A genetic analysis of CRM negative mutants at the td locus of *Neurospora crassa*

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1. INTRODUCTION

The last step in the biosynthesis of L-tryptophan in the fungus *Neurospora crassa* is catalysed by the enzyme tryptophan synthetase. The td locus controls the formation of this enzyme; mutants which possess alterations at the td locus have been isolated. Phenotypically, these mutants have been subdivided into two major categories: mutants which possess a protein serologically related to tryptophan synthetase designated CRM+ and mutants which possess no cross-reacting material (CRM⁻). CRM+ mutants may be further subdivided on the basis of residual enzymatic activity and cofactor requirements (DeMoss & Bonner, 1959; Bonner, Suyama & DeMoss, 1960; Yanofsky, 1960; Suyama, 1960). The occurrence of CRM+ and CRM⁻ mutant types appears to be a generalized phenomenon (Yanofsky & Stadler, 1958; Garen, 1960; Lee & Englesberg, 1962).

It is felt that problems concerning the genetic nature of a mutation which results in a CRM⁻ phenotype be considered in greater detail since approximately half of the two hundred td mutants of *Neurospora* are CRM⁻.

The fact that a mutation is capable of producing such a severe alteration in the ultimate gene product might imply that we are dealing with drastic changes at the td locus. The fact that these primary CRM⁻ isolates are capable of reversion tends to rule out the possibility that deletions of a portion of the td locus could be invoked as the cause of a CRM⁻ phenotype.

Genetic data (Suyama, Lacy & Bonner, 1963) favoured the possibility that CRM⁻ mutations at the td locus represented multiple base pair alterations. This conclusion was based on the following observations.

(1) Primary CRM⁻ mutations are difficult to position within the genetic map. Generally, such mutations map as extended genetic damage. On the other hand, CRM+ mutations are highly amenable to fine structure analyses (Kaplan, Suyama & Bonner, 1963).

(2) Primary CRM⁻ mutants positioned at or near the indole utilizing region of the locus (Bonner, Suyama & DeMoss, 1960) are capable of giving rise to two types of revertants, namely, indole utilizers (Suyama, 1962) and prototrophs. The appearance of this latter class of primary CRM⁻ mutants has provided the basis for the following series of experiments.

The characteristics of the td-tryptophan synthetase system have made it possible for us to construct a model system of multisite mutants which have one lesion within the indole utilizing region of the locus. By starting with a primary CRM⁺ mutant, which has retained the catalytic ability to convert indole to tryptophan, it has been possible to isolate double mutants at the td locus. These multisite mutants were then compared to the wild-type organism in their antigenic relationships. They were further compared to primary CRM⁻ isolates discussed above with respect to their mapping and reversion characteristics.

Experimentally, we are attempting to define the nature of a primary CRM⁻ isolate by comparing the primary isolates to a number of secondarily produced CRM⁻ strains which represent the model system. Additionally, we wish to determine the minimum genetic damage required to produce a CRM⁻ mutant.

2. MATERIALS AND METHODS

(i) *Media*

Cultures of the strains used were grown on agar slants prepared from the minimal medium of Vogel (1956). L-tryptophan (150 μg./ml.) or indole (40 μg./ml.) was added to support growth of mutant strains. Cultures were grown at 30° C.

The details of crossing, media employed, and method of analysis will be described elsewhere (Kaplan, Suyama & Bonner, 1963).

Plating media used in reversion studies are similar to the growth media given above except that 0·05% dextrose is used in place of sucrose and 1% sorbose is added to induce colonial growth.

(ii) *Reversion studies*

Five-day-old conidia of the mutant strains to be tested were suspended in distilled water and filtered through cheesecloth. A sample of the undiluted conidial suspension was overlaid on the appropriate medium as a check for spontaneous reversion. A second aliquot was diluted and various dilutions were overlaid on L-tryptophan supplemented minimal medium to determine the viable count prior to irradiation. Spore suspensions were irradiated at a distance of 15 cm. from a GE-G15T8 Germicidal lamp with continuous stirring for 80 sec. Unpublished experiments have shown that under these conditions, the greatest absolute number of reversions could be obtained. Irradiated cultures were then placed in the dark for 1 hr. After this time, aliquots were overlaid on 5 minimal and 5 indole supplemented plates. An aliquot of the spore suspension was diluted and various dilutions were overlaid on L-tryptophan supplemented plates to determine the percent survival. Plates were

incubated at 30° C. for 48 hr. and then scored for revertants. They were re-scored after an additional 24 hr. incubation.

(iii) *CRM assay*

The method of Suskind, Yanofsky & Bonner (1955) was used to detect and assay CRM. A strain is designated CRM⁻ if it has less than 2·5% the activity of wild type. This level represents the limits of the assay in our hands.

(iv) *Strains*

Td201 is an indole utilizer isolated by Ahmad & Catcheside (1960) and character-ized by Rachmeler & Yanofsky (1961). It was derived from the Emmerson strain 5256 by UV irradiation and originally this strain was designated A78.

The remaining strains used in this study have been described in detail elsewhere (Kaplan, Suyama & Bonner, 1963).

3. RESULTS

(i) *Isolation of the double mutants*

Strain td201 was grown on L-tryptophan supplemented medium. Three-day-old conidia were suspended in distilled water and filtered through cheesecloth. The suspension was then irradiated with UV to a survival of 10%. After 1 hr. in the dark, the conidia were added to 500 ml. of liquid supplemented indole medium. Mutants were concentrated by the filtration technique of Woodward, De Zeeuw & Srb (1953). After filtration for 5 days, the remaining conidia were plated on L-tryptophan supplemented plates. Colonies were cut out and transferred to tryptophan growth tubes. Each colony so isolated was tested for growth on minimal and indole supplemented media. Strains having acquired a specific requirement for tryptophan were classified as presumptive double mutants. These were then back-crossed to the St Lawrence wild type, and tryptophan requiring mutants of both mating types were isolated. In all, five tryptophan-requiring mutants were isolated from the indole utilizing strain td201. These five strains showed an absolute requirement for tryptophan for growth in contrast to the parental strain td201. The strains have been designated 78–101, 78–109, 78–110, 78–137 and 78–174.

(ii) *CRM determinations*

Strains 78–101, 78–109, 78–110 and 78–174 were all found to be CRM⁻. Strain 78–137 was found to be CRM⁺. This strain, like the parental strain, was shown to have high CRM activity in crude extracts. In addition, the mutation introduced into this strain had no effect on the ability of CRM in this strain to be repressed or derepressed by L-tryptophan. The extent of repression and derepression is the same as that observed in the parental strain td201. Because we were able to isolate four CRM⁻ double mutants, we felt that this approach was valid. It was of interest, however, that it was also possible to obtain a CRM⁺ double mutant.

(iii) *Mapping*

If our hypothesis concerning the type of lesion which is responsible for a CRM⁻ phenotype is correct, it would be expected that the secondarily derived CRM⁻ strains should also be difficult to position within the map of the td locus.
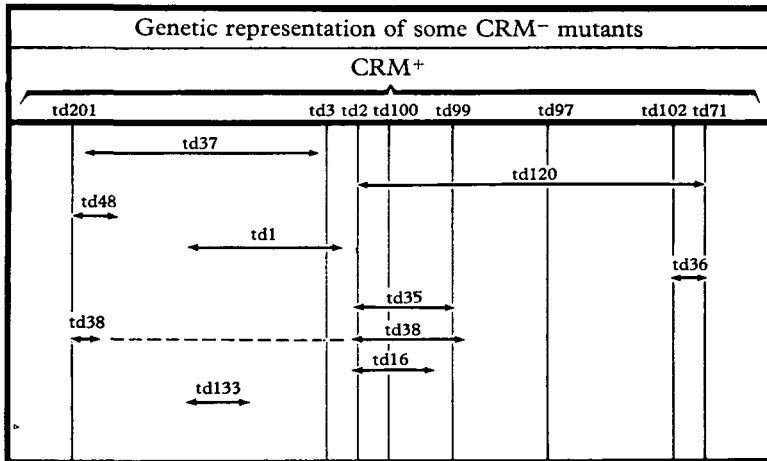

Fig. 1. CRM⁺ markers have been listed above the line. CRM⁻ lesions and the sites they overlap have been listed by arrows.

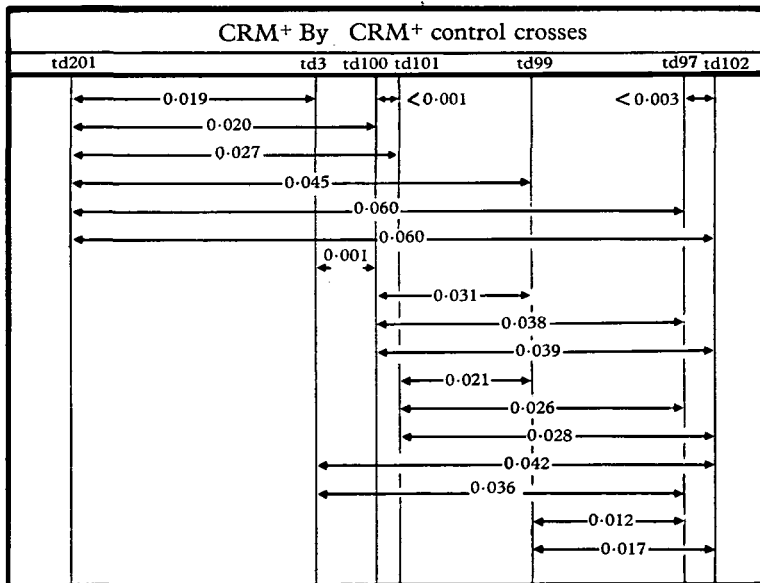
Fig. 2. CRM⁺ markers have been listed above the line. Map distances between markers have been listed by arrows.

Figure 1 schematically shows the mapping features of a few representative primary CRM⁻ mutants. The position of CRM⁺ markers involving the same genetic regions are listed for comparison.

From these results, it can be seen that the CRM⁻ mutants appear to have either extended genetic lesions or multisite lesions. In either case, it becomes quite clear that these mutants are difficult to position with a high degree of accuracy.

In contrast, CRM⁺ mutations are relatively easy to position within the map (Fig. 2). Detailed results of these crosses and those to follow will be presented elsewhere (Kaplan, Suyama & Bonner, 1963). In general, the additivity of distances is good. Each marker can clearly be positioned relative to all markers and each marker appears to represent a point mutation.

The evidence so far presented points out the striking dissimilarity between primary CRM⁺ and primary CRM⁻ mutants when these mutant types are compared in genetic fine structure analysis. These analyses would appear to be in accord with the phenotypic results. Extended genetic damage may account for the apparent complete absence of a protein serologically related to tryptophan synthetase.

(iv) *Double mutants*

The presumptive double mutants were first verified as true double mutants and positioned relative to a single CRM⁺ marker. Figure 3 illustrates how this can be accomplished with only one cross involving each of the double mutants.
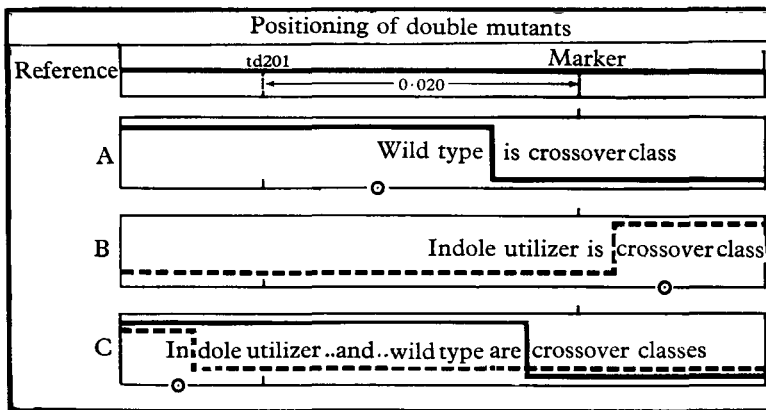

Fig. 3. Explanation in text.

In Fig. 3a, it can be seen that if the newly introduced mutation (⊙) of a double mutant lies between the td201 allele and a known marker allele, wild-type recombinants will result as the only single crossover class that can be recognized.

The second possibility, shown in Fig. 3b, depicts a situation where the second mutation is to the right of td201 and the same td marker. Indole utilizers will be the only recognizable single crossover class.

In Fig. 3c, it can be seen that it is possible to recognize both indole utilizer and wild-type recombinant classes resulting from a single crossover when the second mutation is to the left of both the td201 allele and the td marker allele.

(v) *Three-point crosses*

The previous crosses have demonstrated that CRM⁺ mutations may be positioned relative to any other CRM⁺ mutation. Primary CRM⁻ mutations do not show these properties.

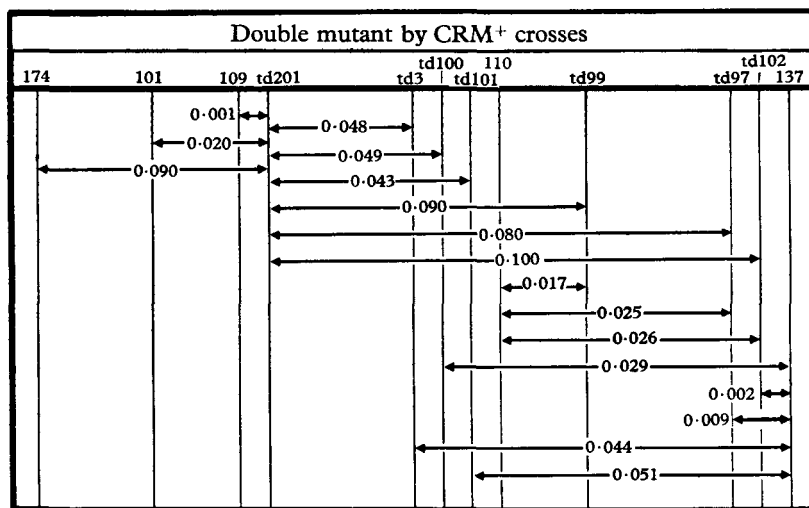Fig. 4. Point mutations have been prefixed with td. The second component of the double mutants has been numbered without a td prefix. The map distances from td201 to the td markers are pooled values from crosses of the double mutants 78-101, 78-109, and 78-174.
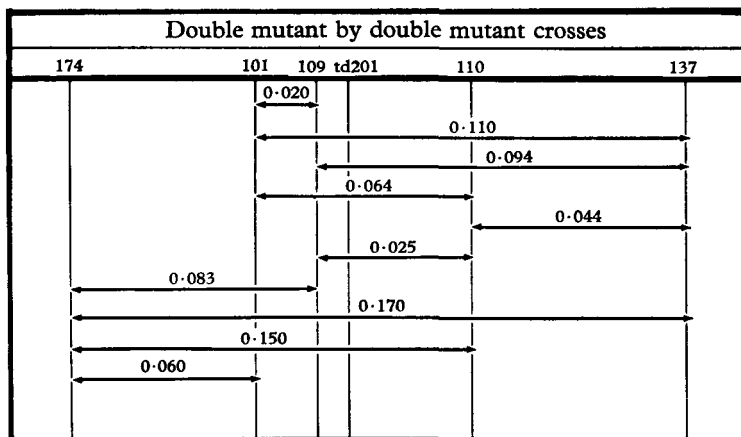

Fig. 5. The double mutant alleles have been listed above the line. Map distances have been represented by arrows.

Figure 4 summarizes the results obtained when the five double mutants were crossed to the CRM⁺ td marker strains listed. From these results three conclusions may be made: (a) All of the alleles map as point mutations. (b) The relative positions of all of the td marker strains are maintained. (c) The double mutants map as would

be expected of two independent point mutations. When contrasted to the secondary CRM⁻ isolates, the primary CRM⁻ isolates do not behave as multisite mutants.

When employing the double mutants in three-point crosses, the distances determined were based upon the frequencies of both prototrophs and indole utilizers. It was, therefore, of interest to test the validity of these mapping data by determining the total distance between alleles of the double mutants on the basis of one recombinant class.

Figure 5 shows the last series of crosses presented. In this series double mutants were crossed by double mutants; indole utilizing recombinants are the only crossover class capable of being isolated. From the three-point crosses discussed above, the recombination frequencies for this series of crosses could be predicted. With only one exception, it can be seen that the recombination frequencies obtained represent the added values of the frequencies listed in Fig. 4.

When a comparison is made between the primary and secondary CRM⁻ mutants, the two appear to represent different types of genetic lesions. The former are not amenable to fine structure mapping employing two-point crosses whereas the latter seem to be highly resolvable when employed in critical genetic analyses. Since the crossing data show that the secondary CRM⁻ isolates are composed of two discrete point mutations, it may be predicted that they should also differ from the primary CRM⁻ mutants with respect to their reversion properties.

(vi) *Reversion studies*

As mentioned above, primary CRM⁻ mutants are capable of being induced to revert to both prototrophs and indole utilizers. The indole utilizers so derived are in turn revertible to prototrophy.

It should be mentioned that the possibility of an outside suppressor was examined in connection with the reversion properties of these primary CRM⁻ mutants. No indication of a suppressor mutation was obtained.

Table 1. *Reversion studies on double mutants, td201 and one representative primary CRM⁻ isolate*

Strain*	Viability		Spontaneous reversion		Induced reversion/10^8: surviving conidia	
	Pre-irrad.	Post-irrad.	Indole	Minimal	Indole	Minimal
td201	5×10^8	1×10^8	0	0	—	5 (25)†
78-109	7.5×10^9	1.5×10^9	0	0	3.3 (250)	0
78-110	4.8×10^{10}	8.7×10^9	0	0	0.6 (240)	0
78-101	2.4×10^{10}	4.2×10^9	0	0	0.1 (20)	0
78-137	1.1×10^{10}	2.1×10^9	0	0	1.0 (103)	0
78-174	2×10^{10}	1×10^{10}	0	0	1.2 (606)	0
td48 CRM negative isolate	2.6×10^8	6.0×10^7	0	0	330 (198)	105 (63)

* See text under 'strains'.
† (N) = actual number observed.

Table 1 is a summary of the reversion data, using UV irradiation, obtained with one such primary CRM⁻ mutant as well as the double mutants. In addition, the result obtained with the point mutant parental strain td201 is listed. With the exception of one strain, approximately 80% kill was obtained. No strains were found to yield spontaneous revertants.

All five double mutants could be induced to revert to growth on indole medium, but no prototrophs were obtained. The parental strain, as expected, reverts to prototrophy. From the reversion frequencies obtained for the double mutants and that obtained for the parental strain, we can calculate that, were a double mutant capable of reverting to prototrophy, it should do so at a frequency between 1 in 10^{14} to 1 in 10^{16}.

The evidence indicates that the primary CRM mutations do not show those properties characteristic of intragenic multisite mutations.

4. DISCUSSION

In the foregoing section, we have dealt with the nature of the genetic lesion responsible for the CRM⁻ phenotype of primary mutant isolates at the td locus of *Neurospora crassa*. From the experimental observations cited above, it is necessary to consider the nature of a mutational event which could result in the complete loss of the protein end product.

Barring the possibility that these primary CRM⁻ negative mutants represent a more exotic mutational event than those considered here, the more likely alternative explanation remains; they are a distinct class of point mutations.

Recent evidence (Freese, 1961; Tsugita & Fraenkel-Conrat, 1962) suggests that a point mutation results from the change of a single nucleotide pair within the DNA.

Forgetting for the moment the nature of the point mutation responsible for a primary CRM⁻ mutant located near the indole utilizing region of the td locus, we are able to suggest an explanation for the peculiar reversion properties of these mutants. From the primary mutant two recognizable changes may occur upon further irradiation. First, a true back mutation ot the original wild-type state and second, a change within the already altered coding frame giving rise to a protein with partial catalytic activity, an indole utilizer. Upon subsequent irradiation of the indole utilizer, a third change occurs within the same coding frame resulting in a wild-type organism. All that need be considered here is the possibility of having two apparently normal enzymes possessing two different amino acids at the same site within the polypeptide chain. Experimentally, this situation has been realized with the A component of *E. coli* tryptophan synthetase (Henning & Yanofsky, 1962).

The data of Henning & Yanofsky (1962) also indicate that the alteration of a single base pair leading to a single amino acid substitution may result in a CRM⁺ phenotype.

The question then remains as to whether or not we can account for a CRM⁻ mutant from our understanding of a point mutation? In consideration of this question, we wish to suggest several alternatives: (1) *Unrestricted alterations*,

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whereby one of a large number of amino-acid replacements results in a CRM⁻ mutant; (2) *Restricted alterations*, whereby a few key amino-acid changes give rise to a CRM⁻ mutant; (3) *Nonsense alteration* as used by Benzer & Champe (1962) to explain the occurrence of ambivalent r_{II} mutants in phage T_4, and (4) *Reading frame alterations*.

The end result of any of these events could be the apparent absence of CRM as determined by the assay procedure. The nature of this assay would not allow us to detect those antigenic regions of tryptophan synthetase not involved in enzymatic activity.

Present studies allow us to state that in a number of td mutants, currently designated CRM⁻, there exists a protein species serologically related to the wild-type enzyme. The nature of the cross-reaction shows some correlation with the position of the mutant within the td locus. These findings (to be published) should enable us to determine the nature(s) of a primary CRM⁻ mutation.

The remaining characteristic of these primary CRM⁻ mutants and perhaps their most interesting feature revolves about their unusual mapping properties in two-point crosses. We can present no reasonable explanation for these observations.

From the above discussion, we may conclude that for the present at least, the structural and functional nature of a CRM⁻ mutation remains an enigma. The data are compatible with the conclusion that we are dealing with a series of point mutations. The possibility that CRM⁻ mutations involve a unique class of multisite or deletion type mutations appears unlikely. Methods presently available should allow us to resolve some of these problems.

5. SUMMARY

The possibility that primary CRM⁻ isolates at the td locus of *Neurospora crassa* are multisite lesions has been examined. By comparing the behavior of primary CRM⁻ mutants to known multisite CRM⁻ mutants in the genetic tests of recombination and reversion, it can be concluded that primary CRM⁻ mutants are not multisite. The genetic implications of a CRM⁻ and CRM⁺ mutant have been discussed.

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