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In Vitro Complementation as an Assay for New Proteins Required for Bacteriophage T4 DNA Replication: Purification of the Complex Specified by T4 Genes 44 and 62

(gene products/mutants/cell lysates)

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ABSTRACT **We have developed an *in vitro* complementation assay for six T4 bacteriophage gene products believed to be components of the T4 DNA replication apparatus. This assay is based upon the fact that DNA synthesis in an infected cell lysate that lacks a given gene product is specifically stimulated by addition of the missing product. By the use of such an assay, two proteins that appear to be the products of T4 genes 44 and 62 have been purified to electrophoretic homogeneity as a single complex of the two polypeptide chains.**

Escherichia coli cells infected with bacteriophage T4 amber mutants in genes 32, 41, 43, 44, 45, or 62 synthesize little or no DNA, even though all four dcoxyribonucleoside triphosphates are present (1, 2). For several of these genes, temperature-sensitive mutations exist for which DNA replication stops abruptly after a shift to the nonpermissive temperature (3, 4). These six gene products may, therefore, be directly involved in building the T4 DNA replication apparatus (4).

The products of T4 genes 43 and 32 are a DNA polymerase (5, 6) and a DNA-unwinding protein (7), respectively; both of these proteins have been purified and characterized in detail (8–11). The DNA-unwinding protein increases the *in vitro* rate of polymerization by the polymerase 5- to 10-fold (12); in addition, these two proteins appear to specifically interact with each other (12).

In order to determine their roles in T4 DNA replication, the products of genes 41, 44, 45, and 62 must also be identified and purified. For this purpose, we have developed an *in vitro* DNA synthesizing system that shows a requirement for these gene products (13). This system consists of concentrated, gently lysed T4-infected cells, in which the endogenous DNA serves as the template. Like the system described by Smith, Schaller, and Bonhoeffer for *E. coli* (14), and by Okazaki and coworkers for T4-infected *E. coli* (15), these cell lysates support a brief period of rapid DNA synthesis when supplied with deoxyribonucleoside triphosphates. Using such a system as an assay, we have purified the products of T4 genes 62 and 44 to homogeneity.

MATERIALS AND METHODS

Bacteria, Bacteriophage, and Enzymes. The host strain for all experiments was *E. coli* D110 (Pol A_1, *end*I^-, *thy*$^-$, *su*$^-$) obtained from Dr. C. C. Richardson (16). The following T4 mutants were obtained from the Cal Tech collection: *am* HL618 (gene 32$^-$), *am* B22 (gene 43$^-$), *am* N81 (gene 41$^-$), *am* N82 (gene 44$^-$), *am* E10 (gene 45$^-$), and *am* E1140 (gene 62$^-$). In addition, T4 phage SP62-*am* N55 (gene ?, gene 42$^-$), whose SP62 genotype causes it to overproduce several early gene products when DNA replication is blocked (J. Wiberg, manuscript in preparation), was generously provided by Dr. Wiberg. Purified T4 DNA polymerase was a gift from Dr. Wai Mun Wang; T4 gene 32-protein was prepared according to Alberts and Frey (10).

Preparation of Infected Cells. E. coli D110 was grown in log phase to a concentration of 4×10^8 cell per ml in M-9 minimal medium supplemented with 0.3% casein hydrolysate, 1 μg/ml of thiamine, and 0.02 mg/ml of thymidine. The cells were infected by addition of the appropriate mutant T4 bacteriophage at a multiplicity of infection (MOI) of 5, followed by incubation for 20 min at 37° [for SP62-*am* N55, a 60-min incubation was used]. The infected cells were harvested by centrifugation at 4°, washed twice in 20% sucrose containing 0.05 M Tris·HCl (pH 7.4)–1 mM Na_2EDTA, and stored as a frozen pellet.

Preparation of Receptor Cell Lysates. A pellet of frozen cells was thawed and evenly suspended in 4 volumes of 25% sucrose–0.05 M Tris·HCl (pH 7.4). After addition of one more volume of 10 mM Na_2EDTA containing 2 mg/ml of egg-white lysozyme (Worthington Biochemical), the mixture was incubated for 30–60 min in an ice bath. 5 more volumes of buffer containing 0.05 M Tris·HCl (pH 7.4)–0.03 M $MgSO_4$ and 1% Brij-58 (Atlas Chemical Industries) were then added, and the incubation was continued for 15–30 min to complete lysis (15, 17). The final concentration of the lysed cells was about 4×10^{10} per ml.

Preparation of Donor Extracts. A lysate prepared as described above was centrifuged at 30,000 × *g* for 15 min to pellet cell membranes and associated DNA. The supernatant was then centrifuged at 165,000 × *g* for 35 min to pellet ribosomes. This second supernatant is called the "extract" (or Fraction I) in our purification scheme.

Complementation Assay. 50 μl of mutant-infected, receptor cell lysate is mixed with 50 μl of donor extract or buffer in an ice bath. A 25-μl aliquot is added to 25 μl of a mixture containing 0.05 M Tris·HCl (pH 7.4), 0.2 mM deoxyadenosine, 2 mM ATP, and 0.04 mM each of dGTP, dCTP, and TTP, and [^{3}H]dATP (250 Ci/mol). After incubation at 37° for 20 min, DNA synthesis is stopped by addition of 50 μl of

TABLE 1. *Extent of in vitro DNA synthesis in cell lysates*

Cell lysate*	cpm	% of Wild type
Wild-type T4†	8750	(100)
T4 Gene 32⁻	1980	22
T4 Gene 41⁻	1700	19
T4 Gene 43⁻	120	1
T4 Gene 44⁻	450	5
T4 Gene 45⁻	810	9
T4 Gene 62⁻	670	8
Uninfected cells	2200	—

* For the particular mutants used and details of the assay, see *Methods*. A filter blank of 50 cpm has been subtracted.

† The level of activity in this lysate may be misleadingly enhanced relative to other lysates, since more T4 DNA template is present due to intracellular DNA synthesis.

0.2 M Na_3EDTA and chilling. The entire mixture (100 μl) is then spotted on a glass-fiber filter (Whatman GF-A), batch-washed in 5% trichloroacetic acid and ethanol (18), and dried and counted by standard techniques. In order to quantitate activity, donor extracts are serially diluted into a buffer containing 0.05 M Tris·HCl (pH 7.4)–5 mM $MgSO_4$–1 mM 2-mercaptoethanol–10% glycerol–100 μg/ml bovine-serum albumin (Cal Biochem); each dilution is then tested by mixing duplicate aliquots with receptor cell lysate as described above.

RESULTS

DNA synthesis in T4-infected cell lysates

DNA synthesis in lysates of T4-infected cells is of brief duration, lasting only about 2 min at 37°. The initial rate of synthesis corresponds to about 10^3 molecules of [^{3}H]dATP incorporated into DNA per cell per sec. The product made is stable for at least 30 min at 37°; it can be degraded by added pancreatic DNase, but not by pancreatic RNase.

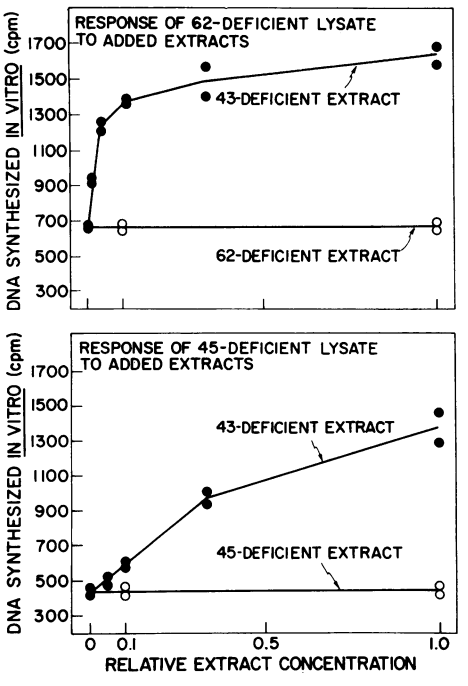

FIG. 1. Complementation of 62-deficient and 45-deficient receptor cell lysates by added donor extracts.

T4 DNA replication *in vivo* exhibits a nearly absolute requirement for the products of T4 genes 32, 41, 43, 44, 45, and 62 (2). *In vitro* DNA synthesis in cell lysates is likewise reduced if cells have been infected with these bacteriophage mutants. As listed in Table 1, the DNA synthesis in these mutant lysates is reduced to anywhere from 20 to 1% of the wild-type level, depending on the particular gene product missing.

In vitro complementation for DNA synthesis

In preliminary tests, purified 32-protein stimulated the amount of DNA synthesis in its deficient lysate 1.7-fold, while purified 43-protein (T4 DNA polymerase) stimulated DNA synthesis in its deficient lysate 1.5-fold. Neither protein stimulated DNA synthesis in the opposite lysate. Individual

TABLE 2. *Purification of 62-complementation activity*

Fraction	Volume (ml)	Protein (mg/ml)	Activity (units/ml)	Specific activity (units/mg)	Activity recovered (%)
I	100	12.4	68,000	5,500	(100)
II	120	1.23	68,000	55,000	120
III	11.5	1.14	240,000	210,000	40
IV	17.3	0.35	86,000	250,000	22

A unit is defined as the amount of activity sufficient to give a 2.0-fold stimulation of DNA synthesis in the standard assay with 12.5 μl of 62-deficient cell lysate. The activities reported were determined by assay of serial dilutions of each fraction with the same receptor lysate on a single day; with these cells, concentrated Fraction IV stimulated synthesis 3.1-fold (see Fig. 4). Gene-44 complementation activity copurified, and gave identical yields at each step (see text). Protein was determined by biuret assay after trichloroacetic acid precipitation of each fraction.

For the purification, 100 ml of extract, prepared as described in *Methods* from SP62-*am* N55-infected cells, was pumped at 80 ml/hr onto a DEAE-cellulose column at 4° (Whatman DE-52, 1 meq/g). The column, equilibrated and rinsed in 0.02 M Tris·HCl (pH 7.4)–5 mM $MgSO_4$–1 mM 2-mercaptoethanol–1 mM Na_3EDTA–10% glycerol, had a packed volume 2.5 times the volume of extract and was 50-cm long. All of the 62-activity emerged in the breakthrough fractions, which were dialyzed into 0.02 M potassium phosphate (pH 7.0)–5 mM $MgSO_4$–1 mM 2-mercaptoethanol–10% glycerol (Fraction II). Fraction II was loaded at 45 ml/hr onto a 18 × 2.5 cm hydroxyapatite column (BioRad), rinsed with the above dialysis buffer containing 0.10 M potassium phosphate, and then eluted by elevation of the potassium phosphate concentration to 0.15 M. This 0.15 M eluate was placed in a dialysis bag, concentrated about 3-fold with solid sucrose, and then dialyzed against 5 mM Tris·HCl (pH 7.4)–0.1 mM $MgSO_4$–1 mM 2-mercaptoethanol–10% glycerol. This material, called Fraction III, was subjected to isoelectric focusing (300 V for 39 hr at 4°), with 10% glycerol–5 mM 2-mercaptoethanol added throughout the column. 11 ml of Fraction III was used with a 110-ml (pH 6–9) gradient in an LKB-8110 Electrofocusing column. The material was focused as recommended by LKB (LKB Produkter, Stockholm, Sweden) in a 1–40% sucrose gradient, with the cathode at the top, and was collected from the top. The pH 8.2 fraction is called Fraction IV. The total amount of Fraction IV isolatable from T4 *am*B22-infected cells (no SP62 genotype) was 10- to 20-fold less, with most of the additional purification needed being obtained in the isoelectric focusing step. When it was desirable to remove ampholines, Fraction IV was readsorbed to hydroxyapatite and batch eluted.

lysates deficient in the products of T4 genes 41, 44, 45, or 62 were then tested for their ability to be complemented by extracts containing these proteins. Extracts were prepared and used for complementation as described in *Methods*. Fig. 1 (*top*) illustrates the level of DNA synthesis in a 62-deficient lysate after addition of various amounts of an extract made from cells containing the product of gene 62. As shown, the donor extract could be diluted 27-fold and still evoke a 2-fold stimulation of DNA synthesis. However, an extract prepared in exactly the same manner from 62-deficient cells had no stimulatory effect. Similar complementation of a 45-deficient lysate by the gene 45 product is shown in Fig. 1 (*bottom*). Analogous complementations have been obtained with receptor cell lysates deficient in the products of genes 44 and 41 (not shown). In each case, the controls show that the stimulation of DNA synthesis observed is due to the addition of the missing gene product.

Reproducible response to these gene products requires the high concentrations of receptor cell lysate used. This suggests that at least some of the factors necessary for DNA synthesis must be present at very high concentration if efficient complementation is to occur (see also ref. 19).

Purification of gene 62 and gene 44 proteins

By use of the complementation response as an assay, 62-activity was originally purified from cells infected with T4 *am* B22 (gene 43), and later from cells infected with the overproducer T4 SP62-am N55. Preparative isoelectric focusing yields the final Fraction IV, isoelectric at pH 8.2. As judged by electrophoresis on polyacrylamide gels containing sodium dodecyl sulfate, followed by protein staining (20), Fraction IV consists almost entirely (>95%) of two proteins, having apparent molecular weights of 34,000 and 20,000, respectively. The recovery and purity at each step of this purification are listed in Table 2 for SP62-*am* 55-infected cells, with details of the procedure given in the legend.

Fraction IV does not stimulate DNA synthesis in lysates deficient in the products of genes 32, 41, 43, or 45 (Table 3). However, both 44-deficient lysates and 62-deficient lysates are complemented. Most significantly, as shown in Fig. 2,

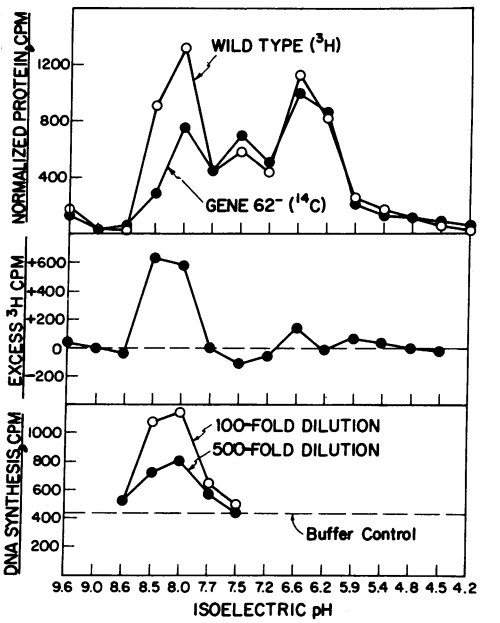

FIG. 3. Isoelectric focusing of T4 gene 62 activity: Fraction IV was derived from Fraction III of a mixed, doubly-labeled extract, prepared as described in the text. Both cultures were labeled from 5 min to 15 min after infection at 25° with radioactive leucine. To insure that the two phages differed only by a single mutation, the wild-type was selected on *E. coli* B as a spontaneous revertant from the *am*E1140 (gene 62$^-$) stock. Electrofocusing was performed in a 10-ml J-tube with a pH 3–10 gradient, as described by Godson (23); the mean pH of each 0.5-ml fraction collected is indicated. In addition to measurement of the 62-protein activity in the complementation assay (*bottom*), aliquots of each electrofocusing fraction were counted by standard radioisotopic techniques (*top*). The excess of ^{3}H-labeled protein derived from these counts is plotted in the *middle panel*. The data shown have been corrected for channel overlaps, and normalized to a ^{14}C:^{3}H ratio of 1.0 in the original extract. A total of about 0.02% of the incorporated [^{3}H]leucine radioactivity was recovered in the active fractions.

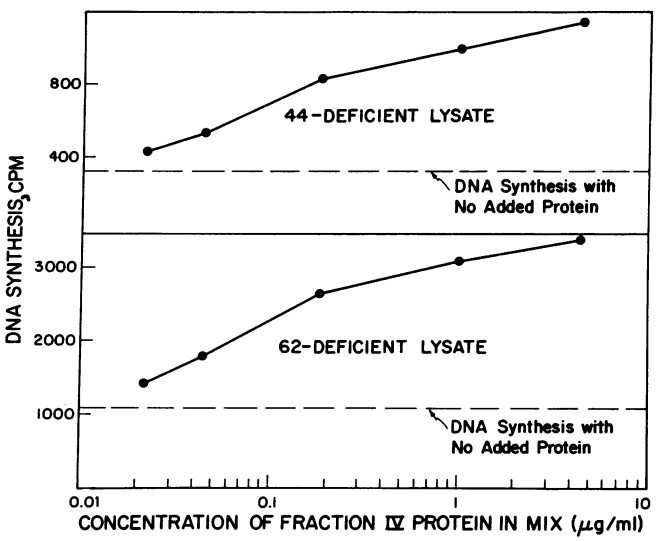

FIG. 2. Response of DNA synthesis in 44- and 62-deficient receptor lysates to dilutions of Fraction IV protein.

the percent stimulation of these two lysates is similar over a 250-fold concentration range of purified complex. Therefore, it appears that one of the two proteins in Fraction IV is the gene 62-protein and that the other is the gene 44-protein.

Attempts were made to separate the two proteins in Fractio IV by taking advantage of the difference in their size. However, on sucrose gradients the proteins sedimented together as a homogeneous complex at 7.1 S. On gel filtration columns the two proteins eluted together at an apparent (spherical) molecular weight of about 300,000 (manuscript in preparation). In all cases, the molar ratio of 34,000-dalton protein to 20,000-dalton protein was constant at about 2:1, as estimated by elution of bands stained with Coomassie Blue from sodium dodecyl sulfate–polyacrylamide gels (21). The sedimentation and gel-filtration data (22) suggest that the complex is actually quite asymmetric, with a molecular weight of about 164,000; the ratio of chains in this complex should therefore be 4:2 (i.e., expected molecular weight of 176,000).

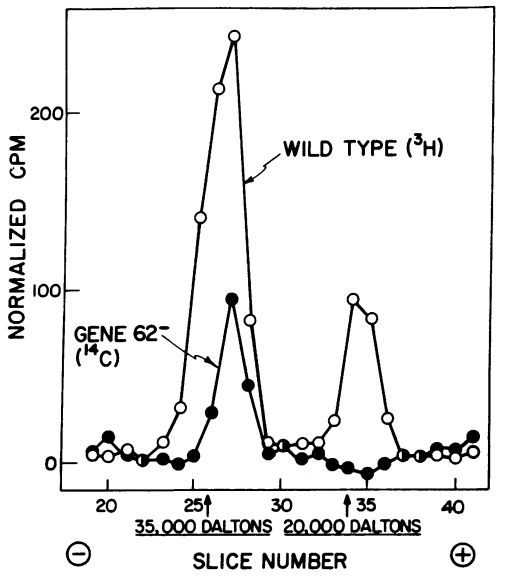

FIG. 4. Electrophoretic analysis of an active isoelectric focusing fraction on a polyacrylamide gel containing sodium dodecyl sulfate. Isoelectric focusing of a doubly-labeled, radioactive extract was performed as for Fig. 3. To remove ampholines, the protein in the fraction with the most complementation activity was precipitated with 5% trichloroacetic acid after addition of lysozyme carrier, and redissolved in gel sample buffer containing 1% sodium dodecyl sulfate. Subsequent sample treatment, electrophoresis, and gel counting techniques were described (24). The counts shown have been corrected for overlap and normalized as for Fig. 3.

In an attempt to determine which protein in Fraction IV is the direct product of gene 62, a double-label experiment was performed: T4 wild-type infected cells were labeled with [^{3}H]leucine after infection, and T4 *am*E1140 (gene 62$^-$)-infected cells were similarly labeled with [^{14}C]leucine; the two cultures were then mixed, and Fraction IV was prepared. Fig. 3 shows the profiles obtained from the final isoelectric focusing column. The bottom panel shows 62-activity as measured with a 62-defective lysate, while the top panel displays the relative amount of ^{14}C- and ^{3}H-labeled protein in each isoelectric fraction. The pH 8.5 and 8.0 fractions are clearly enriched for ^{3}H, indicating that much of the protein they contain was derived only from the wild-type extract. Comparison of the top and bottom panels shows that the ^{3}H-enriched fractions contain the 62-complementation activity.

The active fractions from a comparable isoelectric focusing column were further fractionated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Fig. 4). There are two major peaks of radioactive protein, one at 34,000 daltons and one at 20,000 daltons; *both* of these peaks are greatly enriched for ^{3}H. To explain why *neither* component of the complex was isolated from the 62-defective cells in this experiment, we postulate that a complex is formed *in vivo* that does not readily exchange components *in vitro:* the 62-deficient lysate would thus be unable to contribute its ^{14}C-labeled 44-protein to the ^{3}H-labeled wild-type complex, and this free 44-protein would be lost during the purification. In fact, further experiments suggest that the complex can be formed only with great difficulty from its separate components *in vitro,* since a 1:1 mixture of 62- and 44-deficient donor extracts complements both 62- and 44-deficient receptor lysates only at 100-fold greater concentrations than does a wild-type donor extract (unpublished results).

In recent experiments, done in collaboration with Dr. J. Wiberg, the radioactive proteins in partially fractionated crude extracts have been analyzed by double-label techniques after fractionation on sodium dodecyl sulfate–polyacrylamide gels. Comparing different mutant-infected cells (all of which have the SP62 genotype), we find that a protein with a molecular weight of about 34,000 is missing in a 44-deficient extract, and that a protein with a molecular weight of about 20,000 is missing in a 62-deficient extract. It appears that the heavier component in Fraction IV is the product of gene 44 and the lighter component is the product of gene 62.

DISCUSSION

The *in vitro* DNA synthesis described here is of brief duration, and its relation to true DNA replication is not known. In particular, we do not understand the wide variation in the background levels of (uncomplemented) DNA synthesis in different mutant lysates (Table 1). Perhaps several different partial reactions are possible *in vitro;* clearly, true replication forks need not be operative in any of our complemented lysates. Nevertheless, this system exhibits a requirement for proteins whose precise functions are unknown, but whose involvement in DNA replication has been established from the isolation of conditional lethal mutants. Consequently, we can use this system to provide an assay for isolation of these proteins. This report describes the successful purification of gene-44 and -62 proteins. Dr. Hiroko Hama-Inaba has similarly purified the gene-45 protein to homogeneity using this complementation assay. As in the present case, double-label techniques confirm the identity of the isolated gene-45 protein and attest to the specificity of the assay (H. Hama-Inaba, L. Moran and B. Alberts, manuscript in preparation).

The tight binding of gene-44 and gene-62 proteins found here, as well as the weaker interaction of gene-32 and gene-43 proteins observed previously (12), may be remnants of a much larger structure that constitutes the DNA replication apparatus *in vivo.* It is our hope that, like the ribosome (25), this entire apparatus can be induced to self-assemble *in vitro,* given the proper conditions and suitable concentrations of the appropriate pure proteins and DNA. Whether gene products in addition to those tested in Table 1 will be necessary for such a reconstruction remains an open question: although studies with known *E. coli* DNA mutants have thus far failed to implicate host components in T4 DNA replication (26),

TABLE 3. *Complementation of different mutant lysates by the Fraction IV protein complex*

Receptor cell lysate	DNA Synthesis *in vitro* (cpm) Concentration of Fraction IV protein in the reaction mixture		
	0	0.2 μg/ml	4 μg/ml
Gene 32$^-$	2000	2040	2200
Gene 41$^-$	1700	1940	1770
Gene 43$^-$	170	130	120
Gene 45$^-$	370	350	350
Gene 44$^-$	450	850	920
Gene 62$^-$	660	1000	1740

a requirement for host RNA polymerase (27) and/or T4 "DNA delay" gene products (28) cannot be ruled out.

Determination of the precise role of the 44–62 protein complex in DNA replication may be difficult, inasmuch as it could require purification and characterization of all of the other replication proteins with which this complex interacts.

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