THE DETECTION OF REVERSE MUTATIONS AT THE *mtr* LOCUS IN NEUROSPORA AND EVIDENCE FOR POSSIBLE INTRAGENIC (SECOND SITE) SUPPRESSOR MUTATIONS[1]

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THE *mtr* mutants of *Neurospora crassa* are resistant to the amino acid analogs 4-methyl-tryptophan (4MT) and *para*-fluorophenylalanine (FPA). It has been suggested that the *mtr* locus is the structural gene for a permease which facilitates the uptake of aromatic amino acids (LESTER 1966; STADLER 1966).

The products of forward mutation at *mtr* can be selected on media supplemented with either 4MT or FPA. Reversions to *mtr*+ can also be selected if one employs an *mtr* strain which has a requirement for one of the amino acids whose uptake is controlled by this system (STADLER 1967).

In the experiments reported here, an *mtr* strain which required histidine was treated with a variety of mutagens. Histidine is taken up via two pathways—the tryptophan pathway controlled by the *mtr* locus and a second pathway which has a high affinity for arginine. Because the tryptophan pathway is nonfunctional in an *mtr* strain, histidine is taken up solely by the arginine transport system. Therefore, reversions of *mtr* (and of *hist*) can be selected on a medium supplemented with histidine and an excess of arginine; this mixture completely blocks the uptake of histidine by the arginine pathway (STADLER and KARIYA 1969).

In addition to revertants at the *hist* and *mtr* loci, this procedure has also yielded *mtr* revertants resulting from suppressor mutations at another locus. Four chemical mutagens and ultraviolet (UV) irradiation have been tested for induction of mutations at these three loci. The results have revealed several distinct patterns of specificity.

MATERIALS AND METHODS

Media: mtr cells were selected on either HP4MT (minimal sorbose medium of LESTER and GROSS (1959) supplemented with histidine, pyridoxine and 4MT) or HPFPA (supplements: histidine, pyridoxine and FPA). *mtr*+ cells (of histidine-requiring strains) were selected on HPArg (supplements: histidine, pyridoxine and arginine). The control medium for growth of all cells was HP (supplements: histidine and pyridoxine), and the test medium for histidine requirement was P (supplement: pyridoxine). Concentrations of supplements were as follows: pyridoxine, 5mg/l; 4MT, 70 mg/l (filter sterilized); FPA, 10 mg/l; L-arginine, 60 mg/l; L-histidine, 10 mg/l in HPArg, 50 mg/l in other media.

Strains: The original strain carried the mutants *hist-2* (histidine-requiring) and *pdx-1* (py-

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ridoxine-requiring) and was mating type *A*. Conidia were treated with UV and plated on HPFPA. Resistant colonies were isolated after two days of incubation at 33°C. One of these mutants was designated *mtr26*. It was resistant to both FPA and 4MT and was located between *pdx-1* and *col-4* on linkage group IV (BRINK, unpublished) where the *mtr* locus is situated (STADLER 1966). This strain (*hist pdx mtr26 A*) was the subject of all the reversion experiments to be reported here. The histidine allele was *hist-2* (Y152M43) and was originally isolated following X-irradiation (WEBBER and CASE 1960).

Reversion experiments: Cultures were reisolated from single-cell colonies and grown on fresh slants at room temperature for 7–9 days. Conidia were suspended in phosphate buffer or sterile water and filtered to remove mycelial fragments. The conidial suspensions were treated with mutagens as described below. Chemical treatments were terminated by washing the conidia on millipore filters (5μ) several times with phosphate buffer and resuspending in sterile water. Appropriate concentrations of treated conidia were then incubated in HPArg to select revertants. These plates were incubated for 2 days at 33°C before the revertant colonies were counted and isolated for further study. Dilutions of the untreated and treated conidial suspensions were plated on HP to determine the total numbers of viable cells and the percent survival after treatment.

Mutagen treatments: Some of the treatments have been adapted from procedures described by MALLING (1966). The treatments with chemical mutagens were administered at room temperature in 0.1 M phosphate buffer at pH 7.0 with constant agitation. *Ultraviolet irradiation:* A General Electric germicidal lamp was used at doses giving 25–50% survival (30–45 sec at a distance of 10 cm). Conidial suspensions in sterile water were constantly agitated during the irradiation. The usual precautions were taken to avoid photoreactivation. *Ethyl methanesulphonate (EMS) and Diethyl sulfate (DES) treatments:* Conidia were suspended in buffer to which the mutagen was added to a final concentration of 1.0% EMS or 0.5% DES. Treatment times were 330 min for EMS and either 20 or 40 min for DES. *ICR-170* treatment:* The mutagen was freshly dissolved in water (0.25 mg/l) and added to a suspension of conidia in buffer to give a final concentration of 10 μg/ml. The treatment was administered in dim light for periods of 200 or 400 min. *N-methyl-N'-nitro-N-nitrosoguanidine (NMG) treatment:* A solution of NMG, freshly prepared in buffer, was added to a conidial suspension to give a concentration of 80 μg/ml. Treatment time was 6 min.

Classification of revertants: The revertants were purified by restreaking on HPArg before their classification by growth tests. Drops of conidial suspensions were tested on plates of sorbose medium to distinguish the two basic classes of revertants. The *hist*+ revertants grew in the absence of histidine (on P) and were still resistant to FPA and 4MT while the *mtr*+ revertants required histidine for growth and showed partial or complete sensitivity to the analogs.

The growth tests on the inhibitors provided a positive check of the genetic purity of the *mtr*+ revertants. If any nonreverted nuclei were present, they produced resistant conidia which gave spots of strong growth in the background of nongrowing conidia. In such a case, the revertant was streaked out on HPArg again and reisolated.

Genetic analysis of the revertants: A sample of the *hist*+ revertants was crossed to wild type to determine whether they had resulted from back mutation or suppression. In the case of suppression, the original mutant is expected to reappear among the progeny, while the cross of a back-mutant should yield no mutants. Random ascospores from these crosses were scored for histidine requirement.

The *mtr*+ revertants were analyzed in a corresponding manner. They were crossed to an *mtr*+ strain, and the progeny were scored for *mtr*. The *mtr*+ tester for these crosses was *hist-2 col-4 a*. The presence of *hist* in both parents insured that all the progeny would be *hist*, permitting positive growth tests for *mtr*+ (on HPArg) as well as for *mtr* (on HPFPA or HP4MT). The *col* (colonial morphology) mutant is closely linked to *mtr*, and it can be visually scored in colonies on sorbose plates. In the crosses of those revertants which resulted from suppressor mutations, the segregating *col* marker provided a simple check on the linkage relations of the suppressor and *mtr*. Duplicate platings of ascospores on HP, HP4MT and HPArg (about 100 spores/plate)

* 2-methoxy-6-chloro-9-[3-(ethyl-2-chloroethyl) aminopropylamine] acridine dihydrochloride.

TABLE 1

Expected colony counts on duplicate platings of ascospores from the cross of an mtr+ *revertant* (hist pdx mtr+ A) *to* hist col a

	HP		HP4MT		HPArg	
	col	*col+*	*col*	*col+*	*col*	*col+*
Reversion at *mtr* locus	50	50	0	0	50	50
Reversion by unlinked suppressor	50	50	0	25	50	25

were incubated overnight at 33°C before the colonies were counted and scored for *col*. Expected results of this test for the two kinds of *mtr*+ revertants are shown in Table 1.

Revertants which resulted from events at the *mtr* locus were further screened for the presence of intragenic suppressors by plating large numbers of ascospores from the same crosses on HP4MT to select for rare *mtr* progeny.

RESULTS

The results of the reversion experiments are shown in Table 2. The *mtr*+ reversions include both locus revertants and those arising as a consequence of mutation at an unlinked suppressor locus (su^{mtr}).

Specificity of the mutagens: Both kinds of reversions (*hist*+ and *mtr*+) have shown quite a specific response to the chemical mutagens. ICR-170 produced *no*

TABLE 2

The frequencies of hist+ *and* mtr+ *reversions produced by various mutagens*

Experiment No.	Mutagen and concentration	Time of treatment (min)	Cells per ml $\times 10^{-6}$	Percent survival	Number of surviving cells tested $\times 10^{-6}$	Revertants	
						hist+	*mtr+*
1	none (control)	. . .	. .	100	47.2	0	36 (0.8)
2	UV	. . .	1.2	30	2.2	12 (5.4)	8 (3.6)
3	UV	. . .	1.2	39	4.7	44 (9.4)	92 (19.6)
4	ICR-170 10μg/ml	200	4.9	62	4.5	0	108 (24.0)
5	ICR-170 10μg/ml	400	4.9	47	3.4	0	172 (50.6)
6	EMS 1.0%	330	0.7	68	4.9	4 (0.8)	37 (7.6)
7	DES 0.5%	20	0.7	96	6.9	2 (0.3)	32 (4.6)
8	DES 0.5%	40	0.7	75	5.2	3 (0.6)	53 (10.2)
9	NMG 80μg/ml	6	0.8	45	10.8	152 (14.1)	7 (0.7)

Numbers in parentheses are revertants per 10^6 surviving cells.

histidine revertants while producing very large numbers of *mtr*$^+$ revertants. The alkylating agents EMS and DES caused low frequencies of *hist*$^+$ and rather high frequencies of *mtr*$^+$. Treatment with NMG resulted in many histidine revertants, while the frequency of *mtr*$^+$ was no higher than that of the untreated control.

Analysis of the histidine revertants: A sample of fifteen of the histidine revertants produced by treatment with UV, EMS or DES were crossed to wild type to screen for unlinked suppressor mutations. All the reversions were found to have occurred at the *hist-2* locus. A similar analysis was carried out on eight NMG-induced histidine revertants isolated from another *mtr* strain carrying the same *hist-2* allele, and all were found to be at the locus (BRINK, unpublished).

Analysis of the mtr *revertants:* A sample of *mtr* reversions was genetically analyzed for unlinked or intragenic suppressors. The results are shown in Tables 3 and 4. All the mutagens except NMG produced reversion events both at the *mtr* locus and at an unlinked suppressor locus. The small number of revertants which resulted from the NMG experiment have not been tested.

The data of Table 3 permit us to make rough estimates of the relative frequencies of the two kinds of *mtr*$^+$ revertants resulting from each of the mutagenic treatments. However, the samples of EMS and DES revertants tested were not random ones; a phenotypic signal was used to select for genetic analysis those revertants which were most likely to be at the *mtr* locus. Revertants at the *mtr* locus usually showed restored sensitivity to both FPA and 4MT, while the suppressed *mtr* strains were sensitive to FPA but were usually able to grow on 4MT at the concentration used in our growth tests. Therefore, the calculated frequency of revertants occurring at the *mtr* locus following treatment with either of these mutagens certainly represents an overestimate of the true value.

The samples of *mtr*$^+$ revertants produced by UV and by ICR-170 were unselected, so the observation that over half of them resulted from events at the *mtr* locus is probably accurate. It is clear that both of these mutagens have produced higher *mtr*$^+$:*su*^{mtr} ratios than either of the alkylating agents.

Further analysis of the revertants at the *mtr* locus has shown that in some cases

TABLE 3

The numbers of mtr$^+$ *revertants resulting from reversion at the locus or from mutation at an unlinked suppressor locus*

Experiment No.	Mutagen	Total Number of *mtr*$^+$ revertants	Number genetically tested	*mtr* locus revertants	Suppressor type revertants
1	none (control)	36	6	0	6
2	UV	8	8	3	5
3	UV	92	26	18	8
5	ICR	172	45	24	21
6	EMS	37	10	1	9
7	DES	32	10	3	7
8	DES	53	4	0	4

TABLE 4

Genetic analysis of mtr+ *locus revertants: the production of* mtr *recombinants in crosses of revertants to "wild type"*

Mutagen	Revertant	Selfed cross: Total spores $\times 10^{-3}$	Selfed cross: *mtr*	Cross to "wild type": Total spores $\times 10^{-3}$	Cross to "wild type": *mtr*	Outside markers: P1 *pdx col+*	P2 *pdx+ col*	R1 *pdx+ col+*	R2 *pdx col*
UV	*295*	628	0	352	0	..	..	..	..
UV	*299*	285	0	598	0	..	..	..	..
ICR-170	*1300*	292	0	486	0	..	..	..	..
ICR-170	*1310*	585	0	588	1	..	1	..	..
ICR-170	*1316*	838	0	334	2	1	..	1	..
ICR-170	*1319*	329	0	239	1	..	..	..	1
ICR-170	*1320*	646	0	619	0	..	..	..	..
ICR-170	*1321*	1436	0	647	0	..	..	..	..
ICR-170	*1328*	761	0	220	0	..	..	..	..
ICR-170	*1341*	835	0	471	0	..	..	..	..
ICR-170	*1346*	34	0	459	2	2	..	..	..
ICR-170	*1351*	563	0	539	3	1	1	..	1
ICR-170	*1360*	767	0	602	3	1	2	..	..
ICR-170	*1363*	778	0	391	4	2	2	..	..
ICR-170	*1364*	851	0	556	0	..	..	..	..
ICR-170	*1372*	909	0	389	2	2	..	..	..
ICR-170	*1373*	776	0	467	0	..	..	..	..
ICR-170	*1380*	842	0	279	0	..	..	..	..
ICR-170	*1387*	235	0	278	0	..	..	..	..
ICR-170	*1406*	283	0	266	0	..	..	..	..
ICR-170	*1430*	320	0	465	0	..	..	..	..
ICR-170	*1431*	55	0	383	0	..	..	..	..
ICR-170	*1455*	474	0	404	1	..	1	..	..
ICR-170	*1465*	501	0	410	0	..	..	..	..
ICR-170	*1477*	520	0	335	0	..	..	..	..

"Wild type" refers to the ancestral allele of *mtr+* which was present in the stocks before the forward mutant *(mtr26)* was selected. In these crosses the revertant parent was *hist pdx mtr+ col+ A* and the "wild type" parent was *hist pdx+ mtr+ col a*. An ascospore of the genotype *hist pdx mtr+ col+ a* was isolated from such a cross and was crossed back to the revertant parent for the selfed cross.

a low frequency (up to ten per 10^6 ascospores) of resistant colonies are recovered from revertant × wild type crosses, while selfed crosses of the same revertants failed to produce any resistant colonies at similar or higher plating densities (see Table 4). Similarly, the analysis of many wild type × wild type crosses has also failed to produce any resistant colonies (STADLER and KARIYA 1969).

These rare resistant ascospores may arise from a crossover event between the *mtr26* mutant site and an intragenic suppressor site. However, most of these resistant progeny carry parental combinations of outside markers, indicating that the event was gene conversion rather than reciprocal recombination. It is not yet known whether all the resistant progeny represent the *mtr26* mutant, or whether the second site alteration also results in the mutant phenotype when separated from the original mutant.

Ten of the DES-induced revertants which resulted from unlinked suppressor mutations were tested for cross-suppression of another *mtr* allele (*mtr14*). The result was positive in every case, corresponding to the finding of STADLER (1967), who tested nine suppressors of *mtr* and found that none was allele-specific in its suppression. Uptake studies of these suppressor strains suggested that they represented forward mutation of a gene controlling another uptake system. Genetic tests showed that all nine mutants were at the same locus in linkage group I, linked to mating type. The suppressors isolated in the present study have not been mapped. However, samples of progeny from crosses segregating for some of these suppressors were scored for mating type, and linkage was indicated in every case.

DISCUSSION

The experiments reported here employ a technique which is especially suitable for studies of mutagen specificity, because it permits the selective recovery of mutations at three different loci in a single plating. Each experiment provides its own built-in control. For example, we know that all the conditions required for mutagenesis were satisfied in the ICR-170 experiment which yielded no *hist*$^+$ revertants, because the same plating yielded large numbers of *mtr*$^+$ revertants. Similarly, the negative result with NMG on *mtr*$^+$ reversion is proved to be valid by the numerous *hist*$^+$ revertants which were recovered from the same preparation. The following conclusions can be drawn: (1) The *hist-2* allele *Y152M43* was not reverted at all by ICR-170, but it back mutated with a high frequency following treatment with NMG. On the other hand, allele *mtr26* reverted at the *mtr* locus with high frequency following treatment with ICR-170, while NMG showed no evidence of induction of this type of mutation. (2) Reversions at the *mtr* locus were produced by all mutagens tested with the possible exception of NMG. However, the ratio of *mtr*$^+$:*su*^{mtr} varied for the different mutagens. Following UV irradiation or treatment with ICR-170 the ratio is above 0.5, while treatment with alkylating agents gave a ratio of less than 0.2. (3) Resistant colonies were recovered with a low frequency (less than ten per 10^6 plated ascospores) from crosses of revertants at *mtr* to wild type, but never from selfed crosses of these revertants.

It has been suggested that ICR-170 produces mutations of the frame-shift type (AMES and WHITFIELD 1966), although in Neurospora, MALLING (1967) has demonstrated that this compound can also revert base-substitution mutants with a low frequency, particularly when higher concentrations of the chemical are used. The first mutation experiments with ICR-170 were performed on Drosophila; as a result of these investigations, the compound was assumed to cause mutations via alkylation (CARLSON and OSTER 1967; SNYDER and OSTER 1964), although more recently CARLSON *et al.* (1967) presented evidence that it was also producing frame-shift changes in this organism. Thus, there are two mechanisms of action which have been postulated to account for the mutagenic action of ICR-170. These two hypotheses will be considered in light of the results obtained in this investigation.

Hypothesis 1: ICR-170 produces and reverts only those changes of the base addition/deletion type. Since ICR-170 has caused reversions at the *mtr* locus which are separable by recombination from the original mutant site, one of the main criteria for reversion of frame-shift mutations is satisfied (BRENNER *et al.* 1961; CRICK *et al.* 1961; STRESINGER *et al.* 1966). However, on the basis of this hypothesis the following conditions must also be satisfied:

a) Since *mtr26* was induced by UV irradiation, this agent must be able to cause frame-shift mutation. DRAKE (1963; 1964) has suggested that about half of the UV-induced mutations that he obtained in the *rII* region of phage T4 were of the base addition/deletion type. If *mtr26* is, in fact, a spontaneous mutation which arose on a treated plate, then it may still be a frame-shift change, as DRAKE and MCGUIRE (1967) have found that some of the spontaneous mutations occurring in the *rII* region are due to base addition or deletion, although replication is apparently necessary for their production.

b) The *hist-2* allele *Y152M43* cannot be a frame-shift change as it was not revertible by ICR-170.

c) Since both EMS and DES have reverted *mtr26* at the locus, these agents must be able to delete (or add) a base or bases with low frequency. The possible mechanisms whereby alkylating agents induce mutations have been extensively discussed (for reviews see FREESE 1963; KRIEG 1963a; BROOKES and LAWLEY 1964; and LOVELESS 1966). Generally, it is concluded that these compounds produce mutations of the transition type due to molecular instability created by the alkylation of guanine. However, KRIEG (1963b) has reported several mutants in T4 which may have resulted from base deletion. Similarly, LOPRIENO (1966) has proposed that some methyl methanesulphonate-induced mutants in Saccharomyces may be base deletions because of their nonleakiness and inability to be reverted by mutagens specifically causing base substitution changes. More recently, MALLING and DE SERRES (1968) have suggested that in Neurospora about 10% of EMS-induced mutants in the *ad-3B* region are frame-shifts.

One would expect the second-site change to be as effective as the initial mutant site (i.e. *mtr26*) in drastically altering the structure of the protein product, thereby conferring resistance on the organism. However, tests have not been carried out to confirm this prediction. Studies in Salmonella have shown that both sites are mutant when individually isolated by recombination (RIYASATY and ATKINS 1968).

Hypothesis 2: ICR-170 produces and reverts only base-substitution mutants. In this case, the apparent second-site changes may be similar to those described by HELINSKI and YANOFSKY (1963) for the tryptophan A locus in *E. coli.* Thus, if the initial mutant lesion results in an amino acid substitution, this may be corrected by a second substitution further along the cistron which restores partial or complete activity to the protein synthesized by the particular structural gene.

The patterns of mutagen specificity are difficult to explain by this hypothesis. If NMG is producing mutations via diazoalkane formation (MANDELL and GREENBERG 1961; MARQUARDT *et al.* 1964; ZIMMERMANN *et al.* 1965), then this compound is a much more efficient alkylating agent (at least at the pH used in

these experiments) than either DES or EMS, since both these mutagens reverted *hist* with a low frequency compared with NMG. It is possible that NMG produces base-substitution mutants different from those resulting after treatment with DES or EMS, and this is perhaps consistent with the claim that NMG produces transitions in both directions (ZAMPIERI and GREENBERG 1967; BAKER and TESSMAN 1968). However, this explanation has to be reconciled with the striking fact that NMG was ineffective in producing su^{mtr} mutations. Undoubtedly, the genetic nature of the suppressor locus is important in the interpretation of these results; possibly there are only a small number of sites which can be effectively altered by mutation and these are more frequently mutated by EMS and DES than by NMG.

The assumption that the mutations induced by ICR-170 result from base substitution (presumably via alkylation) poses another complication. Its pattern of mutagenic activity was different from those of all the other agents tested. On this hypothesis we must conclude that the different alkylating agents show three distinct patterns of specificity for mutation at the three loci studied here. Perhaps some secondary cellular phenomenon, rather than the base composition at the mutable sites, accounts for the different yields of mutations produced by these chemicals (see AUERBACH 1966 for review).

Although neither hypothesis is completely compatible with the results obtained, it seems that the frame-shift hypothesis more satisfactorily explains the mechanism of action of ICR-170, at least at the concentrations used in these experiments.

Crosses of the revertants at the *mtr* locus to another mtr^+ strain produced rare *mtr* recombinants, indicating that the reversions had occurred at second sites in the gene. We were only able to perform this genetic test for second-site reversion by virtue of the efficient system for selecting rare mutant spores among many nonmutants. For many genetic loci, such a technique is not available. If intragenic suppression occurs frequently in eukaryotes, then reversion studies in which these suppresssors cannot be recognized may result in spurious interpretations of mutagen specificity at the level of the DNA double helix.

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SUMMARY

Induced mutations were analyzed in a strain of Neurospora which carried a mutant gene (*hist*) resulting in a requirement for histidine and another (*mtr*) which destroyed the activity of a normal uptake system for amino acids. Cells were treated with mutagens and then plated in a medium which selected reversions at both loci. The selected revertants also included mutations at a third locus —a suppressor of *mtr*. The chemical mutagens showed three distinct patterns with regard to the relative frequencies of induced mutations at these three loci. The pattern shown by the alkylating agents DES and EMS was different from that shown by NMG, and both of these patterns differed from that of ICR-170.— A genetic analysis of revertants at the *mtr* locus showed that they had resulted

from events at sites which were separable from the original mutant site. It appears that this was a frame-shift mutant whose reversion was efficiently induced by ICR-170 and by UV, less efficiently by DES and EMS, and not at all by NMG.

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