Immunochemical Distinction between the *Escherichia coli* Polypeptide Chain Elongation Factors T_u and T_s*

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ABSTRACT: An antiserum had been previously prepared against the combined AA-tRNA binding factors T_u and T_s. Here, the specificity of this antiserum against the individual factors T_u and T_s was investigated. It was found that the activity of the antiserum was specifically directed against T_s. This was observed both in immunoprecipitation tests and in immunoinhibition tests. The same specificity was obtained with three independently prepared antisera and the same T factor preparation.

The evidence for the immunochemical distinctness of T_u and T_s is discussed, and also the implications for previous studies with this antiserum.

The elucidation of the role of the separated microbial factors T_u and T_s (Lucas-Lenard and Lipmann, 1966) in polypeptide chain elongation has been progressing in recent years (Ertel *et al.*, 1968a,b; Miller and Weissbach, 1969, 1970; Weissbach *et al.*, 1970; Ravel *et al.*, 1968a,b; Shorey *et al.*, 1969; Skoultchi *et al.*, 1968; Lucas-Lenard *et al.*, 1969). T_u binds to a GTP or GDP moiety, forming a "binary complex," which can interact with AA-tRNA to form a ternary complex. The ternary complex is an intermediate in the binding of AA-tRNA to ribosomes. T_s appears to have a catalytic role in this process (Ertel *et al.*, 1968a; Weissbach *et al.*, 1970; Miller and Weissbach, 1970), and acts by labilizing the bond between T_u and GDP (Miller and Weissbach, 1970), thus facilitating the formation of the ternary complex (Weissbach *et al.*, 1970). Earlier studies made use of the fact that T_u and T_s exist in the form of a stable complex (Nishizuka and Lipmann, 1966; Parmeggiani, 1968; Gordon, 1969; Lucas-Lenard *et al.*, 1969; Miller and Weissbach, 1969), referred to as T (Nishizuka and Lipmann, 1966), which has been purified to homogeneity (Parmeggiani, 1968; Gordon, 1969).

Attempts have been made to obtain an initial evaluation of the role of the T factor in polypeptide chain elongation (Gordon, 1967–1969; Cooper and Gordon, 1969; Ravel, 1967; Lucas-Lenard and Haenni, 1968; Allende *et al.*, 1967), and to investigate its variations between species (Gordon *et al.*, 1969; Krisko *et al.*, 1969) as well as the regulation of its synthesis (Gordon, 1970). Detailed consideration of T_u and T_s separately was deferred, but there is now essentially complete agreement between the results obtained with the T fraction and those obtained with separated T_u and T_s, especially where there appeared to be some measure of discrepancy. Thus, the inability to detect the binary complex on gel filtration, originally attributed to the lability of the binary complex (Gordon, 1968), can now be explained by the ability of T_s to labilize the binary complex (Miller and Weissbach, 1970).

The T fraction dissociates into T_u and T_s in the presence of its substrate(s) (Ravel *et al.*, 1968a; Miller and Weissbach, 1969; Lucas-Lenard *et al.*, 1969), and this has facilitated their separation into functionally and chromatographically distinguishable proteins (Ertel *et al.*, 1968a; Weissbach *et al.*, 1970; Miller and Weissbach, 1969, 1970). This report will describe the more rigorous evaluation of the specificity of an antiserum prepared previously (Gordon *et al.*, 1969) against a homogeneous preparation of T factor (Gordon, 1969). We show here that the antiserum is in fact a highly specific anti-T_s, both in terms of precipitation reactions and in terms of specific inhibition.

Materials and Methods

All the methodology is unchanged from that described previously in other publications from our laboratories. The T_u, prepared by Dr. D. Miller, was homogeneous and crystalline (D. L. Miller and H. Weissbach, to be published), and the T_s, prepared by Dr. J. Hachmann, was approximately 50% pure. The T_u used throughout this study was identical with T_u-GDP described previously (Weissbach *et al.*, 1970). Assays for T_u and T_s were based on the exchange of [^{3}H]-GDP with T_u-GDP as described previously (Ertel *et al.*, 1968; Weissbach *et al.*, 1970) using nitrocellulose filters. The units of T_u and T_s are defined as that amount giving 1 pmole of binding in the assay after 5 min at 0°. T_s was measured as the rate in the presence of excess T_u; T_u as the amount in the presence of excess T_s. The T_u used here had a specific activity of 18,000 units/mg. The T_s was 230,000 units/mg. The reaction mixtures for measuring the exchange of [^{3}H]GDP with GDP bound to T_u contained, in a total volume of 0.2 ml: 0.05 M Tris-Cl (pH 7.5), 0.05 M NH_4Cl, 0.01 M $MgCl_2$, 2.5×10^{-6} M [^{3}H]GDP, and T_u and T_s. Further details are described in the legends. The reactions were stopped by the addition of 0.8 ml of wash buffer (0.01 M Tris-HCl, 0.01 M NH_4Cl, and 0.01 M $MgCl_2$), and the solutions poured through a nitrocellulose filter (Millipore Corp.) and washed with 15 ml of the same buffer. The filter was dissolved in a scintillation fluid (Bray, 1960), and the radioactivity determined. The antiserum was the same as described by Gordon *et al.* (1969), and the turbidimetric assay of immunoprecipitation was as described by Gordon (1970). Each mixture contained 0.1 ml of antiserum

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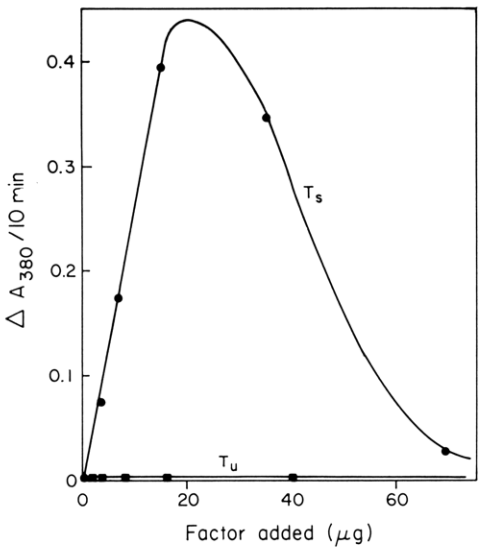

FIGURE 1: Turbidity formation by the T factors and anti-T factor serum. Reaction mixtures were made as described under Materials and Methods, with the amount of T_u (■—■) or T_s (●—●) protein as indicated on the abscissa. The time course of development of the turbidity was followed, and the increment in 10 min was recorded.

(added at zero time), factor protein, 0.1 M NaCl, and 0.01 M potassium phosphate buffer (pH 6.8), as described in the legends. The turbidity was read at A_{380} in a Beckman DB spectrophotometer.

Results

Immunoprecipitation with T_u and T_s. Previous studies described the preparation of a highly specific antiserum directed against the T factor (Gordon *et al.*, 1969), and the separation of T_u and T_s into functionally distinguishable

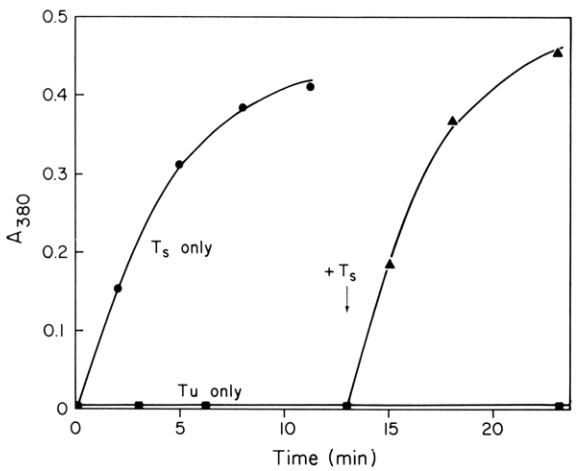

FIGURE 2: Time course of development of turbidity with T factors and the anti-T factor serum. Reaction mixtures were made up as described under Materials and Methods, including (●—●) 17.4 μg of T_s, or (■—■) 0.2, 0.4, 0.8, 1.6, 4.0, 8.0, 16.0, or 40 μg of T_u. To the one containing 8.0 μg of T_u, 17.4 μg of T_s was added at the time indicated by the arrow (▲—▲). Indistinguishable results were obtained with this amount of T_s and 2, 4, or 16 μg of T_u.

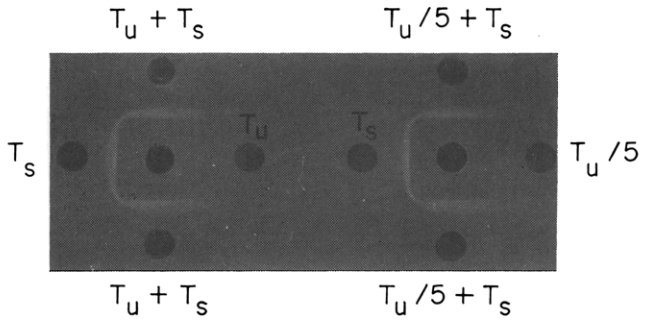

FIGURE 3: Immunodiffusion precipitation of the T factors. The center wells contained anti-T factor serum. Wells labeled T_s contained 0.17 mg/ml, those labeled T_u, 0.8 mg/ml, and those labeled $T_u/5$, 0.16 mg/ml.

proteins (Ertel *et al.*, 1969b; Weissbach *et al.*, 1970; Miller and Weissbach, 1970). The precipitation reaction between this antiserum and the separated T_u and T_s is shown in Figure 1. The T_s reacted as found before for the T factor (Gordon, 1970), but no reaction whatsoever was obtained in the presence of T_u. This was found for a 25-fold range of concentrations of T_u. No cross-reacting material was detected when T_u was tested in the presence of T_s. This was shown in two ways: there was no significant stimulation or inhibition

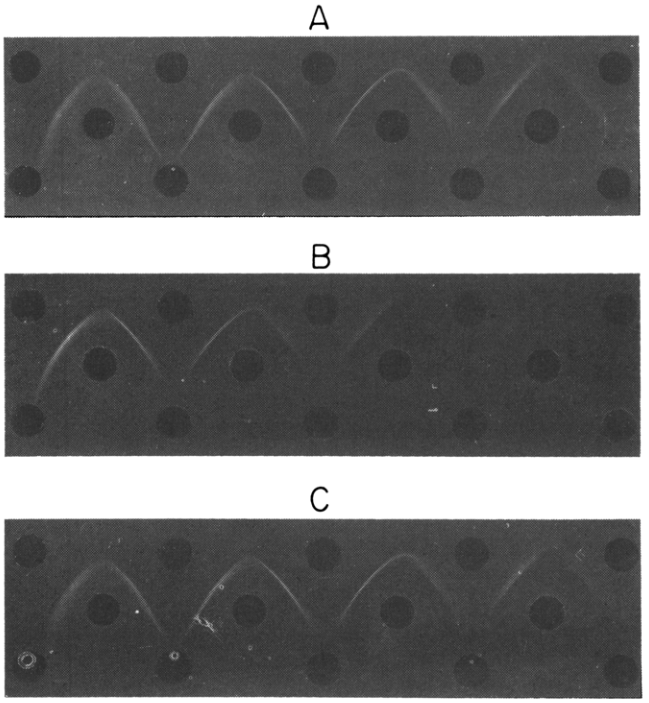

FIGURE 4: Immunodiffusion precipitation of the T factors with antisera from different rabbits. The antisera A, B, and C were all prepared identically (see Gordon *et al.*, 1969), except that A was by intradermal injection and B and C were by subcutaneous and intramuscular injection. In each case, the center row contained the antiserum, the upper wells contained the T_s (from left to right: 1.75, 0.85, 0.42, 0.21, 0.10 mg per ml, respectively), and the lower wells contained the T_u (from left to right: 0.8, 0.16, 0.03, 0.006, and 0.001 mg per ml, respectively).

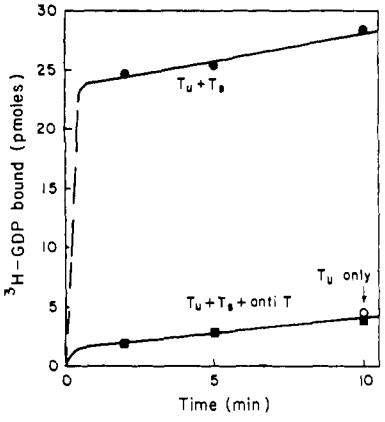

FIGURE 5: Effect of anti-T factor serum on GDP binding at 0°. Binding reactions were as described under Materials and Methods with (■—■) 40 μl of the antiserum, 28 units of T_u, and 60 units of T_s. In addition, assays were run with no antiserum (●—●) or no antiserum and no T_s (○—○). The reactions were stopped at the times indicated on the abscissa.

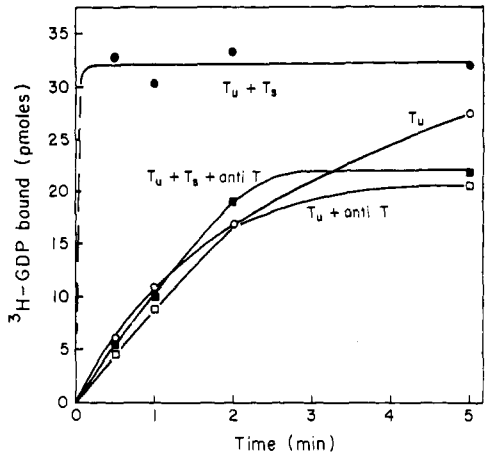

FIGURE 6: Effect of anti-T factor serum on GDP binding at 37°. Reaction mixtures and symbols are the same as in Figure 4, except for the additional experiments with reaction mixtures in which only T_u and the antiserum were present (□—□).

of the development of turbidity, as shown in Figure 2. Again, a range of concentrations of T_u was tested. The same result was obtained with immunodiffusion precipitation analysis on Ouchterlony plates (Figure 3). A single band was obtained with T_s, a reaction of identity with T_u–T_s mixtures, and none with T_u alone. The antiserum prepared against T factor was thus a highly specific anti-T_s by precipitation reactions.

Individual Variability of Antisera. Since individual rabbits vary considerably in their response to antigens, it is possible that the specificity of the antiserum was a result of the particular rabbit. Three individual sera were tested, all made identically, except in the route of entry (see legend to Figure 4). Ouchterlony plates shown in Figure 4 revealed that all three were specifically anti-T_s, except one which showed a barely detectable trace of precipitation in the presence of T_u (not visible in photograph). Thus, the specificity of the antisera reflects the antigenicity of T_s and not the individual variability of antisera.

Immunoinhibition with T_u and T_s. In previous publications it was found that the role of T_s is to render GDP bound to the T_u exchangeable with exogenous GTP or GDP (Weissbach *et al.*, 1970; Miller and Weissbach, 1970). This observation has been used as a functional parameter to define the T_u and T_s activities, and determine whether the results obtained with the precipitation test can be correlated with enzymatic studies. Figure 5 shows that the T_s stimulation of the binding of [^{3}H]GDP to T_u-GDP was inhibited by the antiserum; in the presence of the antiserum, the binding was reduced to the slow rate obtained with T_u alone at 0°. The slow rate of binding in the absence of T_s was stimulated by raising the temperature, and the results in Figure 6 show that at 37° this reaction was not appreciably inhibited by the antiserum. The slight inhibition observed was probably due to nonspecific inhibition, as has been seen with polyphenylalanine synthesis using a control immune serum (Gordon *et al.*, 1969). Again, at 37°, as at 0° (Figures 5 and 6), the T_s stimulation was completely inhibited by the antiserum. That the inhibitory activity was directed specifically to that part of the binding reaction stimulated by T_s was confirmed by the experiment of Figure 7. With increasing amounts of antiserum, the [^{3}H]GDP binding approached the value obtained with T_u alone.

Together, all these results show that the antiserum prepared against the T factor preparation was in fact a highly specific anti-T_s.

Discussion

Immunochemical Distinction between T_u and T_s. Previously, the proposal that T_u and T_s were different was based on their chromatographic separability (Lucas-Lenard and Lipmann, 1966; Ravel *et al.*, 1968b; Ertel *et al.*, 1968a,b; Skoultchi *et al.*, 1968) and distinct properties in function (Ertel *et al.*, 1968b; Weissbach *et al.*, 1970; Miller and Weissbach, 1970). The present studies have shown that they are different proteins with regard to immunochemical specificity.

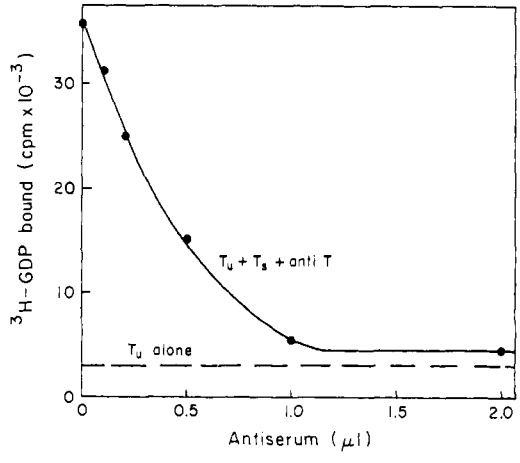

FIGURE 7: Titration of the anti-T factor serum in the GDP binding reaction at 0°. Reaction mixtures were the same as in Figure 5, except that the volume of antiserum was varied as indicated on the abscissa. The dotted line represents the binding with no antiserum and no T_s.

It has always been difficult to verify the role of T_s, since a stringent T_s requirement for the chain elongation process has not been observed. The antiserum, which was previously shown to be a specific inhibitor of the chain elongation process, is shown here to be a specific anti-T_s.[1]

Approximately the same equivalence point for the antiserum was obtained in precipitation tests (0.21 μg of protein/μl of serum, Figure 1) as in inhibition tests (0.26 μg of protein/μl of serum, Figure 7). This confirms that both represent different aspects of the same antibody reaction. It is noteworthy, however, that the precipitation reaction takes many hours at 0°, while the inhibition is essentially instantaneous. This is presumably due to the fact that many more interactions would be required to produce a visible precipitate, whereas a "single hit" would suffice to inactivate an enzyme.

Implications for Previous Studies with the Antiserum. The previous conclusions concerning the species variations of the antigenic determinants of the T factor (Gordon *et al.*, 1969) must now be made more specific, and should only refer to the immunochemical determinants of T_s. In particular, the members of the family *enterobacteriaceae* which were immunochemically indistinguishable by their response to anti-T_s factor serum may in fact be distinguished by an anti-T_u serum.

Previous studies (Gordon, 1970) on the regulation of synthesis of the T and G factors led to the conclusion that they were coordinately regulated with the ribosomes and present in the cell in approximately 1:1 mole/mole stoichiometry with respect to the ribosomes. It was implicit in those determinations that the antiserum used in the quantitation was not specific for T_u or T_s only. It is now evident that the conditions were such that the measurements were specific for T_s. The earlier conclusion must now be made more specific: the *relative* ratio of T_s to ribosomes is constant and independent of the growth rate in cells in log phase. The regulatory characteristics of T_u are therefore still unknown, and this is currently under study.

The *absolute* quantitation of the amount of T in moles per mole of ribosomes was dependent on the calibration of the immunoprecipitation reaction with a homogeneous preparation of T, containing a then unknown ratio of T_u:T_s. More recent experiments (J. Gordon, unpublished) have shown that T factor preparations comparable to those used previously to elicit the antibody, and also to calibrate the immunoprecipitation system, contain T_u and T_s in approximately 1:1 molar ratio. The conclusion that there is present in the cell 1 mole of T_s/mole of ribosomes is therefore probably tenable. These aspects are currently the subject of a more detailed investigation.

[1] Preliminary results with an *in vitro* amino acid polymerization system have confirmed the present results. The antiserum inhibited the T_s stimulation of polyphenylalanine formation in a poly U dependent system. In addition, the inhibition by the antiserum was prevented by the addition of excess T_s, but not by the addition of T_u or G.

Acknowledgments

The continuing encouragement, support, and discussions of Dr. Fritz Lipmann are gratefully acknowledged. The gifts of the purified factors from Drs. D. Miller and J. Hachman are also gratefully acknowledged.

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