A PHAGE-MUTANT DIRECTED SYNTHESIS OF A FRAGMENT OF DIPHTHERIA TOXIN PROTEIN

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SUMMARY. A mutant of converting phage β was isolated by nitrosoguanidine treatment of a lysogenic, toxinogenic strain C7(β) of Corynebacterium diphtheriae. The lysogenic strain obtained by lysogenization of a nonlysogenic, nontoxinogenic strain C7(-) with the phage mutant, produced extracellularly a single major protein species of molecular weight 24,000, having no diphtheria toxicity but having ADP ribosylation activity and cross-reactivity with diphtheria antitoxin. Iron inhibited the formation of the product of the mutant-phage lysogen. The mutation did neither affect the UV inducibility of the prophage nor the phage producing ability of the mutant-phage lysogen.

In an attempt to elucidate the mechanism of regulation of phage-directed synthesis of diphtheria toxin protein, we have isolated a unique mutant of converting phage β. In the present paper we describe the isolation and characterization of the phage mutant, the new lysogen of which appears to produce a small fragment of diphtheria toxin protein.

MATERIALS AND METHODS. Bacterial strains, media employed and the methods for bacterial growth, toxin production and toxin assay and phage techniques were described previously (1-3). Analyses of the extracellular protein products of the lysogens were done using the fractions, precipitable with ammonium sulfate at 70% saturation, of the culture supernatants obtained by incubating the heavy cell suspensions for 2.5 hours under the growth-limiting conditions (3,4). Analytical polyacrylamide gel electrophoresis was performed using 7.5% acrylamide gel by the methods originally described by Davis (5). Molecular weight determinations by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis were carried out as described by Weber and Osborn (6), using bovine serum albumin (Armour Pharmaceutical Co.), pepsin (2x crystallized, Worthington Biochem. Co.) and egg white lysozyme (3x crystallized, Sigma Chem. Co) as standard proteins. ADP ribosylation activity was assayed by measuring incorporation of radioactive label from NAD-(adenosine)-^{3}H (590 μCi/μmole, New England Nuclear) into trichloroacetic acid-precipitable material in the presence of a partially purified preparation of transferase II according to Collier and Kandel (7) with a slight modification.

RESULTS AND DISCUSSION

Isolation and selection of the phage mutant: Cells of a toxinogenic, lysogenic strain C7(β) (wild-type phage lysogen) actively growing in the modified Pope's medium were treated with N-methyl-N'-nitro-N-nitrosoguanidine (Aldrich Chem. Co.) (10 μg/ml, about $4x10^8$ cells/ml) in 0.05 M Tris-maleic buffer pH 6.0 at 37 C for 15 min (surviving fraction about 0.1), washed and diluted in the fresh medium and incubated for 2 hours and then plated on tryptose agar plates for surviving colonies. Each culture of heavy growth in toxin production medium from individual surviving colonies was tested on Ouchterlony's immunodiffusion plates against 5 limit flocculation (Lf) units/ml of horse diphtheria antitoxin, using toxin (5 Lf units/ml) as a reference, for their ability to produce toxin to select "non-toxin producible" lysogens.

Phage derived from "non-toxin producible" lysogens thus obtained were then used to lysogenize a nonlysogenic, nontoxinogenic strain C7(-) and the

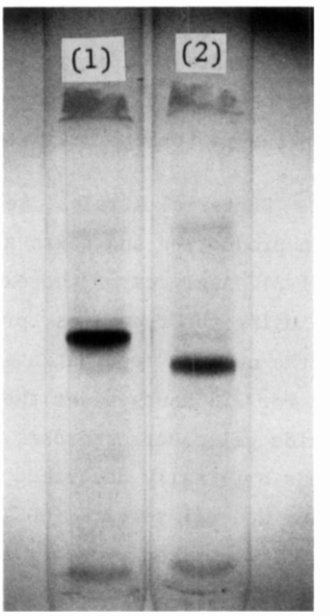

Fig. 1. Electrophoresis in polyacrylamide gel of the extracellular protein fractions of (1) C7(β) and of C7(β-NG2). Total protein applied on each gel was approximately 50 μg. The electrode buffer was 0.05 M Tris, 0.27 M glycine, pH 8.3. Electrophoresis was at a constant current of 2.0 ma/gel for 2.5 hours. Gels were stained in 1% amido black in 7% acetic acid. Migration was from top to bottom.

lysogen newly obtained were again tested as described above for their ability to produce toxin to select phage mutants. In this manner the phage mutant β-NG2 was isolated.

Comparative studies on the properties of the extracellular protein of the mutant-phage lysogen C7(β-NG2) with toxin protein of the wild-type phage lysogen C7(β): In contrast to nonlysogenic strain C7(-), the mutant-phage lysogen was found to produce a comparable amount (about 15 μg/ml) of protein into the culture medium to that by the wild-type phage lysogen. Subsequent analyses by polyacrylamide gel electrophoresis of the extracellular protein product revealed that the mutant-phage lysogen produces a single major protein species of a mobility different from that of toxin (Fig. 1) and that the fraction (8) from the gel corresponding to the single major protein (β-NG2 protein) band has no toxicity detectable by rabbit skin reaction test. Even highly concentrated extracellular protein fraction of C7(-) showed no distinct protein band in gel electrophoresis, indicating the phage-directed synthesis of β-NG2 protein. Thus the properties of β-NG2 protein were further studied on other attributes of diphtheria toxin such as molecular weight, immunochemical specificity and ADP ribosylation activity. By electrophoresis in SDS-polyacrylamide gel, the molecular weight of β-NG2 protein was estimated to be 24,000, while that of C7(β) toxin 65,000 (Fig. 2). Fig. 3 shows the

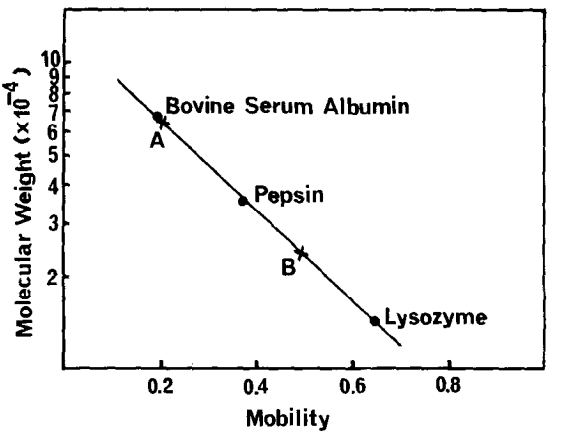

Fig. 2. Log of molecular weight versus mobility by SDS-polyacrylamide gel electrophoresis of C7(β) toxin, β-NG2 protein and standard proteins (bovine serum albumin, mol. wt. 68,000, pepsin, 35,000 and egg white lysozyme, 14,300). (A) C7(β) toxin, (B) β-NG2 protein.

result of the immunodiffusion analysis. As is seen, β-NG2 protein formed a single precipitation band against antitoxin (100 Lf units/ml) and the band fused partly with the band of C7(β) toxin. This indicates the partial identity of the antigenicity of β-NG2 protein with toxin. As illustrated in Fig. 4,

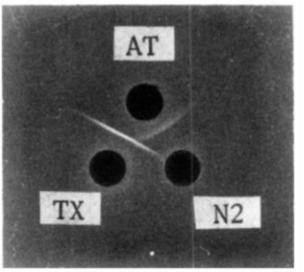

Fig. 3. Immunodiffusion of β-NG2 protein and toxin. TX: C7(β) toxin (100 Lf units/ml), N2: extracellular protein fraction of C7(β-NG2) (200 μg/ml) and AT: horse diphtheria antitoxin No. 68 (100 Lf units/ml).

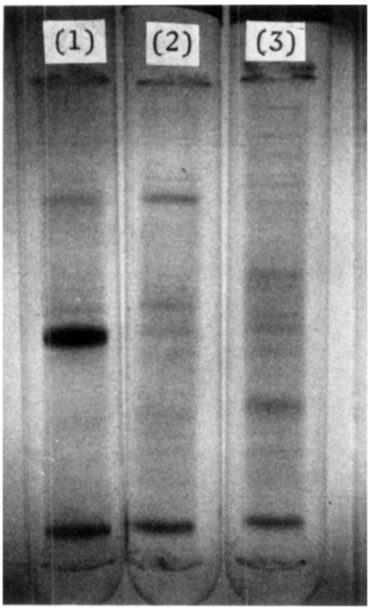

Fig. 4. Electrophoresis in polyacrylamide gel of the extracellular protein fractions of C7(β-NG2) produced in deferrated medium to which (1) No Fe^{++} added, (2) Fe^{++} 3.0 μg/ml added and (3) No Fe^{++} but chloramphenicol 100 μg/ml added. Other experimental conditions are the same as described in the legend for Fig. 1.

the product of C7(β-NG2) showed much higher ADP ribosylation activity than that of the toxin produced by C7(β). And the activity of the product of the mutant-phage lysogen showed almost no change upon mild treatment with trypsin. This is in sharp contrast to the case of C7(β) toxin ("intact toxin" (9,10)), where the activity was greatly enhanced after such treatment to the level of that of the C7(β-NG2) product. Incubation of C7(β) toxin with the cell extracts of the mutant-phage lysogen did not result in any fragmentation of the toxin protein.

Considering from these properties described above, β-NG2 protein appears to be a fragment of diphtheria toxin protein molecule, having similar properties of the N terminal fragment, "Fragment A" recently reported of diphtheria toxin (7,10), suggesting occurrence of a mutation (possibly a nonsense mutation) in the structural gene carried by the phage. The identity of β-NG2 protein with "Fragment A" must await further investigations.

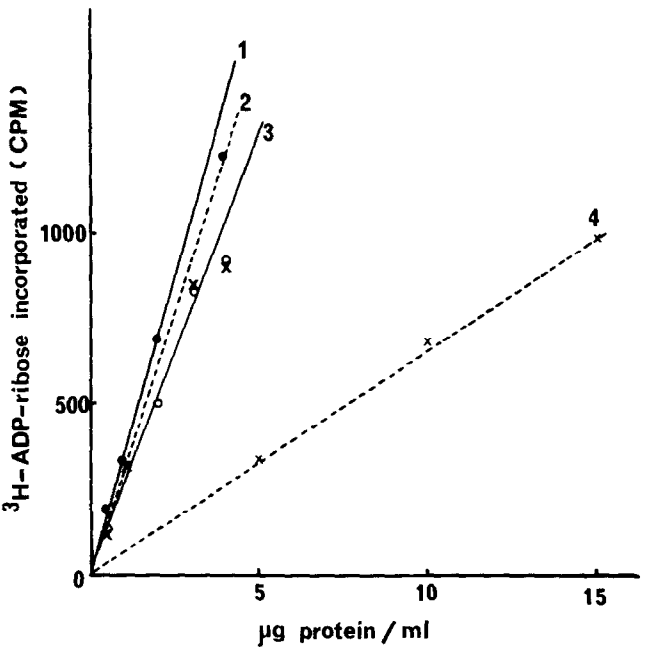

Fig. 5. ADP ribosylation activity of C7(β) toxin and of β-NG2 protein before and after treatment with trypsin. Samples (50 μl) were assayed in the reaction mixtures (final 250 μl) containing 12.5 μmoles of Tris-HCl buffer, pH 8.2, 25 nmoles of EDTA, 10 μmoles of dithiothreitol, 200 μg of partially purified transferase II and 50 pmoles of NAD-(adenosine)-^{3}H. Incubation was at 25 C for 15 min. Trypsin treatment was carried out according to Drazin et al. (9): Toxin or the test protein (0.17 mg per ml, in 50 mM Tris-HCl, pH 8.2-1 mM EDTA) was incubated at 25 C for 30 min with trypsin (1.0 μg/ml).
(1) Treated β-NG2 protein, (2) Treated C7(β) toxin, (3) Untreated B-NG2 protein and (4) Untreated C7(β) toxin.

Effect of iron and chloramphenicol on the production of a fragment (β-NG2 protein) of diphtheria toxin by the mutant-phage lysogen: Chloramphenicol (100 μg/ml) inhibited the production of β-NG2 protein, suggesting de novo synthesis of the protein (Fig. 5). Fig. 5 also shows that the addition of iron (3.0 μg/ml) into the culture medium resulted in almost complete inhibition of the production of the fragment (β-NG2 protein) by the mutant-phage lysogen. Whether this inhibition is related to the processes for the synthesis or for the extracellular release of β-NG2 protein is not known. However, the present result clearly suggests that the N terminal portion, whose size is equal to or less than 24,000 as molecular weight, of whole toxin polypeptide must be involved in the mechanism of inhibition of diphtheria toxin production by iron.

Effect of the mutation in the toxin gene carried by the phage on the phage growth: In Figs. 6A and 6B are illustrated the comparison of C7(β-NG2) with C7(β) in terms of their prophage inducibility and phage producing ability. It may be seen that both the mutant-phage- and the wild-type phage lysogens behaved similarly in ultraviolet (UV) light dose response (Fig. 6A) and also in kinetics of phage production following UV irradiation with the optimal

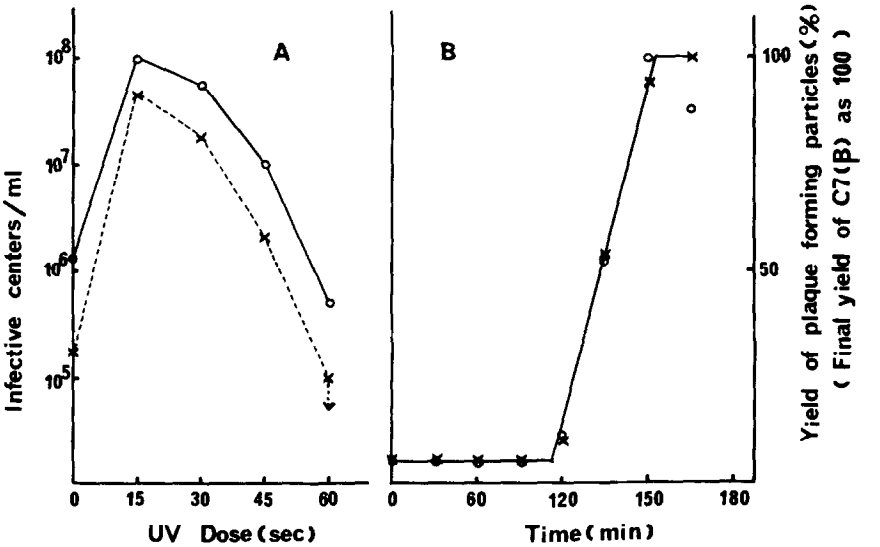

Fig. 6A. Induction of phage formation from prophage β and its mutant β-NG2 by UV irradiation. The numbers of infective centers found when C7(β) (x--x) or C7(β-NG2) (o--o) was plated on C7(-) immediately after irradiation are plotted.

Fig. 6B. One-step growth curves of phage β and its mutant β-NG2 in strains C7(B) and C7(B-NG2) respectively, following induction with UV light.
(x--x): β, (o--o): β-NG2.
Conditions for these experiments are as described previously (2).

dose (Fig. 6B), yielding the comparable average burst sizes. β-NG2 mutation in the toxin gene thus appears not to affect the phage growth, providing an additional evidence for the view (1,11,12) that there exists no direct relation ship between toxin and phage production.

ADDENDUM: During the course of this investigation, Drs. T. Uchida and A.M. Pappenheimer, Jr. kindly informed us their isolation of a phage mutant of β, the lysogen of which produces a nontoxic but enzymatically active cross-reacting material (molecular weight 45,000) of diphtheria toxin before publication of their study (13).

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