

are intrinsically defined, and the opportunity to consider viscosity effects and general equations of state is not lost.

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GENE PRODUCTS OF CRM⁻ MUTANTS AT THE TD LOCUS*

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The fungus *Neurospora crassa* normally possesses an enzyme, tryptophan synthetase (t'sase), which catalyzes the terminal reaction in the biosynthesis of the amino acid tryptophan.

The enzyme is recognizable *in vitro* by virtue of its catalytic activity for three reactions: (1) indole-3-glycerol phosphate (InGP) + L-serine → L-tryptophan, (2) indole + L-serine → L-tryptophan, (3) InGP → indole, and by the fact that injection into rabbits of partially purified preparations elicits the formation of neutralizing antisera. Many mutations leading to a growth requirement for tryptophan are known at the genetic locus (td) which determines the primary structure of the enzyme. Of these mutant strains some, designated CRM⁺, synthesize easily recognizable gene products, in that extracts of such strains react with neutralizing antibodies to t'sase, and of these, some still possess enzymatic activity for either reaction 2 or 3. The other class of mutant strains, CRM⁻, yields extracts with no demonstrable enzymatic or serologic activity, in terms of neutralizing antibodies.

CRM⁺ mutants have been intensively studied both enzymatically and genetically.¹⁻⁵ Analogous studies of CRM⁻ mutants have, until recently, been lacking.^{6,7}

With the large number of CRM⁻ isolates available at the td locus and the generalized occurrence of this mutant phenotype in many gene-enzyme systems, an understanding of the nature of the mutational event leading to a CRM⁻ phenotype is essential to an understanding of the gene-protein relationship. Genetic analyses by means of intragenic three-point crosses and reversion tests performed on a number of CRM⁻ mutants at the td locus reveal these strains as products of point mutations.^{5,6} Speculation on the nature of the point mutation resulting in a CRM⁻ phenotype has been published previously.⁶ Detailed phenotypic

analysis of the CRM⁻ strains has recently become possible with the purification of tryptophan synthetase.^{8, 9} Investigations with antiserum to the purified enzyme have revealed that many CRM⁻ strains do make easily detectable gene products at the td locus.

Materials and Methods.—Strains: A detailed description of the CRM⁻ strains used has appeared elsewhere.^{4, 5} Figure 1 shows the relative position of each strain within the genetic map of the td locus (see also ref. 5). It can be seen that the CRM⁻ mutants are distributed at random throughout the genetic locus. Table 1 lists some of the important properties of the strains used in the present study.

Growth and preparation of strains for serological analysis: Strains were grown in liquid culture at 30°C with shaking, using the minimal medium of Vogel.¹⁰ For growth of td-mutants the minimal medium was supplemented with 150 µg L-tryptophan/ml. A 24-hr, 40-ml culture was used as inoculum for 1-liter cultures. Stocks were maintained in test tube cultures on the appropriate media.

For the preparation of extracts, cultures were grown for 48 hr and harvested by filtration through cheesecloth. The mycelia were washed in distilled water, squeezed dry, frozen, and lyophilized. The dried material was powdered in a Wiley Mill and extracted for 30 min at 0°C with veronal buffer¹¹ (pH = 7.3, μ = 0.15, 10 ml buffer/gm powder). Cell wall debris was removed with a 1.5% protamine sulfate solution, and the supernatant was passed over a 2 × 30-cm G25 coarse-grade Sephadex column (using extraction buffer) with the bulk of the protein being collected in a single fraction. Material so prepared is called crude extract. Details of the scheme for large-scale growth and enzyme purification are to be found in references 8 and 9. Enzyme and CRM assays were performed by the methods of Yanofsky¹² and of Suskind, Yanofsky, and Bonner,¹³ respectively. Protein was determined by the method of Lowry *et al.*¹⁴ Before use, the enzymatic, serological, and growth characteristics of each strain were examined as a precaution to rule out reversion during growth.

Serology: Rabbits were immunized with one primary injection (2.5 mg purified protein) in the toe pad, using Freund's adjuvant. After 6 weeks a booster injection (1.0 mg purified protein in saline) was given intravenously and the serum was collected 1 week later. Quantitative complement fixation (C'-fixation) was performed by the method of Mayer *et al.*¹⁵

A unit of complement (C'H₅₀) is defined as that amount of guinea pig serum required to give 50% hemolysis of a sheep erythrocyte antiserum erythrocyte suspension under standard conditions.¹⁵

Guinea pig complement was obtained as the lyophilized material from Baltimore Biological Laboratories (Westchester, Pa.). Sheep erythrocytes in Alsever's solution and antiserum hemolysin were purchased from Hyland Labs (4501 Colorado, Los Angeles, Calif.).

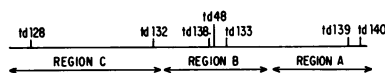


FIG. 1.—A genetic site map of the td locus representing relative positions of the CRM⁻ alleles as determined from three-point crosses. In terms of CRM⁺ mutants, region A has been called the indole-accumulating region, region B the indole-utilizing region, and region C the quiet region.^{1, 5}

TABLE 1
PROPERTIES OF td MUTANTS USED IN THIS INVESTIGATION

Strain	CRM*	Reaction†			Reverts to:	
		1	2	3	Wild type	Indole utilizer
td 139	0	0			+	0
td 140	0	0			+	0
td 133	0	0			0	+
td 48	0	0			+	+
td 138	0	0			+	+
td 132	0	0			+	+
td 128	0	0			+	0
74A (wild type)	+	1, 2, & 3			—	—
td 141	+	2			+	—

* Using the designation of Suskind, Yanofsky, and Bonner (1955).

† Reaction 1: indole glycerol phosphate → L-tryptophan; reaction 2: indole → L-tryptophan; reaction 3: indole glycerol phosphate → indole.

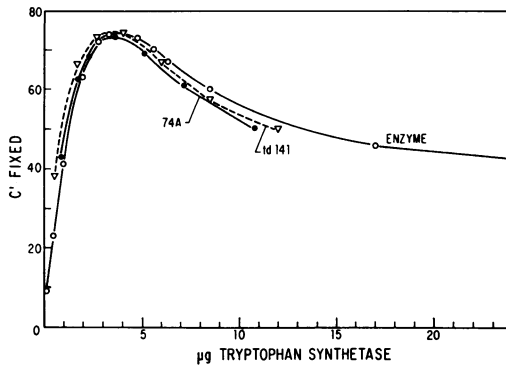


FIG. 2.—Quantitative complement-fixation curves with antiserum to purified tryptophan synthetase at a dilution of 1:90. Complement added at 100 $C'H_{50}$. Enzyme has been purified from strain td 141. 74A and td 141 (see Table 1) are crude extracts, and the micrograms of enzyme were calculated from the specific activities of each preparation and that of the purified enzyme.

Results.—While the absence of enzymatic and serologic cross-reactivity with t'sase neutralizing antibody has, hitherto, rendered ambiguous many efforts to demonstrate a gene product in CRM⁻ strains, there is circumstantial evidence for the existence of such products. Witness that many CRM⁻ strains revert with a respectable frequency to either indole utilization or tryptophan independence. Again, CRM⁻ mutants are capable of complementing with one another or with CRM⁺ mutants (heterocaryon formation) on minimal medium. From such facts it can be inferred that the lesion in the td gene of these CRM⁻ strains is not extensive and that they do form a product. With the purification of t'sase, a screening method for serological cross-reaction between CRM⁻ products and t'sase in terms of all the antibodies to the enzyme, not just neutralizing antibodies, was now set up, employing quantitative C' fixation.

The assay system: To screen CRM⁻ strains for serological cross-reactivity, a standard quantitative C'-fixation curve was obtained with rabbit antiserum to purified t'sase and the enzyme (Fig. 2). With the antiserum at a dilution of 1 to 90, and 100 $C'H_{50}$ in each reaction mixture, a maximum of 75 $C'H_{50}$ was fixed with 3.6 μ g of the enzyme. The curve has proved invariant ($\pm 4\%$) with many repetitions over a period of 6 months. Complete fixation curves were then obtained in triplicate for the purified enzyme from td 141,⁹ crude extracts of td 141, and crude extracts of the wild-type strain 74A from which the derepressed mutant td 141 was isolated. Figure 2 shows the results obtained with each of these preparations. The curves represent a plot of the number of $C'H_{50}$ fixed against the quantity of enzyme in the preparation. In the case of the purified enzyme, this is a weighed quantity. With the crude extracts the values were calculated from the specific activities of the crude preparations and the turnover number of the purified enzyme. Identical curves were obtained for all three preparations, demonstrating the reliability of the method of calculation of the amount of antigen and the method of preparation of the crude extracts. At any point on the curve the reproducibility of the assay has proved to be within ± 3 $C'H_{50}$ units.

The fact that identical results are obtained for each preparation also indicates the immunochemical identity of the antigen in the preparation as measured by this assay system, since the purified enzyme has a 125-fold higher specific activity than the crude td 141, which in turn has a 10-fold higher specific activity than the wild-type preparation. Thus, over a 1500-fold range in protein concentration, identical curves were obtained when the unit plotted on the abscissa is the weight in micrograms of the enzyme in the preparation. This result bears on the relative purity of the antigen and the number of antibody-antigen systems contributing to the C'-fixation curve. Were unrelated antibody-antigen reactions contributing significantly (greater than 5 C'H₅₀) to the amount of complement fixed, these findings would be unexpected.

CRM⁻ screening: The CRM⁻ mutants to be analyzed were chosen as representatives of the three major regions which mutational sites occupy within the td locus. As is seen in Figure 1, td 139 and td 140 are in region A, at one end of the locus, which corresponds to a portion of the indole-accumulating region; mutants td 133, td 48, and td 138, located in region B at the center of the locus, are in the indole-utilizing region; and mutants td 132 and td 128 are in region C at the other end of the locus which represents a mutationally quiet region.^{1, 5} The complement-fixation curves obtained with extracts of these CRM⁻ strains are presented in Figure 3. It is evident from the data that extracts of mutants td 139 and td 140 located in region A did contain cross-reacting antigens and that the antigens differed from one another. Peak fixation for td 139 was 40 C'H₅₀, for td 140, 30 C'H₅₀. The shapes of the curves were similar to each other but different from the standard system; both showed a broad equivalence zone with no region of antigen excess demonstrable with the amounts of antigen employed.

The curves obtained with CRM⁻ mutants td 138 and td 48, located in region B, are shown in Figure 3. Mutant td 138 gave maximum fixation of 30 C'H₅₀ at 1 mg of extract, td 48, 22 C'H₅₀ at 0.5 mg of extract. The shape of the curves for these two mutants is similar in form to the standard system, but differs from those for mutants located in region A. Strain td 133, located in region B, shows no C'-fixing activity over the 32-fold range of protein concentrations used. Nor was C'-fixing activity observed with extracts of td 133 at higher levels of antibody.

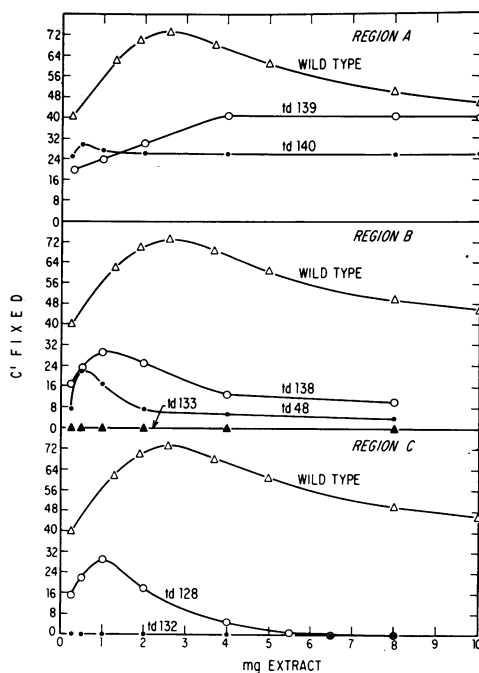


FIG. 3.—Quantitative complement-fixation curves using antiserum to purified tryptophan synthetase (1:90) and 100 C'H₅₀. The abscissa represents milligrams of crude extract. The curve for the wild-type extract has been included as a reference.

Mutant td 128, located in region *C*, also fixed complement (Fig. 3), but the shape of the curve is different from the standard system and from those obtained for mutants in regions *A* and *B*. Td 128 showed a rapid drop in *C'*-fixing activity to zero in antigen excess. Mutant td 132, also plotted in Figure 3, region *C*, showed no activity.

These results demonstrate the presence in five of the seven CRM⁻ strains of a protein species serologically related to the parent molecule. The proteins are not only characteristic of the individual mutants, but those derived from mutations in each region of the locus (*A*, *B*, and *C*) form a class distinct from proteins represented by mutations in the other regions of the locus.

That these results are not attributable to the presence of unrelated antibody-antigen systems common to all of the strains, CRM⁻ and CRM⁺, can be inferred from the following: (1) Each mutant strain used in this investigation has been isolated from the wild-type strain 74A and backcrossed to this wild type. Therefore, the isolation of two strains showing no serologic activity, but isogenic to all of the other strains except at the td locus, indicates the specificity of this system. (2) The maximum levels of fixation differ for different serologically active CRM⁻ mutants. Should a nonspecific system be involved, then each strain would be expected to show the same maximal levels of fixation. (3) The shape of the fixation curves corresponds to the position of the td mutation within the map of the td locus.

Precipitation and neutralization of t'sase by anti-CRM⁻ antiserum: It was observed that all CRM⁻ extracts that showed *C'*-fixation with anti-t'sase antiserum were also precipitated by the antiserum. (CRM⁻ extracts that did not fix complement gave no precipitate.) Precipitates obtained with td 140 (region *A*) and td 128 (region *C*) were washed and injected into rabbits. In preliminary experiments it was shown that the CRM⁻ antisera so obtained would individually precipitate but not neutralize the purified enzyme, and, *in combination*, would neutralize enzyme activity. These results directly demonstrated the antigenic relationship between the CRM⁻ protein and tryptophan synthetase since the rabbits producing these antisera had never been exposed to the competent enzyme.

Discussion.—The investigations were undertaken in order to identify the gene product resulting from a CRM⁻ mutation. With antiserum to purified tryptophan synthetase, it has been possible, by serological methods, to show the existence of gene products as cross-reacting protein antigens in five of the seven CRM⁻ mutants studied. The two CRM⁻ strains without demonstrable cross-reacting antigens are located toward the center of the td locus. The five CRM⁻ strains with activity are scattered throughout the gene, including sites very close to both ends of the locus. It is of interest that all seven CRM⁻ strains used in these studies represent respectable point mutations in terms of their mapping characteristics and reversion properties. However, their gene products, as judged enzymatically and serologically, appear to be more profoundly altered than the products derived from the point mutations leading to the CRM⁺ phenotype. To account for this difference it has been suggested that CRM⁻ strains result from either nonsense mutations¹⁶ or reading-frame alterations,¹⁷ whereas CRM⁺ mutations are merely single amino acid substitutions with the remainder of the proteins intact. The data obtained in these experiments, in particular, the demonstration of antigenic cross-reactivity

in mutants td 140 and td 128 (at opposite ends of the gene), are incompatible with either nonsense mutations or reading-frame alterations as a mechanism to explain all CRM⁻ mutations, if it is assumed that transcription of the genetic material starts at one end of the locus and proceeds uninterrupted to the other end. However, single amino acid alterations remain a possibility.

An alternative explanation consistent with these data is suggested by the enzymatic and genetic similarities of the *E. coli* and *Neurospora* tryptophan synthetase systems. Although the two molecules are indistinguishable enzymatically, in the bacterium two closely linked genes, A and B, prescribe the formation of the dissociable A and B subunits of the *E. coli* enzyme,¹⁸ whereas in the higher organism a single gene prescribes a single protein. Unlike the *E. coli* system, a point mutation in *Neurospora* can lead to the simultaneous loss of all three reactions.

If it is assumed that the *Neurospora* gene has evolved from the *E. coli* genes by the joining of the *E. coli* genes, and if the point of transcription is now taken in the region of fusion (somewhere in the center of the *Neurospora* gene), and if transcription can proceed to the right or to the left independently, then CRM⁻ mutants could be explained by nonsense mutations and/or transcription alterations. CRM⁻ mutants without serological activity then represent a single lesion simultaneously altering left- and right-hand transcription; CRM⁻ strains with antigenic activity reflect transcription alterations either to the left alone or to the right alone. This is supported by the fact that mutants clustered in a given region (A or C) are similar to one another but distinct from those of another region.

These results have demonstrated the usefulness of the present gene-enzyme system for detailed immunogenetic studies in *Neurospora*. Through the use of a complement fixation assay system, it should now be possible to isolate the proteins of CRM⁻ mutants.

Summary.—Complement-fixation analyses have been performed on extracts of CRM⁻ td mutants of *Neurospora*. The results from such analyses demonstrate the existence of protein species serologically related to the parent enzyme. The nature of the cross-reaction appears to be correlated with the position of the CRM⁻ mutation within the map of the td locus.

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ANALYTIC CONTINUATION AND THE SCHWARZ REFLECTION PRINCIPLE

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1. Let I be an open interval on the real axis, G_1 be a region in the upper half-plane, and G_2 be a region in the lower half-plane, such that I is a free boundary arc of both G_1 and G_2 . Suppose that the function $f_1(z)$ is holomorphic in G_1 and $f_2(z)$ is holomorphic in G_2 . Consider the function $f(z) \equiv f_j(z)$ ($z \in G_j$; $j = 1, 2$). T. Carleman,¹ F. Wolf,² and K. Meier³ have shown that under certain conditions it is possible to conclude that f is holomorphic at every point of I or at every point of an everywhere dense subset of I . The conditions in question all involve the behavior of f_1 and f_2 near points of I along vertical straight lines or in Stolz angles. I am first going to prove a theorem of this nature in which it is not necessary to make such stringent assumptions about the mode of approach to points of I .

By an arc Λ_ξ in G_1 (or in G_2) at a point $\xi \in I$ I mean a Jordan arc that extends from a point of G_1 (or G_2) to ξ , and lies, except for ξ , in G_1 (or G_2). Denote by A_∞ the set of points of I at which at least one of the functions f_1, f_2 has ∞ as an asymptotic value, and let \bar{A}_∞ be the closure of A_∞ .

THEOREM 1. *Let S be a subset of I of Lebesgue measure zero and first Baire category. For every $\xi \in I-S$, let Λ_ξ^1 be an arc in G_1 at ξ , Λ_ξ^2 be an arc in G_2 at ξ , ω_ξ be a finite complex number, and put $\varphi(\xi) = \omega_\xi$ ($\xi \in I-S$). Suppose that*

$$(a) \quad \lim_{\substack{z \rightarrow \xi \\ z \in \Lambda_\xi^1}} f_1(z) = \lim_{\substack{z \rightarrow \xi \\ z \in \Lambda_\xi^2}} f_2(z) = \omega_\xi \text{ for every } \xi \in I-S,$$

(b) φ is bounded in some neighborhood of every point of $I-S$,

(c) $\bar{A}_\infty \cap (I-S) = \emptyset$.

Then f is holomorphic at each point of an everywhere dense subset of I .

Proof: Let V_ξ^1, V_ξ^2 be vertical rectilinear segments in G_1, G_2 , respectively, terminating in $\xi \in I-S$. According to my ambiguous-point theorem,⁴ there exists an at most enumerable subset E of $I-S$ such that, for every $\xi \in I-(S \cup E)$, in the notation of cluster-set theory⁵

$$C_{V_\xi^1}(f_1, \xi) \cap C_{\Lambda_\xi^1}(f_1, \xi) \neq \emptyset \neq C_{V_\xi^2}(f_2, \xi) \cap C_{\Lambda_\xi^2}(f_2, \xi).$$

This implies, in view of (a), that ω_ξ belongs to both $C_{V_\xi^1}(f_1, \xi)$ and $C_{V_\xi^2}(f_2, \xi)$ and hence

$$C_{V_\xi^1}(f_1, \xi) \cap C_{V_\xi^2}(f_2, \xi) \neq \emptyset. \tag{1}$$