SUPPRESSION OF A NEUROSPORA *td* MUTANT THAT LACKS CROSS-REACTING MATERIAL

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Received January 28, 1966

THE *td* locus in the fungus *Neurospora crassa* prescribes the amino acid sequence of the enzyme tryptophan synthetase which catalyzes the terminal reaction in the biosynthesis of tryptophan. It has been shown that the enzyme is capable of catalyzing three reactions (YANOFSKY 1960) as shown in Figure 1, and that reaction 1 is the important physiological reaction.

A large number of point mutants have been isolated at the *td* locus. These strains have been conventionally classified as CRM^+ or CRM^- based on the presence or absence in crude extracts of an antigen ("cross-reacting material") that reacts with neutralizing antiserum to tryptophan synthetase. The two classes appear in about equal numbers among the point mutants isolated.

It has been a general observation in the late DAVID BONNER's laboratory and in others that CRM^+ mutants at the *td* locus of Neurospora are readily suppressible and that CRM^- mutants are nonsuppressible. This observation is not in agreement with results found for analogously designated mutants in other organisms (GAREN and SIDDIQI 1962; ALLEN and YANOFSKY 1963) and, if true, would suggest that CRM^- mutants at the *td* locus of Neurospora share some unique property and are fundamentally different from suppressible CRM^- mutants. It should be noted that CRM^- mutants at the *td* locus of Neurospora share one other rather strange genetic characteristic—they tend to map as deletions in two-point intragenic crosses (SUYAMA, LACY, and BONNER 1964) and as point mutations in three-point intragenic crosses (KAPLAN, SUYAMA, and BONNER 1964).

Since it is of considerable interest to find such a large group of mutants which are nonsuppressible, and therefore different from similar mutants in other organisms, and since there is really no clear explanation for these facts, the problem was reexamined.

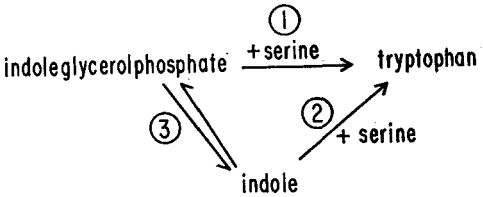

FIGURE 1.—Reactions catalyzed by tryptophan synthetase.

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Strain *td*140, a revertible CRM$^-$ mutant mapping at the extreme right of the locus was selected as the initial test organism. Since it seemed possible that very few of the prototrophs recovered after ultraviolet light (UV) treatment of *td*140 might be the result of suppression, a general method was devised to screen a large number of prototrophs for suppressor mutations. Evidence will be presented that *td*140 can be suppressed by more than one suppressor gene and that the enzyme formed by one of the suppressed strains is similar to wild-type as judged by enzymatic, physical, and immunological criteria.

MATERIALS AND METHODS

Description of mutants: The following mutants were all derived from wild type 74A: *td*140 is a revertible CRM$^-$ mutant; *td*120 a deletion mutant; and *td*141 a CRM$^+$ indole utilizer. All require tryptophan for growth.

Media and crossing: Suppressed strains were maintained on VOGEL's minimal medium (1956) and the mutants on the same medium supplemented with 150 μg L-tryptophan per ml. Strains used as protoperithecial parents were inoculated onto slants containing 2% cornmeal agar supplemented with 0.2% dextrose. When either a *td* mutant or a suppressed strain was to be used as protoperithecial parent, 200 μg of L-tryptophan were added per ml of crossing medium. After 4 to 5 days at 25°C, the cross was completed by addition of approximately 0.5 ml of a conidial suspension from a strain of the opposite mating type. Crosses were then allowed to remain at 25°C until spores were ejected (4 to 6 weeks).

Methods of obtaining suppressors: In order to survey a large number of revertants for suppressor mutations, a method based on plating spores was devised. A fresh conidial suspension of the mutant was placed 15 cm from a General Electric G15T8 germicidal lamp and irradiated for 90 seconds with constant shaking. The conidia were then plated on minimal sorbose medium (Vogel's minimal plus 2% sorbose, 0.5% glucose and 1.5% agar). This was accomplished by adding 1 ml of a suspension of the desired spore concentration to 5 ml liquid minimal sorbose (40°C) which was then layered over a petri plate containing 20 ml cooled minimal sorbose medium. The concentration of conidia was adjusted to yield on the order of 25 or fewer revertants per plate in order to minimize mixing of prototrophs. The colonies to be tested on each plate were transferred to slants containing minimal medium. Since these cultures were derived from multinucleate conidia, they were backcrossed to *td*120, the deletion mutant, to obtain homokaryotic cultures. The ascospores from the cross to *td*120 were suspended in minimal sorbose and heat-shocked for 40 minutes at 60°C, then layered on petri plates containing 25 ml minimal sorbose. Care was taken to obtain plates with only a few colonies to minimize the formation of heterokaryons. One colony from each cross, which was presumably homokaryotic, was then crossed to wild type (74A or 8a) in order to determine whether any *td*$^-$ recombinants (tryptophan requiring colonies) could be obtained. *td*$^-$ recombinants were detected as follows. Ascospores were plated on minimal sorbose medium as described and allowed to grow for 2 days at 30°C. On the third day, a thick suspension of tryptophan supplemented (150 μg/ml) sorbose medium was added and the plates were allowed to remain at 30° for an additional 3 days. The appearance of microcolonies on plates after the addition of tryptophan suggested *td* mutants. This was checked in each case by the transfer of six to ten of these colonies to tryptophan-supplemented medium and a subsequent analysis of their tryptophan requirement in liquid minimal medium.

Preparation of extracts for complement fixation and heat denaturation curves: An extract from a *td*140-suppressed strain was prepared through the first ammonium sulfate step by the procedure of ENSIGN, KAPLAN and BONNER (1964). The precipitate was then dissolved in buffer #2: 0.1 M potassium phosphate (pH 7.8); 10^{-2} M DL serine; 10^{-4} M pyridoxal phosphate; and 2×10^{-2} M EDTA, centrifuged 5 hr at 50,000 rpm (#50 rotor, Spinco model L centrifuge), the pellet discarded, and the supernatant spun an additional 5 hours at the same speed. Tryptophan synthetase was precipitated by the addition of an equal volume of cold saturated ammonium sulfate. The

TABLE 1

Tetrad analysis of td*140 suppressed mutants* × *wild type*

Strain crossed × wild type	Ascus types		
	8T+	6T+ : 2T-	4T+ : 4T-
*td*140 *su*140–3	0	0	3
–10	0	4	1
–15	1	4	1
–16	0	0	4
–19	0	4	1
–23	1	2	0
–25	0	5	0
–32	0	3	2
–33	1	4	0
–35	1	1	1
–41	0	2	1
–42	0	3	2
–46	1	2	1
–52	0	0	5
–55	1	3	1
–61	2	2	1
–64	1	2	0
–81	1	2	3
–88	1	3	2

T+ tryptophan independent; T- tryptophan dependent.
The numbers in the table refer to the number of asci found of each type.

precipitate was dissolved in saline-veronal (0.01 M) buffer pH 7.2 and passed over G25 Sephadex equilibrated with the same buffer. This preparation was then assayed by complement fixation according to the procedure of KAPLAN, ENSIGN, BONNER, and MILLS (1964). For thermostability studies, extracts containing the enzyme were prepared by the method of ENSIGN *et al.* (1964) up through the first ammonium sulfate step. The precipitate was then dissolved in buffer #2 and passed over a G25 Sephadex column equilibrated with the same buffer. Enough ammonium sulfate was added to bring the solution to 5% saturation in order to facilitate removal of denatured proteins formed during the heat denaturation step.

Other procedures: Enzyme assays followed the procedures described by DEMOSS (1962). The sedimentation coefficient value for tryptophan synthetase from the suppressed strain was measured in an YPHANTIS-WAUGH separation cell by the methods of YPHANTIS and WAUGH (1956).

RESULTS

Reversion of td*140:* Conidia of the CRM⁻ point-mutant *td*140, which were UV irradiated and plated on minimal-sorbose medium, gave rise to 344 prototrophs on a total of 15 petri plates. Within two days after plating, 154 colonies had appeared. These were eliminated before conidiation by cutting a small disc around each with a glass tube to which was applied a vacuum. The additional 190 colonies appeared during the next 7 days.

Since it is known that suppressed strains in *N. crassa* are slow growers, this class was most closely examined in the analysis for suppressor genes. A total of 45 slow-growing prototrophs and five fast-growing prototrophs were analyzed

by the plating technique outlined in METHODS. Forty of the 45 slow-growing colonies which were recovered after a cross to *td*120 yielded tryptophan-requiring colonies when crossed to wild type, whereas the five fast growers yielded no such cultures. This indicated at least three different classes of prototrophs: those that grow like wild type (about 50%), those that grow slower than wild type and are the result of suppression (about 40%), and those that grow slower than wild type but are nonsuppressed strains (about 10%).

To test this conclusion, 19 of the suppressed strains were analyzed in the cross to wild type by ordered ascospore analysis (RYAN 1950). As shown in Table 1, 16 of the 19 yielded $6T^+:2T^-$ asci (6 tryptophan-independent spores and 2 tryptophan-dependent spores) showing the independent segregation of two genes whose products interact when present in a common cytoplasm. The even ratio of $4T^+:4T^-$ and $8T^+$ asci suggested that the suppressed cultures were homokaryotic (usually two or three perithecia were analyzed in each cross). These results demonstrate the reliability of this screening method for discovering and isolating large numbers of suppressor mutations for CRM^- lesions at the *td* locus.

Characteristics of td*140 suppressed strains:* The genotypes of the colonies resulting from $6T^+:2T^-$ asci were determined by crossing the slow growing colonies (determined by growth tubes—RYAN, BEADLE, and TATUM 1943) to wild type; those spores pairs which gave rise to td^- progeny were classified as td^- su^-, those in the same ascus which gave only wild-type progeny were classified as td^+ su^- (in this paper su^+ refers to the wild-type allele of the suppressor gene, su^- to the allele capable of suppression). In some cases tryptophan synthetase activity was used to confirm the genetic analysis: td^+ su^- strains showed wild-type activity and td^- su^- strains low specific activity (to be discussed).

Growth rates for td^- su^- strains and td^+ su^- strains were determined on minimal and tryptophan supplemented medium (150 μg/ml). As shown in Figure 2 the td^- su^- cultures show considerable variation in growth rate on minimal medium. In one case, the rate of growth was only 14% of wild type whereas in others it was within 80% of wild type. Unlike other suppressors at the *td* locus, the td^+ su^- strains gave growth rates almost identical to the td^- su^- strains. Also, unlike other suppressed mutants at the *td* locus (YANOFSKY and BONNER 1955), tryptophan did not stimulate growth of the td^- su^- genotype. There was however one excep-

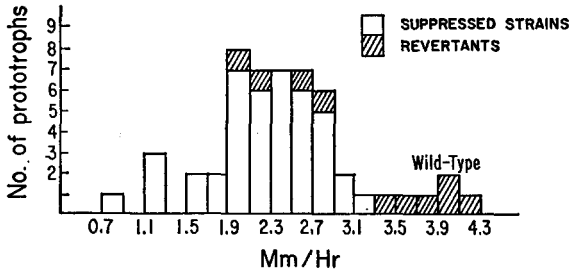

FIGURE 2.—The average linear rate of growth of the *td*140 prototrophs which were obtained on 30 cm growth tubes containing minimal medium.

tional strain isolated (*td*140 *su*140–42) which behaved like the suppressed CRM+ strains described by YANOFSKY and BONNER—the *td*⁻ *su*⁻ genotype grew much slower than the *td*+ *su*⁻ genotype, and the former was stimulated some tenfold by tryptophan when grown on liquid medium.

The number of suppressor genes and their specificity: The different growth characteristics observed for the suppressed strains suggested that several suppressor genes were involved. To test this hypothesis crosses were made between various suppressed strains. Unfortunately, most crosses proved to be infertile, so that sufficient data for analysis is only available for six suppressor strains. The results are shown in Table 2.

If the suppressor genes are unlinked, *td*⁻ *su*⁻ × *td*⁻ *su*⁻ crosses should yield tryptophan dependent colonies and wild-type-like colonies in a 1 to 3 ratio. The ratio from *td*+ *su*⁻ × *td*⁻ *su*⁻ crosses should be 1 to 7 if the suppressor genes are unlinked. Fourteen of the 16 crosses involving the six strains are consistent with the idea that five of the suppressor genes are linked, and that the remaining one is unlinked and therefore represents a different suppressor gene. The two crosses (5 and 9) which are not consistent with this hypothesis possibly indicate, among other things, that the linked suppressor genes are not allelic or that they tend to be unstable. There were no slow growing suppressed strains among those tested.

The question arose whether suppressor genes for *td*140 would suppress any other CRM⁻ mutants. At present only one additional CRM⁻ mutant has been examined—*td*128, mapping at the opposite extreme of the locus. In a cross of *td*140⁻ *su*140–10⁻ × *td*128, two 6T⁻:2T+ asci were observed as well as four 4T⁻:4T+ asci. The appearance of 6T⁻:2T+ asci demonstrated unequivocally that

TABLE 2

Crosses between suppressed strains

Cross	Progeny* T+	Progeny* T-
(1) *td*140 *su*140–10 *A* × *td*140 *su*140–61 *a*	25	0
(2) *td*140 *su*140–81 *A* × *td*140 *su*140–61 *a*	20	0
(3) *td*140 *su*140–23 *A* × *td*140 *su*140–61 *a*	25	0
(4) *td*140 *su*140–61 *A* × *td*140 *su*140–61 *a*	20	0
(5) *td*140 *su*140–61 *a* × *td*140 *su*140–81 *A*	30	2
(6) *td*+ *su*140–23 *a* × *td*140 *su*140–81 *a*	40	0
(7) *td*+ *su*140–10 *A* × *td*140 *su*140–23 *a*	40	0
(8) *td*+ *su*140–61 *A* × *td*140 *su*140–23 *a*	41	0
(9) *td*+ *su*140–61 *A* × *td*140 *su*140–10 *a*	31	1
(10) *td*140 *su*140–15 *A* × *td*+ *su*140–10 *a*	40	4
(11) *td*140 *su*140–15 *A* × *td*+ *su*140–23 *a*	54	3
(12) *td*140 *su*140–15 *A* × *td*140 *su*140–81 *a*	17	4
(13) *td*140 *su*140–15 *A* × *td*140 *su*140–61 *a*	15	0
(14) *td*140 *su*140–15 *A* × *td*140 *su*140–46 *a*	9	3
(15) *td*+ *su*140–46 *a* × *td*140 *su*140–15 *A*	40	2
(16) *td*+ *su*140–46 *a* × *td*140 *su*140–23 *A*	39	0

* T+ tryptophan independent; T- Tryptophan dependent.

*su*140–10 cannot suppress *td*128. A more complete analysis of the specificity of *td*140 suppressor genes is being made.

Properties of the td⁻ su⁻ *enzyme:* For a serological comparison of the gene product of a *td*140 suppressed strain with that of wild type, the active component of *td*140 *su*140–23 was purified fivefold as described in METHODS. This purification results in the elimination of a heavy protein which is found in the mutant *td*140 and which shows complement fixation with the standard anti-tryptophan synthetase antiserum (unpublished results of HARVEY HERSCHMAN and STANLEY MILLS). The specific activity of this preparation for reaction 2 (the conversion of indole to tryptophan) was 0.14 μmole of indole consumed/hr/mg of protein. One μmole indole consumed/hr/mg protein constitutes one unit of enzyme activity and for equivalence with the standard anti-tryptophan synthetase serum 0.9 units are required per ml of serum (KAPLAN, ENSIGN, BONNER and MILLS 1964). In this particular assay, it was predicted that equivalence for the *td*⁻ *su*⁻ preparation would be reached at 5.1 mg of protein. As can be observed in Figure 3, the predicted results were obtained. This indicated that specific activity is a true reflection of the amount of tryptophan synthetase present in the *td*140 suppressed strain. Consequently, it can be concluded that the specific activity per mg of suppressed enzyme is similar to wild type. Since the specific activity in crude extracts is 0.03 and wild type 0.20, it can be concluded that the suppressed strain synthesizes tryptophan synthetase at an efficiency of 15% of wild type. As judged by the complement fixation curve it can also be concluded that the suppressed enzyme is serologically indistinguishable from wild-type.

The thermostability of tryptophan synthetase from *td*140-*su*140–23 was compared to wild type and *td*141, a mutant with a more heat stable enzyme. In the presence of 10^{-4}M pyridoxal phosphate (buffer #2, see METHODS) at 58°, the rate of denaturation for reaction 2 was identical to wild type, although both were clearly less stable than *td*141. Since tryptophan synthetase is less stable in low pyridoxal phosphate, the relative stability of tryptophan synthetase from wild type and the suppressed strain was tested in 10^{-5}M pyridoxal phosphate before and after 24 hours at 3°C. Wild-type extracts retained 42% of their original

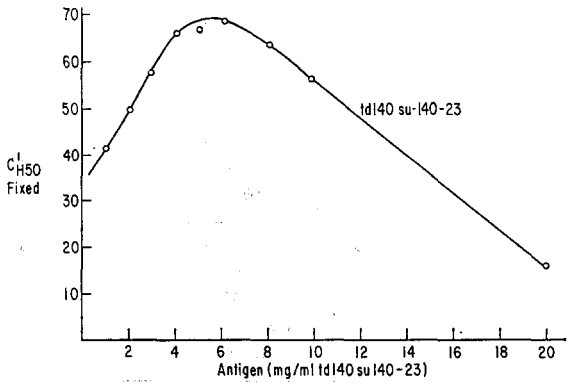

FIGURE 3.—Quantitative complement fixation curve for *td*140 *su*140–23 (a suppressed mutant).

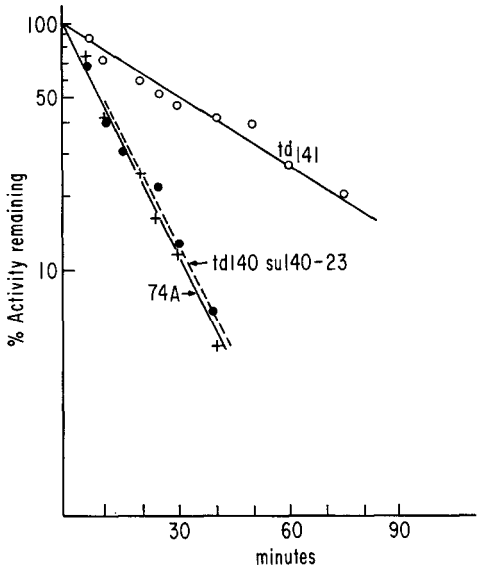

FIGURE 4.—The thermostability of tryptophan synthetase in extracts prepared from *td*141 (a mutant with more heat-stable enzyme than that of wild type), wild type, and *td*140 *su*140–23. 1.5 ml samples of the extracts were placed in a 58° water bath. Two samples were removed for each of the points plotted and placed in a ice bath until they were centrifuged and assayed for enzymatic activity (indole + serine → tryptophan).

activity for reaction 2 and *td*140-*su*140–23, 48%. At 10^{-4}M pyridoxal phosphate each retained better than 95% of their activity under these conditions. That tryptophan synthetase from the suppressed strain is very similar to that of wild type was also shown in the ratio of reaction 1 to reaction 2. The ratio for both wild-type and the suppressed strain was about 1:2 (reaction 1 : reaction 2) which is similar to that reported by DEMOSS (1962).

Since extracts of *td*140 contain no demonstrable enzyme, it was of interest to determine the sedimentation coefficient of tryptophan synthetase from *td*140 *su*140–23. The value as determined by the YPHANTIS-WAUGH separation cell technique was 6.5. This compares favorably to the value of 6.2 reported for wild type by ENSIGN *et al.* (1964).

Specific activities for the suppressed strains examined were similar to other *td*⁻ *su*⁻ mutants described by YANOFSKY and BONNER (1955) and YOURNO and SUSKIN (1965) (~0.03 μmole indole consumed/hr/mg crude extract). Specific activities of the *td*⁺ *su*⁻ strains examined were within the range of wild type.

DISCUSSION

Prototrophs arising after UV treatment of the CRM⁻ mutant *td*140 can be divided into three categories as distinguished by growth rates and genetic analysis. One group, which accounts for approximately half of all the prototrophs, showed growth rates similar to wild type. Suppression was ruled out for those tested in

this group as well as for a minor class of prototrophs (10%) with significantly slower growth rates than wild type. The vast majority of slow growing prototrophs (approximately 40% all prototrophs) were shown to be suppressed strains. These results indicate that at least one CRM$^-$ mutant at the *td* locus of Neurospora is suppressible and that suppression is a highly effective means of obtaining prototrophs. A high frequency of suppressed strains among prototrophs has also been shown in reversion studies involving the A protein (tryptophan synthetase system) in *E. coli* by ALLEN and YANOFSKY (1963).

The observation that individually isolated suppressed strains of *td*140 show considerable variation in growth rate suggested that more than one suppressor gene was involved. An attempt was made to test this hypothesis by making crosses between various suppressor strains. Although most crosses failed, the results do indicate that of the six strains analyzed, five are linked, and possibly allelic, and one is unlinked. These results are similar to those obtained with CRM$^+$ mutants of Neurospora which are suppressible by mutations at several different loci (YANOFSKY and BONNER 1955).

The observation that a CRM$^-$ mutant at the *td* locus is suppressible is in accordance with the results found for analogously designated CRM$^-$ mutants described in other organisms (GAREN and SIDDIQI 1962; ALLEN and YANOFSKY 1963). At this time, it is not clear whether *td*140 or any other CRM$^-$ mutant at the *td* locus is the result of a nonsense mutation such as shown for the *rII* amber mutants of T4 phage by BENZER and CHAMPE (1962) or missense mutations such as demonstrated in *Escherichia coli* for A protein of tryptophan synthetase (YANOFSKY 1963). However, these mutants most closely resemble the CRM$^-$ mutants at the alkaline phosphatase locus of *E. coli* which have been shown to be the result of nonsense mutations (GAREN and SIDDIQI 1962; WEIGERT and GAREN 1965). That is they are nonleaky CRM$^-$ mutants which are revertible and suppressible. Furthermore they map at random along the genetic map.

If *td*140 were a nonsense mutant, three classes of prototrophs might be expected: (1) true revertants (the original nucleotide sequence being restored), (2) other mutational events within the original mutant codon, and (3) suppressors. Class 2 might be expected to give rise to some slow growing revertants since several different amino acids can be substituted at one site (HELINSKI and YANOFSKY 1963; WEIGERT and GAREN 1965). However, second site reversion would not be expected since the retained nonsense codon would lead to peptide fragments (SARABHAI, STRETTON and BRENNER 1964). It is quite possible that the prototrophs obtained from *td*140 fit this classification. The slow-growing, nonsuppressed strains would be exclusively of the second class and the fast growing colonies might be composed of all three classes, although five of the latter were shown to be nonsuppressed strains.

Since it has been shown that *td* mutants of Neurospora which accumulate indole cluster at a different site on the genetic map than those which are capable of utilizing indole for growth (SUYAMA *et al.* 1964; KAPLAN *et al.* 1964), it may be meaningful to note that in those cases where CRM negative mutants have been screened for partial reversion, only those CRM negative mutants mapping at the

"indole utilizer region" revert to "indole utilizers" (Y. SUYAMA, unpublished results). This is readily understood if they are nonsense mutants and the second mutational event took place within the original mutant codon.

One approach to determining whether CRM⁻ mutants at the *td* locus are the result of nonsense mutations is to determine the specificity of the suppressor genes. If in general CRM⁺ mutants at the *td* locus are the result of missense mutations and CRM⁻ mutants the result of nonsense mutations, then suppressor genes for *td*140 should suppress some of the latter, but none of the former (BRENNER, STRETTON, and KAPLAN 1965; GAREN and SIDDIQI 1962; WEIGERT and GAREN 1965). So far only one CRM⁻ mutant, *td*128, has been tested and it was found to be nonsuppressible by a suppressor gene of *td*140.

At the *td* locus of *N. crassa* it is possible to rule our suppression by activation of another pathway for tryptophan biosynthesis because it is not possible to obtain revertants for *td*120, a deletion mutant. Also a CRM⁻ mutant whose gene product is so altered as to be unable to neutralize antiserum against wild-type tryptophan synthetase makes it unlikely that removal of inhibitor would restore activity. Furthermore, the physical, serological and enzymatic evidence presented in this paper suggest that the suppressor gene of *td*140 studied in detail restores a small amount of wild-type-like enzyme (15%). These three facts suggest that the translation process has been altered and as a result the mutant codon is read such that an active enzyme is synthesized at some low rate. Whether a complete but enzymatically inactive protein or a protein fragment is also formed in this strain has yet to be determined.

The author wishes to acknowledge the technical assistance of MRS. MIRIAM BONNER. Special acknowledgment is given to HARVEY HERSCHMAN for his assistance during certain phases of this work.

SUMMARY

It has been a general observation that CRM⁻ mutants (lacking cross-reacting material) at the *td* locus of *Neurospora crassa* are nonsuppressible. However *td*140, a CRM⁻ mutant, is suppressible, in accordance with suppressible CRM⁻ mutants in other organisms. Rather large differences in growth characteristics were encountered among the suppressed strains. Genetic analysis demonstrated at least two different suppressor loci. Extracts from the one suppressed strain studied in detail yielded an active tryptophan synthetase which was indistinguishable from wild-type as judged by physical, enzymatic, and immunological criteria.

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