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# Early Intracellular Events in the Replication of Bacteriophage T4 Deoxyribonucleic Acid

## VI. Newly Synthesized Proteins in the T4 Protein-Deoxyribonucleic Acid Complex[1]

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Shortly after infection of *Escherichia coli* B with T4 phage, the phage deoxyribonucleic acid (DNA) can be isolated as a fast-sedimenting, proteinaceous complex. Formation of the complex is inhibited by the addition of chloramphenicol between 3 and 4 min after infection, suggesting that phage-coded proteins are necessary to form the complex and may contribute to its structure. To determine whether the phage DNA is associated with a random collection of proteins after infection or whether the complex contained a specific set of proteins, total protein from phage-infected cell lysates was compared to complex protein isolated from similar lysates by gel acrylamide electrophoresis. The proteins obtained from complexes exhibited a distinctly different pattern of separation, indicating that the complex contained a specific set of those proteins newly synthesized after infection. The proteins of the complex appear to be associated directly with the DNA rather than with some other component which could impart the characteristic of fast sedimentation to the complex. Fast-sedimenting complexes were isolated from a $^{3}$H-leucine-labeled cell lysate. Part of this material was treated with pancreatic deoxyribonuclease. Deoxyribonuclease-treated and untreated complexes were resedimented in sucrose gradients. Virtually all the untreated complex remained fast-sedimenting, whereas most of the $^{3}$H-leucine label of the deoxyribonuclease-treated material was located toward the top of the gradient. These data suggest a direct association of DNA and protein in the complex.

Soon after infection of *Escherichia coli* B by bacteriophage T4 a fast-sedimenting complex forms between replicative phage deoxyribonucleic acid (DNA) and a protein moiety (5). Addition of chloramphenicol before 4 min after infection inhibits the formation of this fast-sedimenting complex (5); therefore, it is assumed that newly synthesized proteins are necessary for formation of the complex. Furthermore, there exists the possibility that some proteins newly synthesized after infection are actually incorporated into the complex. If newly synthesized proteins are in the complex, it would be of interest to know whether they are representative of all the proteins synthesized after infection or whether they constitute a specialized group of proteins. The purpose of this paper is to examine this point as well as to explore some of the possible modes of association of the DNA and newly synthesized protein moieties of the complex.

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## MATERIALS AND METHODS

The procedures for isotopic labeling and density gradient centrifugation have been described before (4). The lysozyme-Triton X-100 lysis procedure (LTL) was described in the previous paper of this series (5).

TAG medium is of the same composition as TCG except that 19 amino acids excluding leucine were substituted at 5 μg/ml each for the Vitamin Free Casamino Acids. The 19 amino acids were purchased from Nutritional Biochemicals Corp., Cleveland, Ohio, as a kit.

For analysis of samples by polyacrylamide gel electrophoresis, infected cells were sedimented, resuspended, and lysed by the LTL method. Lysates (not exceeding $10^{8}$ cells/ml) were incubated for 10 min at 37 C with 100 μg of pancreatic deoxyribonuclease, mixed with sucrose, and layered on 7.5% gels which were prepared as follows. Four solutions were prepared, stored in the refrigerator, and used daily for preparation of columns: solution I, 16.6% acrylamide (Eastman Organic Chemicals) and 1.33% ethylenedi-

acrylate (Borden Chemical Co.); solution II, 0.2 M sodium phosphate buffer (*p*H 7.0) and 0.2% sodium dodecyl sulfate (SDS); solution III, 1.5% ammonium persulfate; and solution IV, *N,N,N',N'*-tetramethylenediamine (Eastman). For each 10 ml of gel prepared, 4.5 ml of I was thoroughly mixed with 5.0 ml of II. Then 0.5 ml of III and 10 μliters of IV were added, and the solution was poured immediately after mixing into the columns. The buffer used during electrophoresis was 0.1 M sodium phosphate buffer (*p*H 7.0) containing 1% SDS. The columns, supporting stand, and power supply were purchased from Buchler Instruments, Inc. The samples underwent electrophoresis for 14 hr at 20 ma/column [0.9 cm (inside diameter) by 14 cm] at 22 C. At the end of the run, the gels were extracted from the columns by forcing the gels out with a stream of air. The gels were frozen at −20 C. The frozen gels were sliced into sections 1 mm thick by forcing them through a set of rigid razor blades spaced 1 mm apart. Since standard methods of determining the distribution of radioactive labels in polyacrylamide gels were expensive, inefficient, or time consuming, the following proceedure was developed. The slices were transferred onto Fiberglas filters in glass scintillation vials, and 1.0 ml of 2 M $NH_4OH$ was added to each vial. The vials were heated for 2 hr at 80 C to dissolve the gel slices. After the liquid had evaporated to dryness in the heating oven, toluene-based scintillation fluid was added to each vial and the vials were counted for radioactivity in a Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill).

To test the quenching of the label by this procedure, gels were mixed with $^3$H-leucine or $^{14}$C-leucine before solidification. A sample of the $^3$H- or $^{14}$C-leucine was transferred to a scintillation vial before addition of the label to the gel and served as a 100% standard for recovery of the label. After solidification, the gels were extracted from the columns, frozen, and sliced, and the slices were treated with $NH_4OH$ as before. Calculations showed that virtually 100% of the label put into the gel was recovered and that the deviation in counts from slice to slice was less than 10%. Subsequent results indicated that 100% of the input radioactivity incorporated into proteins could be recovered from the columns also.

## RESULTS

To determine whether proteins in the complex were a specific set of those proteins newly synthesized after infection, the following experiment was performed. *E. coli* B23 cells were grown for two generations in high-phosphate TCG, sedimented, and washed in leucine-free high-phosphate TAG. The cells were grown for one-half of a generation to a concentration of $3 \times 10^8$ cells/ml and infected with T4 bacteriophage at a multiplicity of infection (MOI) = 7.0. At 3 min after infection, the culture was divided into two parts: one part contained $^3$H-leucine and the other $^{14}$C-leucine at specific activities of 2 mc/mg. At 8 min after infection, both parts were chilled to 4 C, sedimented, and lysed by the LTL method. The $^3$H-labeled lysate was layered on a 5 to 20% sucrose gradient underlayered with a saturated sucrose "pad" (Fig. 1A). The material reaching the pad was mixed with a sample of the $^{41}$C-lysate, and the mixture was dialyzed against 0.025 M sodium phosphate buffer (*p*H 7.0), sonically treated, and incubated with pancreatic deoxyribonuclease. The $^3$H-lysate and the $^{14}$C-lysate also were mixed together, dialyzed against 0.025 M sodium phosphate buffer (*p*H 7.0), sonically treated, and incubated with pancreatic deoxyribonuclease. The mixture of the $^3$H-lysate and $^{14}$C-lysate was layered on a column of 7.5% acrylamide gel. The mixture of the $^3$H-labeled complex and the $^{14}$C-labeled lysate was layered on an identical column, and the two columns were electrophoresed simultaneously. The results of this experiment are illustrated in Fig. 2 and 3. It is obvious that the positions of the peaks of the $^{14}$C label and $^3$H label of the respective lysates overlap identically, although the ratio of the labels varies somewhat from fraction to fraction (Fig. 2). This result serves as a control for the comparison of $^3$H-labeled complex proteins and the $^{14}$C-lysate; no gross differences in the patterns existed before separation of the complex from the lysate (Fig. 2). However, when one compares the patterns of the same $^{14}$C-lysate label with the label of the $^3$H-complex, one sees a great difference. Some peaks in the $^{14}$C-pattern have been enriched significantly in the $^3$H-pattern, and some of the peaks in the $^{14}$C-pattern are missing almost completely from the $^3$H-pattern. The results of these experiments appear to support the hypothesis that the proteins of the complex are a specialized set of those proteins which are newly synthesized after infection of *E. coli* B with T4 bacteriophage.

At least two general modes of association of the newly synthesized protein and DNA are possible. Either the newly synthesized proteins and the DNA are both attached to some fast-sedimenting macromolecular structure (such as the cell membrane) or they are attached directly to each other. Consider the schemes presented in Fig. 4. In Fig. 4A the protein is associated with the macromolecular structure independently of the DNA. In Fig. 4B, the protein and DNA are attached directly to each other. One should expect that the destruction of the DNA in scheme A would not affect the sedimentation characteristic of the amino acid-labeled moiety. In scheme B, the direct association of the newly synthesized protein and DNA would be extremely sensitive to incubation with deoxyribonuclease. The experiment which discriminated between these two hypotheses was conducted in the following way. $^3$H-leucine was

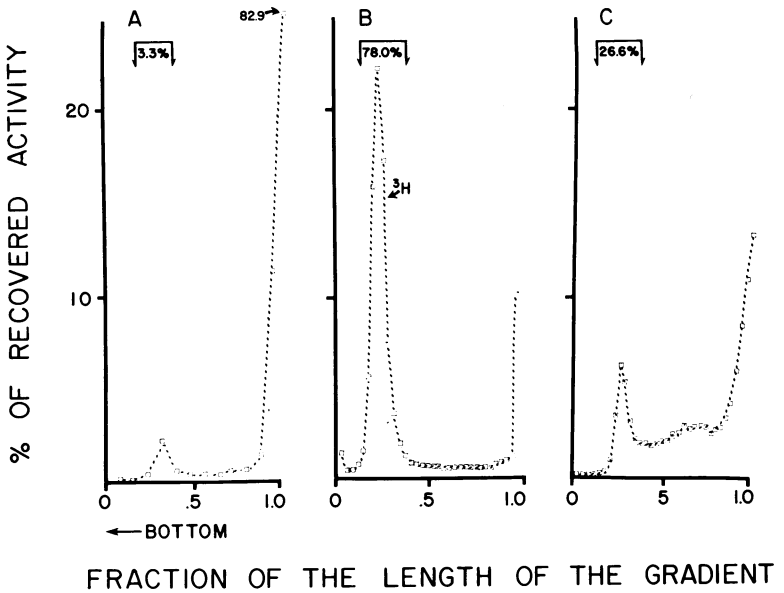


FIG. 1. *Sedimentation of complexes labeled with $^{3}H$-leucine. Escherichia coli B23 cells were infected with T4BO$_{1}^{r}$ bacteriophage in leucine-free TAG. At 3 min after infection, the culture was divided into two parts: $^{3}H$-leucine was added to one part and $^{14}C$-leucine was added to the other. At 8 min after infection, both parts were chilled, sedimented, and lysed by the LTL method. (A) One portion of the $^{3}H$-lysate was layered on a sucrose gradient underlayered with a pad of saturated sucrose and centrifuged for 100 min at 15,000 rev/min in an SW39 rotor, and the distribution of trichloroacetic acid-precipitable label in the gradients was determined. (B) Material reaching the pad was dialyzed against 0.07 M Tris buffer (pH 7.6), incubated for 20 min at 25 C, and reanalyzed on a sucrose gradient. (C) Dialyzed material was incubated with 100 μg of pancreatic deoxyribonuclease per ml for 20 min at 25 C and reanalyzed on a sucrose gradient.*

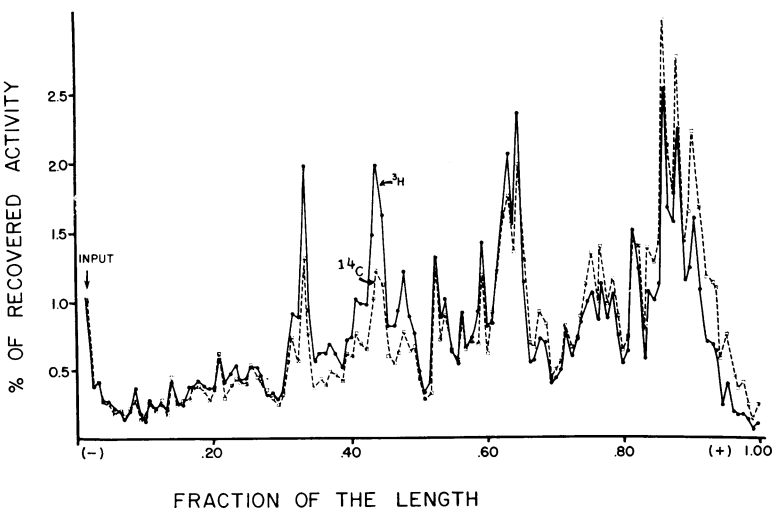


FIG. 2. *Polyacrylamide gel electrophoresis of $^{3}H$-labeled proteins from Triton lysates versus $^{14}C$-labeled proteins from Triton lysates. Portions of the $^{3}H$-lysate and $^{14}C$-lysate described in Fig. 1 were sonically treated, incubated with pancreatic deoxyribonuclease, and analyzed by polyacrylamide gel electrophoresis as described in Materials and Methods.*

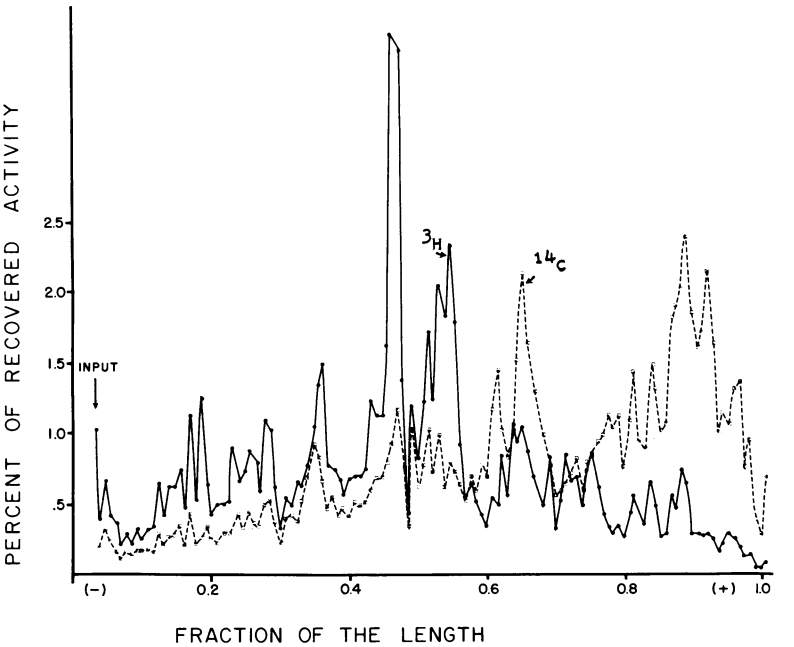


FIG. 3. *Polyacrylamide gel electrophoresis of $^{3}H$-labeled proteins from complexes versus $^{14}C$-labeled proteins from Triton lysates. $^{3}H$-labeled complexes were isolated as described in Fig. 1. A portion of the $^{14}C$-labeled lysate was mixed with the $^{3}H$-labeled complexes, and the mixture was sonically treated, incubated with pancreatic deoxyribonuclease, and analyzed by polyacrylamide gel electrophoresis as described in Materials and Methods.*

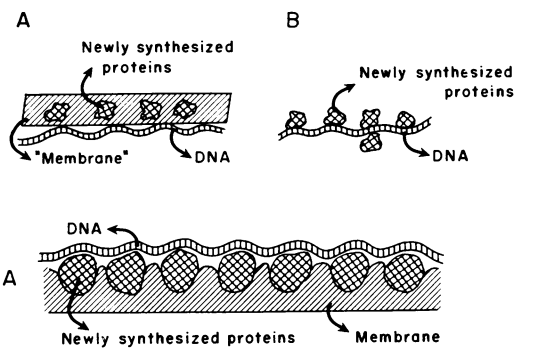


FIG. 4. *Mode of association of newly synthesized proteins and deoxyribonucleic acid in the complex.*

added to infected cells from 2 min after infection to 8 min after infection. The infected cells were lysed by the LTL method and analyzed in 5 to 20% sucrose gradients underlayered with a saturated sucrose pad. Labeled material sedimenting to the pad was isolated and dialyzed against 0.07 M tris(hydroxymethyl)aminomethane buffer (*p*H 7.6); samples were incubated with and without 100 $\mu$g of pancreatic deoxyribonuclease per ml for 20 min at room temperature. This deoxyribonuclease is electrophoretically purified (Worthington Biochemical Corp., Freehold, N.J.) and gives no indication of any proteolytic activity, as assayed by an increase in trichloroacetic acid solubility or change in electrophoretic mobility of labeled proteins. The mixtures then were reanalyzed on sucrose gradients underlayered with a pad. Almost all of the control sample incubated without deoxyribonuclease resedimented to the pad (Fig. 1B). Upon treatment with deoxyribonuclease, 75% of the $^{3}H$-label leaves the bottom of the sucrose gradient and is recovered toward the top of the tube (Fig. 1C). Thus, the fast sedimentation characteristic of most of the moiety labeled with $^{3}H$-leucine is dependent upon the integrity of the DNA in the complex. This supports the assumption that most of the $^{3}H$-label reaching the pad represents some newly synthesized proteins which contribute to the structure of the complex in a direct association with the DNA.

## DISCUSSION

The previous paper of this series described the formation and some of the properties of a DNA-protein complex formed after infection of *E. coli* with T4 phage (5). The proteins of the complex

may possibly consist of those structural or enzymatic proteins necessary for DNA replication. This hypothesis provides an immediate prediction: the newly synthesized proteins in the complex represent a specialized set of those proteins synthesized after infection. To test this prediction, the newly synthesized proteins of the complex were compared with the newly synthesized proteins of the complete cell lysate by polyacrylamide gel electrophoresis. Lysate proteins were labeled with one isotope and complex proteins were isolated from a lysate containing another isotopic label. A mixture of the two types of proteins was analyzed on the same column to compare the distribution of the two protein labels. The conclusion of this experiment was that the pattern of the complex proteins did differ from the pattern of the total lysate proteins. These data seem to indicate that the proteins of the complex may indeed be a specialized set of those proteins newly synthesized after phage infection.

Frankel et al. studied alterations in the membrane proteins of *E. coli* after infection with T4 phage (2). They analyzed $^{14}$C-leucine- and $^{3}$H-leucine-labeled membrane proteins by gel acrylamide electrophoresis. Although there could possibly be a relationship between the membrane proteins described by Frankel et al. and the complex proteins described in this paper, it is difficult to make a comparison, because the isolation of the complex proteins depends upon their association with the phage DNA. Those proteins studied by Frankel et al. were freed from any DNA component before they were isolated (2).

Earhart et al. described what they believe to be a membrane-DNA complex in phage-infected cells (1). The isolation of this complex, according to these authors, depends upon the ability of the portions of the cell membrane to which the DNA is attached to adhere to the hydrophobic surfaces of crystals (1). The authors did not study any proteins which might have been associated with this complex.

The previous paper of this series eliminated the possibility that the protein portion of the complex is attached to one end of the DNA molecules (5). The conclusion of these studies was that the DNA and protein in the complex probably comprised a colinear or "quasi-colinear" structure, the latter type of structure being the more likely in view of the sensitivity of the complex to endonuclease (5). With these data in mind, two possible modes of association between the newly synthesized proteins and the DNA were proposed. (i) The proteins and the DNA are attached independently to some macromolecular structure, such as the cell membrane, which would be responsible for the fast-sedimenting property of the complex, or (ii) the proteins and the DNA are directly associated. Consider the effect incubation with deoxyribonuclease would have on these two different types of association. After destruction of the DNA in the first case, the proteins still should sediment very fast. However, the sedimentation characteristics of the proteins in the second case should be drastically altered after destruction of the DNA. In fact, when complexes labeled with $^{3}$H-leucine are isolated, incubated with deoxyribonuclease, and reanalyzed in a sucrose gradient, most of this label is displaced toward the top of the tube. This indicates that fast sedimentation of the labeled proteins depends on the integrity of the DNA in the complex. In addition to supporting the second model, this experiment indicates that the $^{3}$H-label represents newly synthesized proteins which form an integral part of the protein-DNA complex.

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## LITERATURE CITED

1. Earhart, F., G. Y. Tremblay, M. J. Daniels, and M. Schaechter. 1968. DNA replication studied by a new method for the isolation of cell membrane-DNA complexes. Cold Spring Harbor Symp. Quant. Biol. 33:707–710.
2. Frankel, F. R., C. Majumdar, S. Weintraub, and D. M. Frankel. 1968. DNA polymerase and the cell membrane after T4 infection. Cold Spring Harbor Symp. Quant. Biol. 33:495-500.
3. Kozinski, A. W., and T. H. Lin. 1965. Early intracellular events in the replication of T4 phage DNA. I. Complex formation of replicative DNA. Proc. Nat. Acad. Sci. U.S.A. 54:273–278.
4. Kozinski, A. W., P. B. Kozinski, and R. James. 1967. Molecular recombination in T4 bacteriophage deoxyribonucleic acid. I. Tertiary structure of early replicative and recombining deoxyribonucleic acid. J. Virol. 1:758–770.
5. Miller, Robert C., Jr., and Andrzej W. Kozinski. 1970. Early intracellular events in the replication of bacteriophage T4 deoxyribonucleic acid. V. Further studies on the T4 protein-deoxyribonucleic acid complex. J. Virol. 5:490–501.