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Incorporation of Deoxyribonucleic Acid Precursors by T4 Deoxyribonucleic Acid-Protein Complexes Retained on Glass Fiber Filters

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Bacteriophage T4 deoxyribonucleic acid (DNA)-protein complexes were retained preferentially on glass fiber filters. DNA polymerase activity in the complex was detected through the incorporation of ^{3}H-labeled DNA precursors. The primer-product DNA hybridized with both phage and *Escherichia coli* DNA. Density labeling experiments showed that about 30% of incorporated ^{3}H-deoxyadenosine triphosphate was found in DNA which hybridized with phage DNA; this DNA was found to be covalently attached to the primer DNA.

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 (8, 10). Addition of chloramphenicol to the infected culture before 3 min after infection inhibits the formation of this complex (10); this result indicates that some newly synthesized, phage-coded proteins are necessary for formation of the complex. In fact, ^{3}H-leucine-labeled, newly synthesized proteins are found to be directly associated with the DNA of the complex, and these proteins form a specialized set of those proteins synthesized after infection (9). The following hypotheses can be put forth to explain the role of the complex: (i) the protein moiety serves as a structural support on which the DNA is replicated or (ii) the proteins consist of those enzymes necessary for DNA replication, recombination or both, e.g., DNA polymerase, or (iii) combination of both processes. If the second hypothesis were true, DNA polymerase activity should be detected in the complex.

To examine DNA polymerase activity in the complex, a rapid and reproducible method of isolating large amounts of complex was developed which bypassed the laborious process of sucrose gradient sedimentation; this method involved retaining complexes on glass fiber filters. A double-label experiment showed that parental phage DNA from lysates of infected cells was retained preferentially on glass fiber filters concomitantly with formation of the fast-sedimenting complex; i.e., if reference ^{3}H-DNA was added to lysates of cells infected with ^{32}P-labeled phage, after filtration ^{32}P from cells lysed at about 5 min after infection was retained preferentially on the fiber glass filters. Before 5 min after infection, both ^{3}H and ^{32}P passed through the filter; after 5 min after infection, ^{3}H still passed through the filter, but ^{32}P was retained. Since parental DNA was in the complex at 5 min after infection, it seemed obvious that the complex was being retained preferentially on the fiber glass filter. The experiment was conducted in the following manner: *E. coli* B23 was grown to 3×10^{8} cells/ml in high phosphate TCG (5). The cells were infected at a multiplicity of infection (MOI) of 5 phage per bacterium (MOI = 5.0) with bacteriophage T4 labeled with ^{32}P at a specific activity of 0.5 mCi/mg of P. At intervals after infection, 10.0-ml samples of infected cells were chilled, sedimented, and lysed by the lysozyme-Triton method (10). Mercaptoethanol (ME) was added to a final concentration of 0.01 M. The lysates were stored for 12 hr at 4 C. (After this treatment there were no surviving cells.) ^{3}H-reference DNA was added to the lysates, and the mixtures were filtered at a flow rate of 2 ml/min through glass fiber filters (Reeve Angel 934AH; 2.4 cm) in 10 ml of 0.05 M tris(hydroxymethyl)aminomethane (Tris), 0.05 M NaCl, 0.015 M ethylenediaminetetraacetic acid (EDTA), 0.01 M ME adjusted to *p*H 8.0 with HCl. The filters were washed twice with 10 ml of the same solution, dried, and counted as usual. Table 1 lists the results of this experiment; these results demonstrate that preferential retention of

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intracellular, ^{32}P-labeled DNA occurs concomitantly with the formation of the fast-sedimenting complex (10). Some ^{32}P-labeled DNA from Triton-lysates obtained at 3 min after infection is retained on the filters, but this retention is nonspecific because ^{3}H-reference DNA is retained, also, under these conditions. Addition of 1% sodium dodecyl sulfate (SDS) to these 3-min lysates eliminates this retention but does not affect the preferential retention of ^{32}P at later times. Incubation with SDS is necessary to descriminate between the fast-sedimenting complex and the other T4 intermediate called FSI (10). These results are in excellent agreement with efficiencies of extraction of intracellular DNA with phenol (8) and sucrose gradient sedimentation data (10). Furthermore, the absolute amount of ^{32}P retained on the filters agreed very well with previous estimates of the fast-sedimenting complex; e.g., at 10 min of the infection, about 75% of the ^{32}P was retained.

Knowing that the complex was adsorbed on fiber glass filters, the next step was to detect DNA polymerase activity in the complex. This was accomplished by incubating filters charged with the complex in the presence of labeled DNA precursors. Charged filters (i.e., filters through which cell lysates had passed) were placed in 0.5 ml of a solution containing 0.07 M Tris, 0.02 M $MgCl_2$, 0.01 M ME, and 3×10^{-5} M deoxyribonucleoside triphosphates [deoxyadenosine triphosphate (dATP), deoxycytosine triphosphate (dCTP), deoxythiamine triphosphate, and deoxyguanosine triphosphate] adjusted to *p*H 8.0 with HCl. A 2.5-μCi amount of ^{3}H-labeled dATP or dCTP was added to the reaction mixtures at a specific activity of 5 Ci/mmole of deoxyribonucleoside triphosphate. The suspensions were incubated at 37 C, and the reaction was terminated by the addition of EDTA to 0.025 M and chilling to 4 C. The filters then were washed on a sintered glass disc with a solution containing 0.05 M Tris, 0.05 M NaCl, 0.02 M EDTA (*p*H 8.0). The filters were dried and counted as usual. Table 2 lists the results of this experiment. Incubation of the charged filters with deoxyribonuclease before or during the reaction eliminates incorporation of the label completely; treatment with deoxyribonuclease destroys the product completely. Filters charged with lysates of uninfected cells incorporate at least 10 times less label than infected cells. Incubation of the charged filters in the absence of $MgCl_2$ or one of the nucleotides inhibits the reaction completely. Some variations in the preparation of infected cells before lysis were examined in the course of these experiments. Host cells were ultraviolet irradiated (germinated lamp General Electric; dose = 30 lethal hits/bacterium) in an attempt to decrease a possible contribution from host DNA-protein complexes. Other cells were incubated for long periods of time in chloramphenicol, at 100 μg/ml to allow maximum host DNA degradation before lysis of the cells; here again, the hope was to eliminate DNA-protein complexes assumed to exist in the host before infection.

To determine whether the product of the reaction included *E. coli* DNA or T4 bacteriophage DNA, the synthetic product was extracted from the filters and banded in Cs_2SO_4 density gradients (Fig. 1A). ^{3}H-labeled DNA isolated from the gradient of Fig. 1A was tested for its ability to hybridize with purified *E. coli* or T4 bacteriophage

TABLE 1. *Efficiency of retention of intracellular, ^{32}P-labeled, parental DNA on fiber glass filters*

Time after infection	Efficiency of retention[a]
Triton lysate (3 min)	1.52
Triton lysate (6 min)	131
Triton lysate (8 min)	245
Triton lysate (10 min)	201
Triton lysate (15 min)	322
SDS[b] lysate (3 min)	0.48
SDS lysate (8 min)	155

[a] Efficiency of retention = (^{32}P retained on filter/^{3}H retained on filter)/(^{32}P before filtering/^{3}H before filtering).

[b] Sodium dodecyl sulfate.

TABLE 2. *Incorporation of ^{3}H-dATP by complexes retained on filters*

Host inactivated by ultraviolet light	Deoxyribonuclease	Phage	Chloramphenicol	Time of removal of infected cells	Counts incorporated
			min	*min*	
+				10	1.54×10^4
+		$T4BO_1^r$		10	2.20×10^5
−		$T4BO_1^r$	10–60	60	1.56×10^5
−		$T4BO_1^r$		10	1.01×10^5
−	Added during reaction	$T4BO_1^r$		10	7.23×10^2
−	Added after reaction	$T4BO_1^r$		10	1.01×10^2

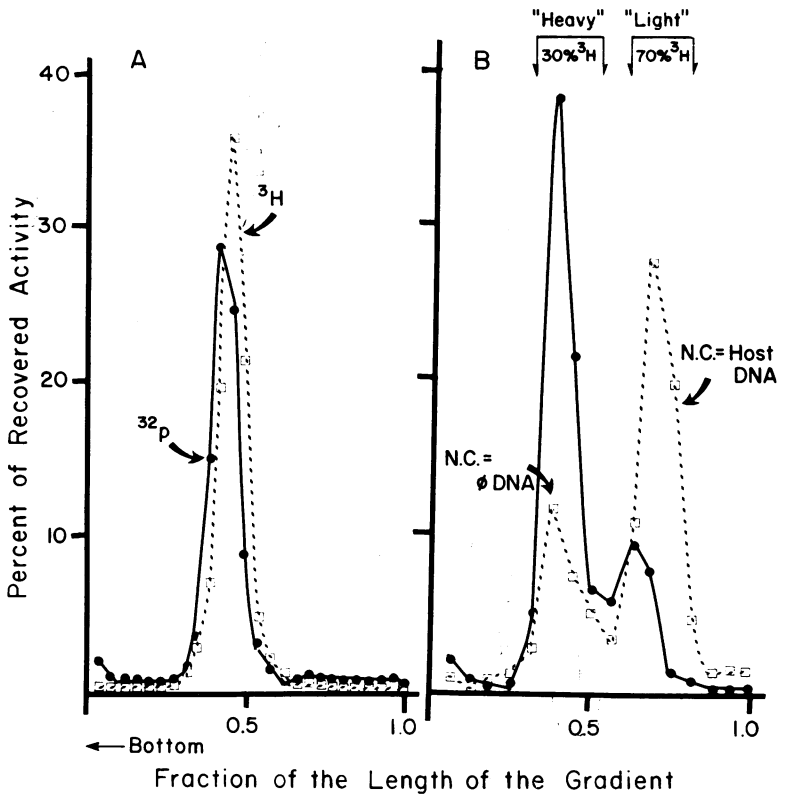

FIG. 1. *Cs_2SO_4 density gradient analysis of ^3H-labeled product DNA. (A) Charged filters were incubated in the standard reaction mixture including ^3H-dATP. The reaction was stopped with 0.025 M EDTA, and the filters were washed free of unincorporated label. The filters were incubated with Pronase (1 mg/ml) for 4 hr, and DNA was extracted from the filters with phenol. During centrifugation, the filters settled to the phenol phase. DNA in the aqueous phase was supplemented with ^{32}P-labeled reference T4-bacteriophage DNA and analyzed in a Cs_2SO_4 density gradient. Fractions containing ^3H-labeled, newly synthesized DNA were isolated; the contents were dialyzed against 10^{-3} M EDTA and tested for their ability to hybridize with E. coli DNA and T4 bacteriophage DNA by standard procedures (2, 6). The synthetic product also was tested for its ability to hybridize with either of the two separated, isolated strands of bacteriophage T4 DNA (4). (B). E. coli B23 was grown to 3 × 10^8 cells/ml in high phosphate TCG and infected (MOI = 15) with phage labeled with 5-BUdr and ^{32}P (specific activity, 0.01 mCi/mg of P). The infected bacteria were incubated for 10 min at 37 C, and then chloramphenicol (100 μg/ml), 5-BUdr (200 μg/ml), uracil (50 μg/ml) and 5-FUdr (5 μg/ml) were added to the medium. The culture was incubated for 60 min. Therefore, most of the replicating phage pool of DNA was labeled with 5-BUdr at a time when no more host DNA was synthesized. The infected cells were lysed and used to charge filters which then were incubated with ^3H-dATP as usual. The synthetic product was extracted from the filters by the Pronase-phenol procedure, and extracted DNA was mixed with ^{32}P-labeled reference phage DNA of heavy and light densities. The mixture was analyzed by Cs_2SO_4 density gradient centrifugation. The curves of Fig. 1B represent the distribution of the same labels as in Fig. 1A. N.C. stands for mitocellulose-mediated hybridization assay (2).*

DNA (*data not shown*). The results indicated that the synthetic product obtained from filters charged with lysates of infected cells hybridized with both *E. coli* DNA and T4 bacteriophage DNA. This fact is reflected in the displacement of the ^3H peak from the ^{32}P reference, because T4 DNA and *E. coli* DNA have different densities. Furthermore, the product hybridized with both strands of the T4 DNA.

Since the efficiencies of annealing of both phage DNA and *E. coli* DNA were not known exactly, there was no way to determine what net proportion of the synthetic product was actually phage DNA. To solve this problem, a method was needed to separate newly-synthesized phage DNA from the host DNA product. The experimental approach adopted was to label the complexed phage DNA used for charging filters with a density label [5-bromodeoxyuridine (BUdr)] in an unlabeled host; then the synthetic product could

be isolated in a Cs_2SO_4 density gradient in two peaks, one heavy peak of phage DNA and one peak at the *E. coli*, light location. The results of this experiment are illustrated in Fig. 1B. As expected, two peaks of ^3H were found in the gradient. The material in each of the two peaks was isolated, dialyzed against 10^{-3} M EDTA, mixed with ^{32}P phage DNA, and tested for its ability to anneal with host and phage DNA. Table 3 lists the results of the hybridization experiment. As predicted, the heavy ^3H-DNA from the Cs_2SO_4 density gradient anneals only with phage DNA, and the light ^3H-DNA anneals only with host DNA. By comparing the amount of ^3H in the two peaks in the Cs_2SO_4 gradient, one concludes that about 30% of the ^3H-dATP incorporated is found in phage DNA.

Phage DNA from the heavy fraction of the Cs_2SO_4 density gradient was heated for 5 min at 100 C in standard saline-citrate containing 1% formaldehyde and banded in a CsCl density gradient to see if the ^3H-labeled product DNA was covalently attached to the heavy primer; if the product DNA (produced through incorporation of "light" precursors) was covalently attached to the primer, then one should expect that the density of the ^3H-labeled material should not change significantly after such heat treatment (conditions which definitely separate the two strands of bihelical T4-phage DNA). If, one the other hand, the product DNA was not covalently attached to the primer, the ^3H label should be displaced toward the top of the CsCl density gradient after heat denaturation. After isolation of the heavy peak from a Cs_2SO_4 density gradient and reanalysis in a CsCl density gradient, the ^3H-labeled product DNA species were found to overlap with admixed, ^{32}P-labeled, 5-BUdr-substituted, T4-reference phage DNA. After heat treatment, the ^3H-labeled product remained at the heavy location and was not displaced toward the top of the gradient. These results indicated that the product DNA was covalently attached to the heavy phage DNA used to charge the fiber glass filters; this result is similar to that obtained by Aposhian and Kornberg with purified, T4-induced DNA polymerase and denatured DNA as a primer (1).

TABLE 3. *Hybridization of synthetic DNA with host or phage DNA*[a]

Method of preparation[b]	DNA used to charge filter	$^3H/^{32}P$	^3H input
			%
"Heavy" peak	Phage	0.98	31
	Host	0.32	0
	Blank	0.36	0
	Input	0.66	100
"Light" peak	Phage	0.07	1
	Host	0.98	30
	Blank	0.25	0
	Input	0.79	100

[a] For a complete discussion of the hybridization technique and treatment of the data, see reference 6.

[b] *See* Fig. 1B.

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