both 5′- and 3′-flanking regions were sequenced in the Mauriceville strain, known to be divergent at the sequence level. It was reasoned that the regions flanking the *mtr* gene should be under relatively little selective pressure and the differences found in these regions could be used to determine the unbiased spectrum. One potential problem with this analysis would be the presence of another gene in the *mtr* region. This was deemed unlikely based on two lines of evidence. First, Northern analysis using the entire 2.8-kb subclone as a probe demonstrated only a single transcript (KOO and STUART 1991). Second, all six possible reading frames were examined and the *mtr* 3′-flanking region had multiple stop codons in all frames, arguing against the presence of another gene in this region. In addition, only three sequence differences were found within the 1472-bp *mtr* ORF (including the 59-bp intron), while 13 sequence differences were found in the 1205 bp of flanking DNA, arguing that the flanking sequences are under less selective pressure than the *mtr* ORF.

Of the 13 sequence differences in the flanking regions, there were 9 base substitutions, 3 frameshifts, and 1 small insertion/deletion. The three frameshifts and the one small insertion/deletion (5 bp) were all associated with monotonic runs (Table 3). Although the number of sequenced mutants is small, they do suggest that the spectrum dependent on an *mtr* phenotype biases against base substitution mutations; 48% (15/31) in spectrum dependent on *mtr* phenotype *vs.* 69% (9/13) in spectrum independent of *mtr* phenotype. There are several caveats, however. One caveat is the assumption of selective neutrality for the flanking regions. Although they are clearly hypermutable compared to the *mtr* ORF, there are certainly some selectively important promoter regions in the 5′ sequences where deletions could be selected against. Also, the observed sequence differences do not appear to be randomly distributed within the flanking regions (Figure 3), possibly arguing for a selective influence. A second potential problem with this type of analysis is the recovery of unstable mutants such as tandem duplications, which have a reversion frequency considerably higher than the frequency with which they occur. This mutational class could be underestimated, with only relatively recent events being observed. A third problem is the correct assignment of deletion/insertion. Because the founder strain is not known, it is not possible to distinguish between deletions and insertions.

Reversion behavior: The relative ease of performing reversion tests with various mutagens makes this approach for identifying mutational category appealing. However, the small number of mutagens with absolute specificity limit the usefulness of this type of analysis. In our study two mutagens, UV and ICR 170, were tested for their reliability in correctly predicting mutational class. ICR 170 is a known frameshift mutagen with absolute specificity in *S. cerevisiae* (MATHISON and CULBERTSON 1985; HAMPSEY *et al.* 1986) and *N. crassa* (BURNS *et al.* 1986), causing insertions of a C:G base pair at a monotonic run of two or more C:G base pairs. This mutagen effectively reverted −1 frameshifts at monotonic runs of three C:G base pairs and also reverted −1 frameshifts nearby these monotonic runs (BURNS *et al.* 1986). Potentially, this mutagen could effectively revert many −1 frameshifts by acting at sites near the original mutation, restoring the proper reading frame, albeit with some changes in amino acid composition.

The other mutagen, UV, does not have absolute specificity in *S. cerevisiae*, producing various types of base substitutions, frameshifts, and multiple base pair changes (LEE *et al.* 1988). However, 29 of 35 UV-induced mutants in that study were single base substitutions. This

may allow it to be used as a relatively accurate predictor of base substitution mutations. Using these two mutagens it was possible to tentatively assign mutants to one of three mutational categories (base substitutions, deletions and frameshifts).

The reversion behavior of the sequenced mutants is shown in Tables 2 and 5. Arbitrary reversion frequencies were chosen to assign the categories (see MATERIALS AND METHODS) to give the maximum correct assignment of the known mutants, resulting in the best possible predictive ability. Even so, the ability of the reversion tests to correctly predict mutational class was poor. Only 13 of 23 UV-reverted mutants turned out to be base substitutions, and, among 16 mutants which failed to revert with either UV or ICR 170, only six were deletions. However, ICR 170 revertibility correctly predicted frameshifts in 10 of 11 cases. One small deletion (19 bp) was reverted by ICR-170, presumably by the ability to correct the reading frame by acting at one of the two adjacent monotonic runs of C:G base pairs present in the deletion mutant. Instability (spontaneous reversion) was an accurate predictor of tandem duplications (4/4), except in the presence of the mutator *mut-1*.

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

LITERATURE CITED

ALBERTINI, A. M., M. HOFER, M. P. CALOS and J. H. MILLER, 1982 On the formation of spontaneous deletions: the importance of short sequence homologies in the generation of large deletions. Cell **29:** 319–328.

BURNS, P. A., J. H. KINNAIRD and J. R. S. FINCHAM, 1984 *Neurospora crassa* suppressors act on amber. Neurospora Newsl. **31:** 20.

BURNS, P. A., J. H. KINNAIRD, B. J. KILBEY and J. R. S. FINCHAM, 1986 Sequencing studies of ICR-170 mutagenic specificity in the *am* (NADP-specific glutamate dehydrogenase) gene of *Neurospora crassa*. Genetics **113:** 45–51.

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

DEGNEN, G. E., and E. C. COX, 1974 Conditional mutator gene in *Escherichia coli*: isolation, mapping, and effector studies. J. Bacteriol. **117:** 477–487.

DRAKE, J. W., 1991 A rate of spontaneous mutation common to DNA-based microbes. Proc. Natl. Acad. Sci. USA **88:** 7160–7164.

FUKUCHI, K.-I., G. M. MARTIN and R. J. MONNAT, JR., 1989 Mutator phenotype of Werner syndrome is characterized by extensive deletions. Proc. Natl. Acad. Sci. USA **86:** 5893–5897.

GIROUX, C. N., J. R. A. MIS, M. K. PIERCE, S. E. KOHALMI and B. A. KUNZ, 1988 DNA sequence analysis of spontaneous mutations in the SUP4–0 gene of *Saccharomyces cerevisiae*. Mol. Cell. Biol. **8:** 978–981.

GURR, S. J., S. E. UNKLES and J. R. KINGHORN, 1987 The structure and organization of nuclear genes of filamentous fungi. Spec. Publ. Soc. Gen. Microbiol. **22:** 93–139.

HAMPSEY, D. M., R. A. KOSKI and F. SHERMAN, 1986 Highly mutable sites for ICR-170-induced frameshift mutations are associated with potential DNA hairpin structures: studies with *SUP4* and other *Saccharomyces cerevisiae* genes. Mol. Cell. Biol. **6:** 4425–4432.

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

KOO, K., and W. D. STUART, 1991 Sequence and structure of *mtr*, an amino acid transport gene of *Neurospora crassa*. Genome **34:** 644–651.

KRAMER, B., W. KRAMER, M. S. WILLIAMSON and S. FOGEL, 1989 Heteroduplex DNA correction in *Saccharomyces cerevisiae* is mismatch specific and requires functional *PMS* genes. Mol. Cell. Biol. **9:** 4432–4440.

KRAMER, W., B. KRAMER, M. S. WILLIAMSON and S. FOGEL, 1989 Cloning and nucleotide sequence of DNA mismatch repair gene *PMS1* from *Saccharomyces cerevisiae*: homology of PMS1 to procaryotic MutL and HexB. J. Bacteriol. **171:** 5339–5346.

KUNZ, B. A., M. G. PETERS, S. E. KOHALMI, J. D. ARMSTRONG, M. GLATTKE *et al.*, 1989 Disruption of the *RAD52* gene alters the spectrum of spontaneous *SUP4-o* mutations in *Saccharomyces cerevisiae*. Genetics **122:** 535–542.

KUNZ, B. A., L. KOHALMI, X. KANG and K. A. MAGNUSSON, 1990 Specificity of the mutator effect caused by disruption of the *RAD1* excision repair gene of *Saccharomyces cerevisiae*. J. Bacteriol. **172:** 3009–3014.

KUNZ, B. A., X. KANG and L. KOHALMI, 1991 The yeast *rad18* mutator specifically increases G·C → T·A transversions without reducing correction of G-A or C-T mismatches to G·C pairs. Mol. Cell. Biol. **11:** 218–225.

KYTE, J., and R. F. DOOLITTLE, 1982 A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. **157:** 105–132.

LEE, G. S.-F., E. A. SAVAGE, R. G. RITZEL and R. C. VON BORSTEL, 1988 The base-alteration spectrum of spontaneous and ultraviolet radiation-induced forward mutations in the *URA3* locus of *Saccharomyces cerevisiae*. Mol. Gen. Genet. **214:** 396–404.

LEGERTON, T. L., and C. YANOFSKY, 1985 Cloning and characterization of the multifunctional *his-3* gene of *Neurospora crassa*. Gene **39:** 129–140.

LIBERFARB, R. M., and V. BRYSON, 1970 Isolation, characterization, and genetic analysis of mutator genes in *Escherichia coli* B and K-12. J. Bacteriol. **104:** 363–375.

MATHISON, L., and M. R. CULBERTSON, 1985 Suppressible and Nonsuppressible +1 G:C base pair insertions induced by ICR-170 at the *his4* locus in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **5:** 2247–2256.

METZENBERG, R. L., J. N. STEVENS, E. U. SELKER and E. MORZYCKA-WROBLEWSKA, 1985 Identification and chromosomal distribution of 5S rRNA genes in *Neurospora crassa*. Proc. Natl. Acad. Sci. USA **82:** 2067–2071.

MICHAELS, M. L., C. CRUZ and J. H. MILLER, 1990 *mutA* and *mutC*: two mutator loci in *Escherichia coli* that stimulate transversions. Proc. Natl. Acad. Sci. USA **87:** 9211–9215.

NGHIEM, Y., M. CABRERA, C. G. CUPPLES and J. H. MILLER, 1988 The *mutY* gene: a mutator locus in *Escherichia coli* that generates G·C → T·A transversions. Proc. Natl. Acad. Sci. USA **85:** 2709–2713.

PALL, P. L., 1969 Amino acid transport in *Neurospora crassa*. Biochim. Biophys. Acta **173:** 113–127.

PENDYALA, L., J. SMYTH and A. M. WELLMAN, 1979 Nature of 6-methylpurine inhibition and characterization of two 6-methylpurine-resistant mutants of *Neurospora crassa*. J. Bacteriol. **137:** 248–255.

PHEAR, G., and M. MEUTH, 1989 A novel pathway for transversion mutation induced by dCTP misincorporation in a mutator strain of CHO cells. Mol. Cell. Biol. **9:** 1810–1812.

REENAN, R. A. G., and R. D. KOLODNER, 1992a Isolation and characterization of two *Saccharomyces cerevisiae* genes encoding homologs of the bacterial Hex A and MutS mismatch repair proteins. Genetics **132:** 963–973.

REENAN, R. A. G., and R. D. KOLODNER, 1992b Characterization of insertion mutations in the *Saccharomyces cerevisiae MSH1* and *MSH2* genes: evidence for separate mitochondrial and nuclear functions. Genetics **132:** 975–985.

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

SCHAAPER, R. M., and R. L. DUNN, 1987 Spectra of spontaneous mutations in *Escherichia coli* strains defective in mismatch correction: the nature of *in vivo* DNA replication errors. Proc. Natl. Acad. Sci. USA **84:** 6220–6224.

SCHAAPER, R. M., and R. L. DUNN, 1991 Spontaneous mutation in the *Escherichia coli lacI* gene. Genetics **129:** 317–326.

SCHAAPER, R. M., B. N. DANFORTH and B. W. 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.

STADLER, D., and E. CRANE, 1979 Analysis of lethal events induced by ultraviolet in a heterokaryon of *Neurospora*. Mol. Gen. Genet. **171:** 59–68.

STADLER, D., H. MACLEOD and M. LOO, 1987 Repair-resistant mutation in Neurospora. Genetics **116:** 207–214.

STADLER, D., H. MACLEOD and D. DILLON, 1991 Spontaneous mutation at the *mtr* locus of Neurospora: the spectrum of mutant types. Genetics **129:** 39–45.

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.

STUART, W. D., K. KOO and S. J. VOLLMER, 1988 Cloning of *mtr*, an amino acid transport gene of *Neurospora crassa*. Genome **30:** 198–203.

TREFFERS, H. P., V. SPINELLI and N. O. BELSER, 1954 A Factor (or mutator gene) influencing mutation rates in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **40:** 1064–1071.

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

WILLIAMS, L. J., G. R. BARNETT, J. L. RISTOW, J. PITKIN, M. PERRIERE *et al.*, 1992 Ornithine decarboxylase gene of *Neurospora crassa*: isolation, sequence, and polyamine-mediated regulation of its mRNA. Mol. Cell. Biol. **12:** 347–359.

WILLIAMSON, M. S., J. C. GAME and S. FOGEL, 1985 Meiotic gene conversion mutants in *Saccharomyces cerevisiae*. I. Isolation and characterization of *pms1-1* and *pms1-2*. Genetics **110:** 609–646.

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

APPENDIX

A consequence of the mutational study at the *mtr* locus was the discovery that the *mtr* ORF, as predicted by KOO and STUART (1991), was incomplete. In sequencing the entire region, three differences from the published sequence were found, including two single-base frameshifts that allowed for the formation of one single larger ORF that included the two distinct smaller ORFs predicted by KOO and STUART (1991) and the region between these ORFs (Figure 1A). The two frameshifts have opposite polarity and flank the site of what had appeared to be the termination codon of the upstream ORF. We have confirmed that this region between the two proposed ORFs was contained within the *mtr* ORF by demonstrating amber suppression of a putative amber mutant located between the two smaller ORFs.

Deletion mapping results (Figure 1B) revealed that a large fraction of the *mtr* mutants mapped upstream of the 845-bp *mtr* ORF (vertical lines in Figure 1A) proposed by KOO and STUART (1991), which included a 59-bp intron near the 3′ end. Several of these mutants could be explained by the existence of a 174-bp upstream ORF (diagonal lines), also proposed by KOO and STUART (1991). However, this still failed to account for the 17 mutants that mapped to deletion *1X*, located between the two ORFs.

Sequencing the wild-type gene: Sequence analysis of the entire subcloned *mtr* region, from bp −297 to bp 2377 as shown in Figure 1A, yielded three differences when compared to the sequence published by KOO and STUART (1991). These included a +1 frameshift at position 135–136, a −1 frameshift at position 353, and a base substitution (T *vs.* G in previous sequence) at position 1460. The two frameshift changes result in a single ORF (Figure 1, solid bar) of 1472 bp containing the majority of the upstream ORF, all of the main ORF (including the 59-bp intron previously proposed), and the region between the proposed ORFs. Using this alternative explanation, all 34 sequenced *mtr* mutants are located within the *mtr* ORF. The 8-base substitution *mtr* mutations in the region between the two ORFs previously proposed all result in either missense or termination codons, consistent with the idea that this region is within the *mtr* ORF.

Amber suppression of an *mtr* mutant: To provide direct evidence that a single larger *mtr* ORF existed, suppression of

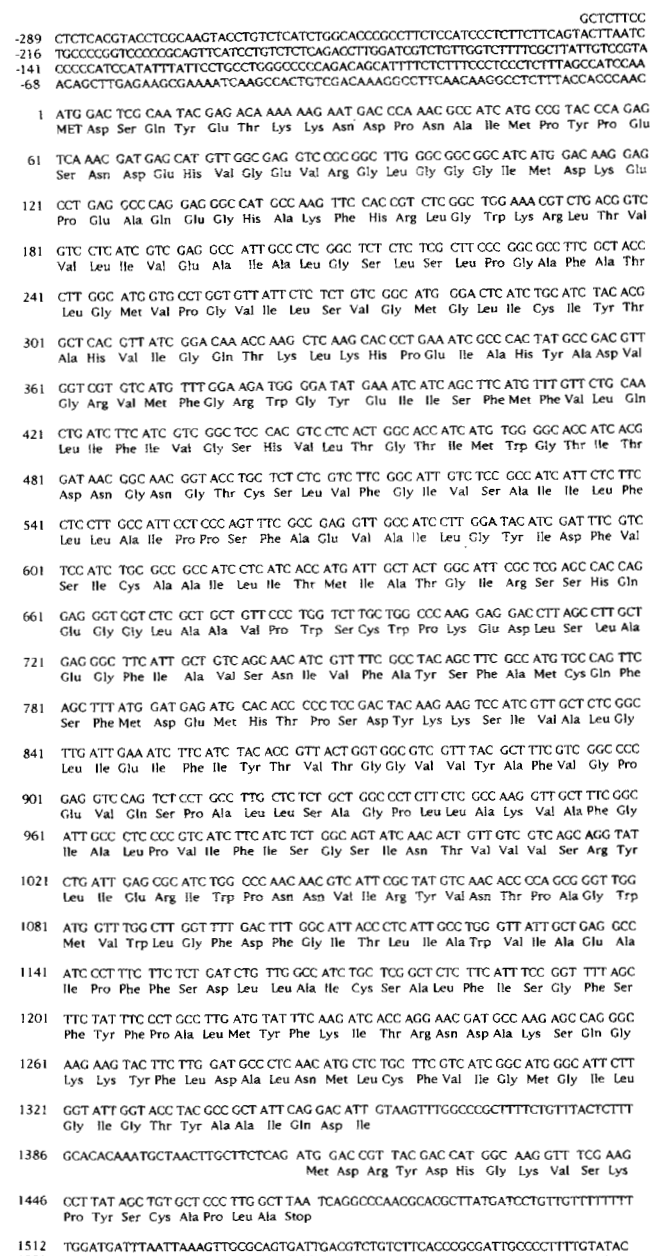


FIGURE 4.—DNA sequence of *mtr* region and newly prosed ORF. Location of intron shown as previously proposed (KOO and STUART 1991), and is based on splice consensus sequences. The accession number for the sequence is L34605.

a putative amber mutation mapping to deletion 1X was demonstrated. Sequence analysis of this allele revealed a G → A transition at bp 383 that resulted in a change from tryptophan to amber nonsense codon in the suspected reading frame. The cross of strain *col-4 ssu-1 ad-2^s trp-2^s a* to strain *mtrM2 ad-2^s trp-2 A* was done, in which an amber suppressor (*ssu-1*) could be followed by suppression of a suppressible *ad-2* allele (suppressible alleles denoted by superscript *s*) and the *mtr* mutant could be followed by the closely linked (<1 m.u.) *col-4* allele (see MATERIALS AND METHODS). The informative progeny class contained the non-suppressible *trp-2* allele, the *mtr* mutant, and the suppressor. This class of progeny showed reduced growth on the *mtr* selective medium (Fpa + Ant) and was also able to grow on the counter-selective medium (0.2 Trp + 2 Arg), indicating partial suppression of the *mtr* mutation. This suppression was dependent on the presence of *ssu-1* ; similar *mtr* progeny lacking the suppressor failed to grow under the counter-selective conditions. This result provided direct evidence that this site was included in the *mtr* ORF (Figure 4).

We conclude that the simplest explanation is the presence of a single ORF, with the translational start at or near the beginning of the upstream ORF proposed by KOO and STUART (1991). This would result in an ORF of 1472 bp including most of the upstream ORF and all of the main ORF (including the 59-bp intron) proposed by KOO and STUART (1991), along with the region between the ORFs. The choice of the translational start site is based on the similarity to the *N. crassa* translational start consensus sequence, ATCA(C/A)(C/A)ATG (LEGERTON and YANOFSKY 1985). The sequence at the proposed translational start site for *mtr* is CCCAACATG.

Hydrophobicity analysis of the previous *mtr* ORF predicted six or seven putative transmembrane stretches (KOO and STUART 1991). The newly proposed *mtr* ORF predicts 11 or 12 transmembrane stretches based on the algorithm by KYTE and DOOLITTLE (1982). The newly proposed ORF contains 471 amino acids, with a predicted molecular mass of 51,128 D.

The translational start site chosen as most likely based on the consensus sequence may not be correct. Two other methionines are present downstream, before the first putative transmembrane stretch, at residues 16 and 37. No *mtr* mutants were recovered upstream of these residues. The DNA sequences at these sites are GCCATCATG and GGCATCATG, respectively. Both contain some similarity to the consensus sequence. The methionine at residue 16 is particularly attractive when the codon usage is studied. The first 15 residues (5′ of residue 16) include five codons which are almost completely absent in the remaining portion of the gene and rarely found in other Neurospora genes (GURR *et al.* 1987). This is not the case for the residues between 16 and 37, where only one rare codon is found.

However, a study of the frequency of nucleotides surrounding the ATG initiating codon of 52 *N. crassa* protein-coding regions (WILLIAMS *et al.* 1992) reveals a strong bias for an A residue at the −3 position, an A or C residue at the −2 position, and a G residue at the +4 position. Based on these criteria, the ATG codon chosen as the start of *mtr* is the most favorable of the three.