Role of Genes 46 and 47 in Bacteriophage T4 Reproduction

I. In Vivo Deoxyribonucleic Acid Replication

JUNKO HOSODA, ELAINE MATHEWS, AND BARBARA JANSEN

Space Sciences Laboratory, University of California, Berkeley, California 94720

Received for publication 17 May 1971

Functional proteins coded by genes 46 and 47 are required for (i) continuation of deoxyribonucleic acid (DNA) synthesis in the late period of T4 infection and (ii) production of normal, late replicating DNA which contains strands with a sedimentation coefficient in alkaline sucrose greater than that of mature DNA (73S). Continued DNA synthesis in the late period in the absence of functional genes 46 or 47 can be achieved by inhibiting late protein synthesis either by using bacteriophage with a second mutation in gene 55 or by adding chloramphenicol to the culture before the decline in the rate of DNA synthesis. However, when functional 46/47 proteins are absent throughout infection, no strands with a sedimentation coefficient greater than 73S (in alkaline sucrose) are produced. This is the case even when DNA synthesis is allowed to continue. DNA arrest is accompanied by conversion of rapidly sedimenting, replicating DNA to slower sedimenting forms. When 46/47 is absent from the beginning of infection, the conversion product has a smaller sedimentation coefficient than mature DNA both in neutral and alkaline sucrose. When DNA arrest occurs midway in infection by heat-inactivating the ts46 enzyme, the conversion product has a sedimentation coefficient (i) the same as mature DNA in both neutral (63S) and alkaline sucrose if capsid assembly is allowed to take place and (ii) close to 63S in neutral sucrose but heterogeneous and relatively greater (up to 100S) in alkaline sucrose if capsid assembly is inhibited. The structure of this DNA is unknown.

The functions of both genes 46 and 47 are required for deoxyribonucleic acid (DNA) replication and recombination in bacteriophage T4 and for host DNA degradation. When either 46 or 47 is not functional, DNA is synthesized at a normal rate during the early infection period but synthesis stops in the late period (11, 33). Partial impairment of either or both genes causes a decided reduction in genetic recombination among progeny phage (4, 5). It has been suggested that both 46 and 47 functions are necessary for expression of exonuclease activity responsible for degradation of host DNA after T4 infection (8, 23, 33). This exonuclease is probably able to digest T4 DNA (17, 19). There has, as yet, been no in vitro evidence reported to substantiate the existence of this exonuclease or to show whether its regulation by genes 46 and 47 is direct or indirect.

The work reported in this paper was done primarily with one temperature-sensitive mutant and several amber mutants in gene 46 and designed to elucidate the precise nature of the mutants' DNA

replication defects in vivo. An amber mutant and a temperature-sensitive mutant in gene 47 were used in several cases to confirm some specific characteristics found with the gene 46 mutants. The effects of late protein synthesis and capsid assembly on DNA synthesis by 46 mutants were also examined by adding secondary mutations to the phages to inhibit these processes.

While this manuscript was in preparation, other investigators reported work related to DNA replication by 46/47 amber mutants (26-28). Most of their data, where it overlaps our own material, was not contradictory to our conclusions. Some of the results presented here have been reported previously (Hosoda and Mathews, Fed. Proc. 28:861, 1969).

MATERIALS AND METHODS

Phage and bacterial strains. T4 mutants used are in Table 1. For amber mutants, *Escherichia coli* B or B23 was used as nonpermissive host and *E. coli* CR63 was used as permissive host.

Media and buffers. Compositions of TCG medium,

TABLE 1. *Role of genes in bacteriophage T4 reproduction*

Mutant ^a	Defective gene(s) ^b	Function affected ^c
N50	20am	Capsid assembly
B17	23am	Main capsid precursor protein
B3	46am	DNA arrest, recombination, nuclease (?)
N130	46am	DNA arrest, recombination, nuclease (?)
L166	46ts	DNA arrest, recombination, nuclease (?)
A456 × 5	47am	DNA arrest, recombination, nuclease (?)
CB62	47ts	DNA arrest, recombination, nuclease (?)
BL292	55am	Late mRNA transcription
B3 + N50	46am, 20am	
B3 + BL292	46am, 55am	
L166 + B17	46ts, 23am	
L166 + BL292	46ts, 55am	
CB62 + B17	47ts, 23am	

^a Amber mutants and temperature-sensitive mutants were obtained from R. Edgar, except A456 × 5, which is a gift from E. Kutter. Recombinants were prepared in this laboratory.

^b In this paper, multiple mutants are represented by gene numbers and type of mutation is given in parentheses, instead of using individual phage names.

^c DNA, deoxyribonucleic acid; mRNA, messenger ribonucleic acid.

TCG low-phosphate medium, and TCG buffer were reported previously (18, 19). Composition of saline sodium citrate (SSC) buffer was 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0. The composition of column buffer CB-0.1 was 0.02 M tris(hydroxymethyl) aminomethane (Tris)-hydrochloride (pH 7.4), 0.001 M MgCl₂, and 0.1 M NaCl. CB-0.2 and CB-0.4 had the same composition as CB-0.1 except NaCl was 0.2 and 0.4 M, respectively.

Chemicals. Most of the chemicals used in these experiments were reported previously (19). In addition, polyethylene glycol (PEG) 6000 was from Matheson, Coleman and Bell, and Sarkosyl NL30 (sodium sarkosinate) was a gift from Geigy Industrial Chemicals, Inc. Cs₂SO₄ was from Gallard Schlesinger Chemical Manufacturing Corp.

Preparation of ³²P-T4. *E. coli* cells were grown to a concentration of 5 × 10⁸ cells/ml in TCG-low-phosphate medium containing ³²P-phosphate (4 to 24 μCi/μmole) and infected with an average of three

phage particles per bacterium. The culture was incubated for 120 min at 30 C and lysed with chloroform. Cell debris was removed by centrifugation at 6,000 rev/min for 10 min after the culture was treated with ribonuclease and deoxyribonuclease (10 μg/ml each) for 30 min at 37 C. Phage preparations were purified with one of the following procedures. (i) Column chromatography: 1 to 5 ml of lysate containing blue dextran dye was applied to a Sephadex G-75 column (1.2 to 2 by 30 cm). Phage were eluted with CB-0.1. The fraction containing the dye marker was applied to a diethylaminoethyl (DEAE) cellulose column (0.5 by 6 cm). The column was washed with CB-0.2 and phage were eluted with CB-0.4. (ii) PEG precipitation (34): NaCl was added to a final concentration of 0.5 M in the lysate. PEG 6000 was then added to a final concentration of 6% (w/v). The mixture was immediately put into ice for 60 min. Phage were precipitated by centrifugation at 10,000 rev/min for 30 min. The supernatant fluid was decanted off, and the interior surface of the centrifuge tube was wiped with a sterile cotton swab to remove any residual supernatant fluid. The pellet was resuspended in 0.5 to 1 ml of TCG buffer and residual debris was removed by centrifugation at 5000 rev/min for 10 min. Recovery was 80% of the infective phage in the lysate.

Method of infection and measurement of the thymidine incorporation rate. The method of infection and measurement of the thymidine incorporation rate were reported in previous papers (18, 19). A second dose of five phage particles per bacterium was given between 4 and 20 min after infection to achieve lysis inhibition. In the case of ³²P-phage, either no secondary phage was added or nonradioactive phage of the same genotype was used, as indicated in the figure legends.

Measurement of total radioactive thymidine incorporated into DNA. To 0.5- to 1-ml portions of phage-infected cultures, a mixture of radioactive thymidine (³H-TdR) and deoxyadenosine was added at indicated times. A 10-μliter sample of each labeled culture was removed with a disposable micropipette (diSPo pipet, Scientific Products, Div. of American Hospital Supply Corp.) and applied to a Whatman no. 3MM filter paper disc (2.3 cm) which was dropped into an ice-cold 5% trichloroacetic acid solution containing 25 μg of TdR per ml. The discs were washed with 5% trichloroacetic acid and then with 95% alcohol, dried, and counted in toluene-liquifluor in a scintillation counter (Nuclear-Chicago Corp.).

Preparation of extracts from phage-infected cells for sedimentation in sucrose or Cs₂SO₄ gradients. Unless otherwise stated, a slightly modified lysozyme-sarkosyl extraction method (14) was used. From labeled, phage-infected cultures, 0.05- to 0.2-ml portions were added to an ice-cold stop mixture to final concentrations of 0.05 M ethylenediaminetetraacetic acid (EDTA, pH 8.5), 0.05 M NaCl, and 1 mg of lysozyme/ml. The sample-stop mixture ratio varied from 1:1 to 1:9 to dilute the DNA to avoid overloading the sucrose gradients. Samples (10 to 50 μliters) of the mixture were pipetted onto filter paper discs, fixed, dried, and counted to determine total isotope incorporation into

the DNA. After the mixture was kept in ice for 20 min a 0.25 volume of 5% sarkosyl was added and the mixture was returned to the ice bath for another 30 min. Reduction of the sarkosyl concentration from 3 to 0.5% did not change the extraction efficiency.

Phenol extraction of DNA. In some experiments, DNA was extracted from phage suspensions or from lysozyme-sarkosyl-treated cell lysates by gently rolling for 30 min at 10 C with an equal volume of freshly distilled phenol saturated with borate buffer (0.03 M sodium borate, pH 9.0, 0.05 M EDTA, 0.2 M NaCl; reference 16). The extraction was repeated twice, and phenol was removed by overnight dialysis at 4 C against an appropriate buffer.

Preparation of ³H-E. coli DNA. An overnight culture of *E. coli* B3 (thymine-requiring strain) was diluted 50 times with TCG medium fortified with ³H-TdR (50 μ Ci, 5 μ g/ml) and grown to a concentration of 10^8 cells/ml. DNA was extracted from the culture by successive treatment with EDTA, lysozyme, sarkosyl, and phenol by a method similar to that used for extraction of DNA from phage-infected cultures. The extract was dialyzed against and kept in SSC containing 2×10^{-2} M EDTA.

Sucrose gradient zone sedimentation. Unless otherwise indicated, 4.6 ml of 5 to 20% linear sucrose gradients (alkaline 0.9 M NaCl; 0.05 M EDTA, pH 12.0; neutral 0.1 M NaCl; 0.05 M EDTA, pH 8.5) were formed in cellulose nitrate Spinco centrifuge tubes [Beckman Instruments, Inc.; 1/2 by 2 inches (ca. 1.3 by 5.1 cm)]. A 0.5-ml layer of Angio-Conray (containing 20% sucrose and adjusted to pH 8.0 for neutral gradients and pH 11.5 for alkaline gradients) at the bottom of the tubes served to stop rapidly sedimenting material. For sedimentation in alkaline gradients, 0.1 ml of 0.3 M or 1 M NaOH was layered on top of the gradient, and 0.1 ml of the cell extract was overlaid on the alkaline layer. Gradients were refrigerated (10 C) for 30 min prior to centrifugation. For sedimentation in neutral gradients, the sarkosyl extract was heated at 65 C for 10 min and cooled to room temperature before application to the gradient. Centrifugation was done with the SW 39, SW 65, or SW 50.1 rotor in a Spinco model L-2 or L-3 ultracentrifuge. Methods of applying samples and collecting and counting fractions were reported previously (18).

Standard DNA markers and superimposition of DNA profiles. In most experiments, labeled T4 phage were added to the stop mixture, and their DNA was extracted with the sample to serve as molecular weight standards. In the graphs, arrows denote the position of T4 standard DNA. In some cases, profiles from different gradients were superimposed by aligning the reference peaks.

Centrifugation in Cs₂SO₄ gradient. Solutions of buffered Cs₂SO₄ solution and DNA were mixed to give 4 ml of a solution with a refractive index of 1.369 (corresponding to a density of about 1.44 g/ml) containing 0.01 M Tris-hydrochloride (pH 7.4) and 0.02 M EDTA. This solution was centrifuged at 30,000 rev/min for 70 hr at 10 C in a no. 50 angle rotor with adaptors for 4-ml tubes. The bottoms of the tubes were punctured with a pin and about 50 fractions were collected on no. 3MM filter discs.

Hydroxyapatite column chromatography. Hydroxyapatite column chromatography of DNA samples was carried out as described by Oishi (25).

Pronase treatment. In the presence of sarkosyl, 1 mg of Pronase per ml was added to cell lysates treated with lysozyme and sarkosyl as described above, and samples were incubated overnight at 37 C. In the absence of sarkosyl, 100 μ g of heat-treated Pronase per ml (37 C, 120 min; 80 C, 2 min) (35) in Tris-hydrochloride buffer (0.01 M, pH 8.0, containing 0.01 M EDTA) was added to phenol-extracted DNA in the same buffer and incubated for 30 min at 37 C.

Mechanical shearing of DNA. Phenol-extracted DNA [a mixture of ³H-(46ts, 23am) DNA from cell lysates and ³²P-T4 DNA from phage] was diluted in SSC buffer to a final concentration of 2 μ g/ml. The DNA solution (4 ml) was sheared in a Sorvall Omnimixer with microattachment at 24 v for 30 min in the cold.

RESULTS

Rate of thymidine incorporation by *E. coli* B infected with a temperature-sensitive mutant in gene 46. Under nonpermissive conditions, DNA synthesis by conditional mutants in genes 46 and 47 stops prematurely (DNA arrest; references 11, 33). It has been shown that DNA arrest of B3, an amber mutant in gene 46, occurs during the same time period when late protein synthesis starts (17, 19). To examine the dependence of DNA synthesis on the gene 46 function, the rates of thymidine incorporation by L166 (46ts)-infected cells at the permissive and nonpermissive temperatures were measured by pulse-labeling the cultures with ³H-TdR.

Figure 1 shows that when L166 (46ts) was grown at 25 C (permissive temperature), thymidine incorporation started around 10 min after infection and the rate increased until 30 min (curve 1). TdR incorporation continued at a constant rate thereafter if lysis was inhibited by adding a secondary dose of phage. When L166 (46ts) was grown at 42 C, TdR incorporation started at about 3 min after infection, but it began declining by the 10th min and nearly stopped by the 30th min (curve 2). When the culture was transferred from 42 to 25 C at 16 min after infection, thymidine incorporation resumed after a brief lag, showing reversibility of the temperature-induced arrest (curve 3).

Figure 1b shows the results of similar experiments with (46ts, 23am), a phage carrying a temperature-sensitive mutation in gene 46 and an amber mutation in gene 23, B17, which prevents capsid formation (curves 4 and 6); curves 5 and 7 show the results with a phage carrying only B17 (23am). The synthesis rate at 25 C was the same for both, showing that the L166 (46ts) mutation did not impair DNA synthesis at this temperature.

FIG. 1. Temperature effect on the rate of radioactive thymidine incorporation by cultures infected with phage containing the L166 (46ts) mutation. (a) Downshift from 42 to 25 C. *Escherichia coli* B cultures were infected with L166 (46ts) and three series of 0.5-ml samples were pulse-labeled with ^{14}C -TdR (0.2 μCi , 4 $\mu\text{g}/\text{ml}$). Curve 1, infection at 25 C, 2-min pulse; curve 2, infection at 42 C, 1-min pulse; curve 3, infection at 42 C, from 0 to 16 min and 25 C thereafter, 2-min pulse. (Times are the mid-points of the pulse periods.) (b) Upshift from 25 to 42 C. *E. coli* B cultures at 25 C were infected with (46ts, 23am) (curves 4 and 6) or with B17 (23am) (curves 5 and 7). At 35 min after infection (curves 4 and 5) or at 60 min after infection (curves 6 and 7), a portion of each culture was transferred from 25 to 42 C. Samples (0.5 ml) were pulse-labeled with ^{14}C -TdR (0.1 μCi , 4 $\mu\text{g}/\text{ml}$) for 2 min.

When the cultures were transferred from 25 to 42 C at 35 min or at 60 min, the rate increased immediately. The B17 (23am)-infected culture continued TdR incorporation at this increased and constant rate of approximately five times as much as that at 25 C (curves 5 and 7). However, the rate of (46ts, 23am)-infected cultures gradually decreased (curves 4 and 6). Responses of the L166 (46ts)-infected cultures to transfer from 25 to 42 C were similar to those of the (46ts, 23am)-infected cultures except the rate decrease after transfer seemed slightly faster with L166 (46ts).

The rate of thymidine incorporation may not strictly parallel the rate of DNA synthesis, since it also depends on other factors such as the rate of TdR entry to the cell, pool size of DNA precursors in the cells, etc. However, the decreased rate of ^3H -TdR incorporation by (46ts)-infected cells at the high temperature where the rate by non-

temperature-sensitive phage-infected cells increases must be reflecting inhibition of DNA synthesis by (46ts) phage. In the case of (46am) mutants, the rate of TdR incorporation measured by pulse-labeling (17, 19) corresponded fairly well to the increase in the total amount of DNA measured by indol-hydrochloride color reaction (33; J. Hosoda, E. Mathews, and B. Jansen, unpublished data). Therefore, it seems reasonable to conclude from the data presented in Fig. 1 that (i) DNA arrest of the 46 mutant is reversible and (ii) 46 function is required for maintaining DNA synthesis beyond the onset of late protein synthesis, which is 18 to 20 min at 25 C (17).

Effect of Cam and an amber mutation in gene 55 on DNA arrest. It has been reported that phenotypic expression of DNA arrest by N130 (46am) is inhibited by an amber mutation in gene 33 or in gene 55 (which prevents transcription of the late messenger RNA) or by addition of chloramphenicol (Cam) to the cultures (7). In our experiments, DNA arrest in both temperature-sensitive and amber mutants in gene 46 was inhibited by a mutation in gene 55 and by Cam.

Tables 2 to 5 demonstrate the rates of ^3H -thymidine uptake under various conditions, as calculated from the increase in radioactivity of cultures continuously labeled with ^3H -TdR. At 30 C, cultures infected with T4 continued TdR incorporation at an essentially constant rate. Phage carrying the L166 (46ts) mutation incorporated TdR at almost the same rate or at a slightly reduced rate as those infected with non-temperature-sensitive phages (Table 2). When a L166 (46ts)-infected culture was transferred from 30 to 42 C at 35 min after infection, the rate of TdR incorporation dropped after a brief period of rapid uptake (Table 3). However, TdR incorporation by (46ts, 55am), a mutant that does not synthesize late proteins, continued after the upshift at the same rate as BL292 (55am). Thus, DNA arrest after heat inactivation of the L166 protein was caused by some part of the late, protein synthesis process. When Cam was added to L166 (46ts)-infected cultures prior to the transfer, TdR incorporation continued at a much higher rate and for a longer time than in its absence, although DNA arrest was not completely prevented (Table 3). At 30 C, late protein synthesis started approximately 12 min after infection (7). Cam addition after a considerable quantity of late proteins had been synthesized (30 min) was as effective in prolonging DNA synthesis as Cam addition at a time when no accumulation of late protein would be anticipated (10 min).

When an L166 (46ts)-infected culture was kept at 42 C for 30 min, DNA synthesis almost stopped (Table 4). Upon subsequent downshift to 30 C,

TABLE 2. Average rate of ^3H -TdR incorporation at 30 C^a

Expt	L166 (46ts)	L166 (46ts) Cam at 10 min	L166 (46ts) Cam at 30 min	(46ts, 55am)	BL292 (55am)	B17 (23am)	(46ts, 23am)
1	33	30	32	37	42		
2						27	28

^a Deoxyribonucleic acid of *Escherichia coli* B cultures infected with various phage was continuously labeled by adding ^3H -TdR at times indicated. Portions (10 μ liters) of labeled cultures were withdrawn at 5- to 20-min intervals and trichloroacetic acid-insoluble radioactivity of the cultures was plotted versus time. The rates of incorporation (increment of the counts incorporated by 10- μ liter cultures per min of incubation) were calculated and are presented in Tables 2 to 5. Cultures at 30 C were labeled with ^3H -TdR (2.5 μCi , 0.5 $\mu\text{g}/\text{ml}$) from 8 min after infection. To some of the cultures, chloramphenicol (Cam, 100 $\mu\text{g}/\text{ml}$) was added at 10 or 30 min after infection. Figures represent average rate during 20- to 110-min period.

TABLE 3. Rate of ^3H -TdR incorporation after the transfer from 30 to 42 C at 35 min of infection^a

Time after transfer (min)	L166(46ts)	L166(46ts) Cam at 10 min	L166(46ts) Cam at 30 min	(46ts, 55am)	BL292 (55am)
2	98	100	87		68
10	22	92	75	121	82
20	22	46	110	63	56
35	1	4	10	36	44

^a Cultures at 30 C were labeled with ^3H -TdR (2.5 μCi , 0.5 $\mu\text{g}/\text{ml}$) from 8 min after infection. To some of the cultures, chloramphenicol (Cam, 100 $\mu\text{g}/\text{ml}$) was added at 10 or 30 min after infection. A part of each culture was transferred to 42 C at 35 min after infection.

DNA synthesis resumed after a long lag period (Table 5). When Cam was added at the time of the downshift to prevent synthesis of new 46 protein, DNA synthesis resumed at a rate equal to 40 to 60% of the rate observed in a similar culture in the absence of Cam. Therefore, at least part of this resumed synthesis is attributable to reactivation of previously synthesized 46ts enzyme. With later temperature downshifts, a gradual decline of infective centers was observed (Table 6). This and an increasingly longer lag period for resumption of DNA synthesis may be caused by more irreversible inactivation of the protein or by more

TABLE 4. Rate of ^3H -TdR incorporation at 42 C^a

Time after infection (min)	L166(46ts)	(46ts, 55am)
7	38	28
15	12	95
25	1	95

^a Cultures at 42 C were labeled by adding ^3H -TdR (10 μCi , 5 $\mu\text{g}/\text{ml}$) at 4.5 min after infection.

TABLE 5. Rate of ^3H -TdR incorporation after transfer from 42 to 30 C at 30 min of infection^a

Time after transfer (min)	L166(46ts)	L166(46ts) + Cam	(46ts, 55am)
35	12	4	85
55	32	19	73
80	51	33	81

^a Cultures at 42 C were transferred to 30 C at 30 min after infection. ^3H -TdR (10 μCi , 5 $\mu\text{g}/\text{ml}$) was added at the time of transfer; chloramphenicol (Cam, 100 $\mu\text{g}/\text{ml}$), when added, was added at the time of transfer.

TABLE 6. Reversibility of L166 (46ts)^a

Condition of incubation	Burst size
30 C from 0 to 120 min.....	240
42 C from 0 to 30 min.....	0.02
42 C from 0 to 60 min.....	0.4
42 C from 0 to 10 min, 30 C from 10 to 130 min.....	200
42 C from 0 to 30 min, 30 C from 30 to 150 min.....	16
42 C from 0 to 60 min, 30 C from 60 to 180 min.....	6

^a *Escherichia coli* B cultures were infected with L166 (46ts) at a multiplicity of five phage per bacterium. At 4 min, anti-T4 serum ($K = 3$) was added, and, at 8 min, the cultures were diluted 10^4 times. The temperature shift was accomplished by diluting infected cultures into 100 volumes of broth at 30 C. Chloroform was added at the end of the incubation period. After proper serial dilution, phage in the lysates were titrated on *E. coli* B at 30 C.

extensive damage to the DNA during prolonged incubation at 42 C. (46ts, 55am) continually synthesized DNA when grown at 42 C from the beginning of infection (Tables 4 and 5) and continued synthesis at approximately the same rate after transfer to 30 C.

Experiments with B3 (46am)-infected cultures at 25 C (Fig. 2a) demonstrate that Cam must be

added before DNA arrest begins. At 25 C, DNA arrest started at 20 min. When Cam was added at 15 min, the rate of TdR incorporation became constant and DNA arrest was prevented. When Cam was added at 20 min, the rate declined for 5 min and remained constant thereafter. Cam addition later than 20 min had almost no effect on DNA arrest.

Figure 2b demonstrates that the additional amber mutation BL292 (55am) allowed DNA synthesis to continue longer in its presence than in its absence, but limited DNA arrest did still occur. An additional mutation N50 (20am) which prevents capsid assembly had no effect on DNA arrest.

It has been reported that a multiple mutant

FIG. 2. Effect of chloramphenicol (Cam) or additional mutations on the rate of TdR incorporation by B3 (46am). (a) Effect of Cam: *Escherichia coli* B23 cultures at 25 C were infected with B3 (46am) and samples were transferred to flasks containing Cam (100 μ g/ml) at 15, 20, 25, and 30 min after infection. Samples (0.5 ml) of the cultures were pulse-labeled with 14 C-TdR (0.2 μ Ci, 2 μ g/ml) for 2 min. Curve 1, no Cam; curve 2, Cam added at 15 min; curve 3, Cam added at 20 min; curve 4, Cam added at 25 min; curve 5, Cam added at 30 min. (b) Effect of additional mutations: *E. coli* B cultures at 25 C were infected with various mutants. Samples (0.5 ml) were pulse-labeled with 14 C-TdR (0.1 μ Ci, 2.4 μ g/ml) for 2 min. Curve 1, B3 (46am); curve 2, (46am, 20am); curve 3, (46am, 55am); curve 4, BL292 (55am).

containing an amber mutation in gene 46 did not shut off host DNA synthesis (A. Cascino, S. Riva, and E. P. Geiduschek, *in preparation*). The possibility that DNA synthesized by 46 mutants in the presence of Cam or by 46-55 recombinants may be host DNA was excluded by experiments presented in Fig. 3. The difference in buoyant density in Cs_2SO_4 between *E. coli* DNA (1.426 g/ml) and glucosylated-T4 DNA (1.443 g/ml) is large enough (12) that they are clearly separable by equilibrium density gradient centrifugation (Fig. 3a). Figures 3b and c show, respectively, that DNA synthesized by B3 (46am) from 40 to 80

FIG. 3. Equilibrium sedimentation in Cs_2SO_4 gradients. DNA to be tested was labeled with ^3H -TdR and mixed with ^{32}P -T4 DNA (density marker) and centrifuged in Cs_2SO_4 by the method described in Materials and Methods. Solid lines, ^3H -counts; broken lines, ^{32}P -counts. (a) *Escherichia coli* DNA. (b) B3 (46am) DNA synthesized during the late infection period in the presence of chloramphenicol (Cam). Cam (100 μ g/ml) was added at 15 min to a B3 (46am)-infected culture at 25 C. DNA was labeled from 40 to 80 min by adding a mixture of ^3H -TdR (50 μ Ci, 0.8 μ g/ml) and deoxyadenosine (100 μ g/ml). (c) (46ts, 55am) DNA synthesized at 42 C. A (46ts, 55am)-infected culture was transferred from 25 to 42 C at 35 min. DNA was labeled with ^3H -TdR (50 μ Ci, 0.8 μ g/ml) from 50 to 80 min.

min after infection at 25 C in the presence of Cam and DNA synthesized by (46ts, 55am) later than 15 min after the temperature upshift have buoyant densities identical to T4 DNA.

Analysis of DNA by zone sedimentation in sucrose gradients. DNA synthesized in the absence of functional gene 46 was labeled with ^3H -TdR, extracted from infected cells and cosedimented with ^{32}P -labeled mature T4 DNA (extracted from phage) in neutral and alkaline sucrose gradients. In several experiments, cells were infected with ^{32}P -labeled phage to trace the fate of parental DNA.

(i) **Early replicating DNA and its conversion to slower sedimenting forms in the late period.** DNA from cultures infected with various phage at 25 C was extracted at 20 min or later and sedimented in neutral sucrose gradients. The results are summarized in Table 7. Several examples of sedimentation profiles are demonstrated in Fig. 4.

As indicated in Fig. 2b and in our previous paper (19), the rate of TdR incorporation by (46am)-infected cells was normal up to 20 min at 25 C. DNA extracted at 20 min sedimented heterogeneously (Fig. 4a and c) and the amount of rapidly sedimenting DNA was 50 to 70% regard-

less of whether or not 46/47 was functional (Table 7). Up to 20 min at 25 C, which corresponds to the early infection period, the absence of the 46 function apparently did not reduce the rate of DNA synthesis and did not significantly alter the structure of the DNA synthesized.

In confirmation of the report of Frankel (14), B17 (23am), which has a normal 46/47 function but cannot make capsids, produced a rapidly sedimenting fraction which increased quantitatively after 20 min. When the phage had an amber mutation in gene 46 or 47, these rapidly sedimenting fractions of parental and newly synthesized DNA were greatly reduced in DNA that was extracted at 40 min, the time when DNA synthesis ceased (Fig. 4b and Table 7). In the case of wild-type phage, the rapidly sedimenting fraction in the late infection period was converted to mature-size DNA when head assembly started (14). The conversion of (46am) DNA from rapidly sedimenting to slowly sedimenting forms in the late infection period was not due to head assembly because the (20am) mutation, known to prevent capsid assembly, did not prevent conversion (column 1, Table 7). The sedimentation rates of (46am) (Fig. 4b) or

TABLE 7. *Distribution of parental and newly made deoxyribonucleic acid (DNA) in neutral sucrose gradients^a*

Phage name	Genes affected	Cam ^b	Time of extraction (min)	Labeling period (min)	Per cent of DNA sedimented more rapidly than mature DNA	
					Parental DNA	Newly made DNA
B3 + N50	46am, 20am	—	20		50	
			40		23	
			20	12-20		60
			40	12-20		14
A456	47am	—	20	12-20		66
			40	12-20		24
B3	46am	+	20	12-20		64
			40	12-20		34
			40	36-40		47
			50	40-50		60
B3 + BL292	46am, 55am	—	20		68	
			40		44	
			20	12-20		65
			40	12-20		33
B17	23am	—	40	20-40		40
			20	12-20		61
			40	12-20		78
			40	20-40		87

^a Experimental conditions were approximately the same as those in Fig. 3.

^b Chloramphenicol (100 $\mu\text{g}/\text{ml}$) was added at 15 min.

(47am) DNA after conversion were less than that of mature DNA.

The addition of an amber mutation in gene 55 or of Cam at 15 min partially inhibited DNA conversion from rapid to slow sedimenting (Fig 4d and Table 7). Late protein synthesis was responsible for DNA arrest of 46 (47) mutants and also is somehow related to the conversion of 46 (47) DNA to slow-sedimenting forms. However, late observations of Frankel with the same phage

(46am) DNA synthesized in the absence of late protein was generally slower sedimenting than normal late-replicating DNA, and a considerable portion was slower sedimenting than mature DNA. Both the slower sedimenting fractions and the fractions which sedimented more rapidly than mature DNA were capable of incorporating ^3H -TdR throughout the sustained synthesis period. In the absence of 46/47 function, a considerable portion of late-replicating DNA seems to be a smaller or less complicated structure than normal replicating DNA.

When a (46ts, 23am)-infected culture was kept at 42 C until DNA synthesis stopped (30 min), and then downshifted to 30 C, DNA synthesis resumed after a long lag period (20 to 40 min). Figure 5a shows that (46ts, 23am) DNA synthesized at 42 C and extracted at 30 min sedimented more slowly than mature DNA although its average sedimentation rate was greater than the corresponding (late) DNA of amber 46/47 mutants. As Fig. 5b shows, most of the (46ts, 23am) DNA, labeled after synthesis resumed and extracted 90 min after the temperature transfer, sedimented at the same rate as mature DNA and a small quantity sedimented more rapidly. Because this phage cannot make head-precursor proteins, this mature-sized DNA could not have been DNA from newly made phage, but more likely was a replicating DNA which had the same sedimentation rate as mature DNA. This abnormal replicating DNA may be related to the decreasing production of infective centers by L166 (46ts) when it was incubated longer than 30 min at 42 C.

Figure 5c demonstrates that the BL292 (55am) mutation completely blocks conversion of L166 (46ts) DNA from the rapid- to the slow-sedimenting form at 42 C.

(ii) **Dependence of the production of strands sedimenting faster than mature DNA in alkaline sucrose on the 46/47 function.** Even though 60% of the replicating DNA extracted after 20 min at 25 C sedimented more rapidly than mature DNA in neutral sucrose, it was not faster sedimenting than mature DNA when examined after denaturation in alkaline sucrose. This was true whether the phage had a normal 46/47 function or not. Figure 6 (curves 1 and 3) demonstrates that DNA extracted at 20 min from (46am, 20am) and B17 (23am)-infected cultures was slower sedimenting than mature DNA. During incubation from 20 to 40 min, a considerable portion of (23am) DNA became faster sedimenting than mature DNA (curve 4), whereas the sedimentation profile of (46am, 20am) DNA did not change significantly (curve 2). Our data on the sedimentation behavior of B17 (23am) DNA is in agreement with previous

FIG. 4. Analysis of (46am, 20am) and (46am, 55am) DNA by zone sedimentation in neutral sucrose gradients. *Escherichia coli* B cultures were infected with (46am, 20am) or ^{32}P -(46am, 55am). Newly synthesized DNA was pulse-labeled with ^3H -TdR (50 μCi , 0.8 $\mu\text{g}/\text{ml}$) and extracted either at the end of the labeling period or after chasing with nonradioactive TdR (100 $\mu\text{g}/\text{ml}$). (a) (46am, 20am), DNA labeled from 12 to 20 min, extracted at 20 min; (b) (46am, 20am), DNA labeled from 12 to 20 min, chased 20 min, and extracted at 40 min. (c) ^{32}P -(46am, 55am), DNA labeled from 12 to 20 min, extracted at 20 min. (d) ^{32}P -(46am, 55am), DNA labeled from 12 to 20 min, extracted at 40 min. Centrifugation: a and b, 37,000 rev per min for 180 min; c and d, 45,000 rev per min for 100 min. Solid lines, ^3H -DNA; broken lines, ^{32}P -parental DNA.

FIG. 5. Neutral sucrose sedimentation analysis of DNA synthesized by various mutants extracted before and after temperature downshift. *Escherichia coli* B cultures were infected at 42 C with (46ts, 23am) or (46ts, 55am) and transferred to 30 C at 30 min. DNA was labeled by adding portions of the cultures to test tubes containing a mixture of ^3H -TdR (50 μCi , 0.8 $\mu\text{g}/\text{ml}$) and deoxyadenosine (250 $\mu\text{g}/\text{ml}$) and extracted immediately after the labeling period. Extracts (0.1 ml), containing DNA extracted from the numbers of bacterial cells indicated in brackets, were sedimented in neutral sucrose gradients in an SW 50.1 rotor at 45,000 rev/min for 50 min. (a) (46ts, 23am) DNA [2.2×10^7 cells], labeled from 4 to 30 min at 42 C, extracted at 30 min. (b) (46ts, 23am) DNA [10^7 cells] labeled from 30 to 120 min at 30 C, extracted at 120 min. (c) (46ts, 55am) DNA [10^7 cells] labeled from 4 to 30 min at 42 C, extracted at 30 min.

(14). (46am, 20am) DNA extracted at 40 min may be a collection of linear molecules shorter than mature DNA. The distribution of ^3H activity in neutral and alkaline gradients was similar with the greatest activity at a position denoting 0.5-unit length (1, 10). DNA synthesized by single (46am) mutants (B3 and N130) and a (47am) mutant (A456) had sedimentation properties similar to (46am, 20am) DNA, but, after prolonged incubation, DNA from these mutants sedimented more slowly than did (46am, 20am) DNA in both neutral and alkaline gradients (not shown).

Without the 46 function, strands which sediment faster than mature DNA after denaturation were not produced when DNA synthesis was maintained by inhibiting late protein synthesis.

FIG. 6. DNA synthesized by various amber mutants; sedimentation analyses in alkaline sucrose gradients. *Escherichia coli* B cultures were infected at 25 C with various phages and labeled with ^3H -TdR (50 $\mu\text{Ci}/\text{ml}$). DNA was extracted either immediately after pulsing or after chasing with nonradioactive TdR (100 $\mu\text{g}/\text{ml}$). Sedimentation was in alkaline sucrose gradients. (a) (46am, 20am): curve 1, labeled from 12 to 20 min; curve 2, labeled from 12 to 20 min and chased from 20 to 40 min; centrifuged at 32,000 rev/min for 120 min. (b) B17 (23am): curve 3, labeled from 12 to 20 min; curve 4, labeled from 12 to 20 min and chased from 20 to 40 min; centrifuged at 32,000 rev/min for 120 min. (c) (46am, 55am), labeled from 25 to 40 min; centrifuged at 45,000 rev/min for 70 min. (d) B3 (46am) + chloramphenicol (Cam): Cam (100 $\mu\text{g}/\text{ml}$) was added at 15 min. DNA was labeled from 40 to 50 min; centrifuged at 36,000 rev/min for 75 min.

(46am, 55am) DNA, synthesized and extracted in the late period (Fig. 6c), or B3 (46am) DNA, synthesized in the presence of Cam and extracted in the late period (Fig. 6d), sedimented more slowly than mature DNA in alkaline sucrose. As shown in Table 2, the temperature-sensitive mutant (46ts, 55am) was capable of continuing to incorporate TdR at 42 C at the same rate as it did at 30 C. However, DNA synthesized at 42 C was more slowly sedimenting than mature DNA in alkaline sucrose (Fig. 7b) and very rapidly sedimenting in neutral sucrose (Fig. 5c). The appearance of strands sedimenting faster than mature DNA in alkaline sucrose was clearly demonstrated after reactivation of the 46ts protein by a temperature downshift of (46ts, 23am)- or (46ts, 55am)-infected cultures. Some single strands from both temperature-sensitive mutants labeled before and after the temperature shift were more rapidly sedimenting in alkaline sucrose than were mature strands (Fig. 7a and b). Figure 7c represents control DNA strands from BL292 (55am)-infected cells and shows that this mutation, which inhibits late protein synthesis, does not prevent accumulation of strands which sediment faster than mature DNA in alkaline sucrose. Therefore, this fast-sedimenting strand production was not a late function.

(46ts, 55am) Parental strands clearly became rapidly sedimenting (Fig. 8) after the temperature downshift. The presence of rapidly sedimenting (46ts, 23am) parental DNA was not clearly demonstrable.

Sedimentation properties of DNA extracted after temperature upshift from cells infected with temperature-sensitive 46/47 mutants. Cultures infected with phage containing a temperature-sensitive mutation in gene 46 or 47 were grown at a permissive temperature (25 C) until late-replicating DNA was formed and then transferred to a nonpermissive temperature (42 C). Sedimentation properties of DNA extracted after incubation at 42 C were different from those of DNA extracted before the upshift and from those of DNA synthesized during a corresponding period by phage with normal 46/47 function. These properties varied depending upon whether late protein synthesis or head formation was inhibited.

(i) **46/47 DNA when late proteins were synthesized but no capsids were assembled.** When (46ts, 23am)-infected cultures were transferred from 25 C to 42 C, a considerable quantity of DNA was produced before synthesis ceased. DNA extracted from (46ts, 23am)-infected cells at the time of the temperature upshift and 5 min after the shift both had a very high sedimentation rate in neutral sucrose (Fig. 9a and b), but their sedimentation behavior in alkaline sucrose was strikingly differ-

ent (Fig. 9e and f). DNA extracted before the shift was very heterogenous in alkaline sucrose and did not form any definite profile peaks, whereas DNA extracted after the shift was more homogenous and formed an asymmetric peak with a sedimentation rate of 100S. After longer incubation at 42 C, the DNA became slow-sedimenting in neutral sucrose, forming a homogenous peak close to the position of mature DNA (Fig. 9c and d), but there was no significant profile change in alkaline sucrose (Fig. 9g and h). Temperature-induced conversion of DNA from the fast- to the slow-sedimenting form in neutral gradients apparently did not include extensive accumulation of single-strand breaks. DNA represented in Fig. 9 was labeled before 40 min, but DNA labeled

FIG. 7. Alkaline sucrose sedimentation analysis of DNA extracted before and after temperature downshift. Extracts were made in the same way as in the legend of Fig. 5 and sedimented in alkaline sucrose gradients at 45,000 rev/min for 35 min. (a) (46ts, 23am) DNA: curve 1, labeled from 4 to 30 min at 42 C, extracted at 30 min [2.2×10^7 cells]; curve 2, labeled from 4 to 30 min at 42 C, extracted at 120 min after 90-min chasing at 30 C with nonradioactive TdR (100 μ g/ml) [10^7 cells]; curve 3, labeled from 30 to 120 min, extracted at 120 min [5×10^6 cells]. (b) (46ts, 55am) DNA: curve 4, labeled from 4 to 30 min at 42 C, extracted at 30 min [10^7 cells]; curve 5, labeled from 4 to 30 min, extracted at 120 min [5×10^6 cells] after 90-min chasing; curve 6, labeled from 30 to 120 min, extracted at 120 min [5×10^6 cells]. (c) BL292 (55am) DNA: labeled from 4 to 30 min at 42 C, extracted at 30 min (curve 7) or at 120 min (curve 8) [5×10^6 cells] after chasing for 90 min at 30 C.

FIG. 8. Sedimentation analysis of ^{32}P -labeled parental DNA extracted before and after temperature downshift. *Escherichia coli* B cultures were infected at 42 C with ^{32}P -(46ts, 23am) or ^{32}P -(46ts, 55am) at a multiplicity of six phage per bacterium. Unadsorbed phage were removed at 2 min by filtration. A secondary dose of nonradioactive phage (six particles per bacterium) was added at 15 min to inhibit lysis. The cultures were transferred to 30 C at 30 min. Extracts (0.1 ml) were centrifuged at 45,000 rev/min for 70 min in alkaline sucrose gradients. (a) (46ts, 23am) DNA and (b) (46ts, 55am) DNA: curves 1 and 3, extracted at 30 min; curves 2 and 4, extracted at 120 min.

after 40 min had similar sedimentation profiles. Similar behavior was observed when the temperature upshift occurred at 60 min after infection rather than 35 min.

(46ts, 23am) DNA extracted 15 or more min after the temperature upshift sedimented faster in alkaline gradients than would be expected from its sedimentation rate in neutral sucrose. Phenol extraction or treatment with Pronase made DNA somewhat heterogeneous in alkaline sucrose but did not change the profile in neutral sucrose (cf. DNA in Fig. 9d and h with control in Fig. 10). Even though its sedimentation rate in neutral sucrose was fairly homogenous and very close to that of mature DNA, this DNA is not a true linear duplex as is mature DNA.

The introduction of double-strand breaks by mechanical shearing eliminated fast sedimentation in alkaline sucrose. As indicated in Fig. 10, (46ts, 23am) DNA sheared into quarter-length molecules formed the same sedimentation profile as sheared mature DNA in both neutral and alkaline gradients.

(47ts, 23am) responded to temperature upshift in basically the same way as (46ts, 23am), although CB62 (47ts) was much leakier than the

L166 (46) mutation. ^3H -labeled DNA of (47ts, 23am)-infected cells extracted at the time of temperature upshift sedimented to the bottom of a neutral gradient and was heterogeneously distributed in an alkaline gradient, as was the corresponding (46ts, 23am) DNA. In neutral sucrose, DNA extracted 15 min after the temperature shift formed a homogenous peak close to the one formed by mature T4 DNA. However, in alkaline sucrose gradients, a large percentage of it sedimented faster than mature T4 DNA.

DNA extracted from a B17 (23am)-infected culture incubated from 0 to 35 min at 25 C and 35 to 50 min at 42 C sedimented to the bottom in neutral sucrose and more heterogeneously than the corresponding (46ts, 23am) DNA in alkaline sucrose (data not presented).

(46ts, 23am)-DNA extracted 15 min after the temperature up-shift (lysozyme-sarkosyl followed by phenol extraction) had the same density as mature T4 DNA in neutral and alkaline CsCl gradients. It eluted from an hydroxyapatite column at the same salt concentration as glucosylated native T4 DNA. If this (46ts, 23am) DNA contained single-stranded portions, they were not abundant enough to cause a change in density in CsCl gradients.

(ii) **L166 (46ts) DNA when head formation was not inhibited.** When a L166 (46ts)-infected culture was transferred from 25 to 42 C at 35 min, DNA synthesis ceased after a period of rapid synthesis as was the case with (46ts, 23am). However, virtually all of the DNA extracted 15 min after the upshift, whether synthesized before or after the shift, had exactly the same sedimentation coefficients as mature DNA in both neutral and alkaline gradients (Fig. 11).

Thirty per cent of this DNA was in intact phage (not extractable with cold neutral sarkosyl). Both before and after the transfer, a considerable quantity of strands with a sedimentation coefficient greater than 73S in alkaline sucrose had been produced and was somehow reduced to the 73S form during the 15 min of incubation at 42 C.

(iii) **(46ts, 55am) DNA when no late proteins were synthesized.** (46ts, 55am)-infected cultures grown at 25 C for 35 min and then transferred to 42 C did not stop DNA synthesis after the shift. DNA extracted 15 min after transfer was as rapidly sedimenting in neutral sucrose as that extracted before the transfer. However, this DNA formed an asymmetric peak with a sedimentation coefficient of 100S in an alkaline sucrose gradient as did the corresponding (46ts, 23am) DNA. (46ts, 55am) DNA was even more homogenous than (46ts, 23am) DNA, and more than 50% of the radioactivity was between the 80S and the 100S positions. The late-replicating DNA, when

FIG. 9. Sedimentation analysis of (46ts, 23am) DNA extracted before and after temperature upshift. (46 ts, 23am)-infected cells grown at 25 C were transferred to 42 C at 35 min. DNA was labeled by adding a mixture of ^3H -TdR (20 μCi , 0.4 $\mu\text{g}/\text{ml}$) and deoxyadenosine (250 $\mu\text{g}/\text{ml}$) at 12 min. Nonradioactive TdR (100 $\mu\text{g}/\text{ml}$) was added at 40 min. DNA was extracted at 35 min (a and e), 40 min (b and f), 50 min (c and g), and 80 min (d and h). Samples (a to d) were centrifuged in neutral sucrose gradients at 36,000 rev/min for 75 min (a) or for 35 min (b to d). Samples e to h were centrifuged in alkaline sucrose gradients at 45,000 rev/min for 75 min (e) or for 35 min (f to h). [4×10^7 cells (a and e); 4×10^8 cells (b to d and f to h)]. Solid lines, ^3H -DNA; broken lines, ^{32}P -DNA (standard).

46/47 was not functional, seemed to be preferentially converted to 100S strands. When late protein synthesis was taking place, late-replicating DNA was converted to 63S/100S DNA. When late protein synthesis was inhibited, preferential production of 100S strands continued without conversion of the replicating DNA to slower sedimenting forms.

DISCUSSION

DNA arrest. In confirmation of a previous publication by Bolle et al. (7), DNA arrest in the absence of 46/47 function was clearly induced by late protein synthesis. When late protein synthesis was inhibited, both 46am and 46ts mutants did not exhibit their usual DNA arrest behavior under nonpermissive conditions, and DNA synthesis continued. Buoyant density profiles in Cs_2SO_4 , of DNA labeled during this sustained synthesis in

the absence of 46 function, proved this DNA was glucosylated T4 DNA, not host DNA.

There are two series of reactions which are characteristic of the late infection period. One is substantial transcription of r-strand (A. Guha et al., *in preparation*) and the other is a series of reactions leading to DNA maturation. It seems likely that DNA arrest in the absence of 46/47 function is caused by one or more of the enzymes involved in one or both of these processes. Cam added at a time when a considerable amount of late protein had been accumulated was equally effective in preventing DNA arrest as Cam added at a time when no late proteins were accumulated. This suggests that the unidentified late protein which induces DNA arrest is labile or quantitatively used up and therefore does not accumulate.

Effect of 46/47 mutation on the structure of vegetative DNA. The 46/47 mutation not only

FIG. 10. Effect of mechanical shearing on the sedimentation properties of (46ts, 23am)-DNA extracted after temperature upshift. DNA of (46ts, 23am)-infected cultures, grown from 0 to 35 min at 25 C and from 35 to 50 min at 42 C, was labeled with ^3H -TdR as in the experiment represented in Fig. 9. DNA extracted with phenol from lysozyme-sarkosyl lysates (0 C) was mixed with ^{32}P -DNA extracted from T4 phage and sheared as described in Materials and Methods. (a and b) DNA before shearing; (c and d) DNA after shearing. Samples a and c were centrifuged in neutral gradients at 36,000 rev/min for 100 min; samples b and d were centrifuged in alkaline gradients, b at 36,000 rev/min for 100 min and d at 45,000 rev/min for 120 min. Solid lines, ^3H -DNA; broken lines, ^{32}P -DNA.

FIG. 11. Sedimentation analysis of L166 (46ts) DNA extracted before and after temperature upshift. L166 (46ts)-infected cells grown at 25 C were transferred to 42 C at 35 min. DNA was labeled by adding a mixture of ^3H -TdR (30 μCi , 0.6 $\mu\text{g}/\text{ml}$) and deoxyadenosine (200 $\mu\text{g}/\text{ml}$) at 12 min. DNA was extracted at 35 min (a and c; 9×10^8 cells) and 50 min (b and d; 4×10^8 cells). Samples a and b were centrifuged in neutral sucrose, c and d in alkaline sucrose. Centrifugation was for 35 min at 45,000 rev/min. Solid lines, ^3H -DNA; broken lines, ^{32}P -DNA (standard).

induces DNA arrest but also greatly alters the structure of vegetative DNA (phage DNA in the host cells as opposed to mature DNA in the phage). Figure 12 summarizes sedimentation properties of DNA at various stages of replication.

Mature DNA molecules of T-even phage are linear, double-stranded molecules and the genetic maps of T2 and T4 are circular (3, 29, 30). Physical studies of T2 have shown circularly permuted nucleotide sequences in mature T2 DNA molecules (32) that are terminally repetitious (24). The sedimentation coefficient of mature DNA in the native state is 63S (9), and in the denatured state it is 73S (31). After normal T4 infection, most of the newly synthesized and a part of the parental DNA sediment much more rapidly than mature T4 DNA in neutral sucrose gradients (13) (Fig. 12, step 1). Sedimentation rates of this replicating DNA after denaturation vary depending on the time of extraction after infection. During the early infection period, most strands (early-replicating DNA) had a sedimentation rate less than mature DNA (M-DNA). In late infection period, strands were produced with a sedimentation rate up to two times greater than that of M-DNA (14) (Fig. 12, step 2; late-replicating DNA). M-DNA is produced from this late-replicating DNA. Linear DNA synthesis and linear phage production continue through most of the late period, keeping the late-replicating DNA pool at a constant size.

Change from early-replicating to late-replicating, newly made DNA depends on genes 46 and 47. Denatured parental DNA strands also become faster sedimenting only when 46 and 47 are functional. In the absence of either gene 46 or 47, DNA synthesis ceases, and early-replicating DNA is converted to DNA with a sedimentation rate less than M-DNA in both the native and denatured state (probably a collection of linear duplex molecules shorter than M-DNA) (Fig. 12, step 4). If late protein synthesis is inhibited, production of early-replicating DNA continues.

Heat inactivation of 46ts in the late period does not prevent DNA maturation when capsid assembly is not inhibited, but all vegetative DNA is exhausted because no new DNA is being synthesized. If late protein synthesis is inhibited, production of late-replicating DNA continues even when 46ts is heat inactivated. Late-replicating DNA synthesized in the absence of 46 function has a more homogenous sedimentation rate in the denatured state (80S to 100S) than normal late-replicating DNA. Heat inactivation of 46ts (or 47ts) when only capsid protein production is inhibited (other late proteins are produced) results in an unusual DNA structure. Its sedimentation rate in the native state is close to that of M-DNA but exceeds that of M-DNA in the denatured state (Fig. 12, step 5). Introduction of double-strand breaks by shearing the DNA into quarter-length segments eliminates this relatively rapid sedimentation in alkaline gradients. The DNA does not contain enough single-stranded portions to be detectable by CsCl density gradient centrifugation. This 63S/100S DNA, currently under further investigation, may have cross-linkage between strands.

Late protein seems to be responsible for conversion of both early- and late-replicating DNA to the slower sedimenting forms found in the absence of 46/47 function (Fig. 12, steps 3-5). There is some evidence that conversion is related to DNA maturation. Experiments undertaken after the text of this manuscript was completed prove that conversion of the rapidly sedimenting 46ts and 46am DNA to slower sedimenting forms is completely blocked when gene 49 is not functional (Hosoda, *in preparation*). Gene 49 is known to be related to head maturation (21), and it regulates synthesis of late protein(s) which converts replicating T4 DNA into 63S DNA *in vitro*. (Frankel, *in preparation*). When genes 46/47 are functional, they probably compete with M-DNA formation to maintain or recreate the replicating DNA pool.

Both 46 and 47 functions seem to be necessary for expression of an exonuclease responsible for

FIG. 12. Sedimentation properties of various DNA. Abbreviations: N, sedimentation rate in native state; A, sedimentation rate in denatured state; M-DNA, mature T4 DNA.

the degradation of host DNA after T4 infection (8, 23, 33). As we have further suggested, the 46/47-related nuclease activity might also be able to digest T4 DNA starting from single-stranded breaks (19; N. Prasad and J. Hosoda, *Fed. Proc.* 30:1264, 1971).

When 46/47 was only partially functional, there was a marked reduction of recombinants among progeny phage, indicating that 46/47 function is necessary for recombination (4, 5). Recently, Broker and Lehman indicated that many parental strand fragments from a ligase⁻-DNA polymerase⁻-(lig⁻, pol⁻)-T4 mutant associated to form branched hybrid molecules which they regard as molecular recombinant intermediates. Mutation in gene 32, 46, or 47 prevented branched-hybrid molecule formation (T. Broker and I. R. Lehman, *J. Mol. Biol.*, *in press*). Mutants in gene 32 are also known to inhibit recombination (4, 5). After heat inactivation of (32ts, 23am), in the absence of capsid assembly, the late replicating DNA was converted to 63S (M. Curtis and B. Alberts, *personal communication*).

Electron microscopy has revealed the rapidly sedimenting vegetative T4 DNA to be a gigantic structure (20) containing some strands longer than mature size, condensed areas (20), possible forks, and single-stranded regions (22). The 32, 46, and 47 roles in progeny recombination, in formation of branched-hybrid molecules and in making late-replicating DNA may share a common mechanism(s). Study of the contributions of these genes to the structure of vegetative (and replicating) DNA may eventually lead to understanding the procedures of mature → vegetative → mature DNA.

It is not clear when 46/47 proteins are synthesized. In the early infection period, the sedimentation coefficients in alkaline sucrose of both parental and newly synthesized DNA did not exceed the coefficient of mature DNA even when 46/47 genes were normal. However, this may not be related to the time of 46/47 protein synthesis. Probably the 46/47 proteins are early proteins. A maturation-defective mutant which does not synthesize late proteins still produces strands with a sedimentation coefficient greater than 73S in alkaline sucrose.

Reversibility of DNA arrest. Experiments with L166 (46ts) and with (46ts, 23am) proved that (i) DNA arrest by these mutants is reversible and (ii) L166 (46ts) enzyme activity is recoverable after heat inactivation. However, the degree of reversibility of DNA arrest decreased gradually as the incubation time at the nonpermissive temperature was increased. When the cultures were downshifted at 30 min (DNA synthesis com-

pletely stopped), synthesis resumed only after a very long lag period. The replicating DNA produced after resumption was not structurally normal. As long as 90 min after the downshift, only a small fraction of the DNA was more rapidly sedimenting than mature DNA. Accumulative damage to the DNA may occur during incubation at the nonpermissive temperature. As other investigators have shown (26, 28), the DNA of 46/47 amber mutants does become detached from the cell membrane, and the long lag period before resumption of synthesis may be required for reattachment before synthesis reinitiates.

ACKNOWLEDGMENTS

We are indebted to C. Shalitin, D. Shah, T. Broker, and R. Frankel for preprints of their manuscripts, to B. Alberts and J. Soder for aid in preparation of this manuscript, and to T. H. Jukes for his interest and support of our research.

This work was supported by U.S. Public Health Service research grant GM-16841-01 from the National Institute of General Medical Sciences, American Cancer Society research grant E-517A, and National Aeronautics and Space Administration grant Ngr 05-003-020 to the University of California, Berkeley.

LITERATURE CITED

1. Abelson, J., and C. A. Thomas, Jr. 1966. The anatomy of the T5 bacteriophage DNA molecule. *J. Mol. Biol.* 18:262-291.
2. Anraku, N., Y. Anraku, and I. R. Lehman. 1969. Enzymatic joining of polynucleotides. VIII. Structure of hybrids of parental T4 DNA molecules. *J. Mol. Biol.* 46:481-492.
3. Baylor, M. B., A. Y. Hessler, and J. P. Baird. 1965. The circular linkage map of bacteriophage T2H. *Genetics* 51:351.
4. Berger, H., A. J. Warren, and K. E. Fry. 1969. Variations in genetic recombination due to amber mutations in T4D bacteriophage. *J. Virol.* 3:171-175.
5. Bernstein, H. 1968. Repair and recombination in phage T4 I, genes affecting recombination. *Cold Spring Harbor Symp. Quant. Biol.* 33:325-331.
6. Bode, V. C., and A. D. Kaiser. 1965. Changes in the structure and activity of DNA in a superinfected immune bacterium. *J. Mol. Biol.* 14:399-417.
7. Bolle, A., R. H. Epstein, W. Salser, and E. P. Geiduschek. 1968. Transcription during bacteriophage T4 development: requirements for late messenger synthesis. *J. Mol. Biol.* 33:339-362.
8. Bose, S. K., and R. J. Warren. 1969. Bacteriophage-induced inhibition of host function. II. Evidences for multiple sequential bacteriophage-induced deoxyribonuclease responsible for degradation of cellular deoxyribonucleic acid. *J. Virol.* 3:549-556.
9. Burgi, E., and A. D. Hershey. 1961. A relative molecular weight series derived from the nucleic acid of bacteriophage T2. *J. Mol. Biol.* 3:458-472.
10. Burgi, E., and A. D. Hershey. 1963. Sedimentation rate as a measure of molecular weight of DNA. *Biophysical Journal* 3:309-321.
11. Epstein, R. H., A. Bolle, C. M. Steinberg, E. Kellenberger, E. Boy DeLa Tour, R. Chevalley, R. S. Edgar, M. Susman, G. H. Denhardt, and A. Lielausis. 1963. Physiological studies of conditional lethal mutants of bacteriophage T4D. *Cold Spring Harbor Symp. Quant. Biol.* 28:375-394.
12. Erickson, R. L., and W. Szybalski. 1964. The Cs₂SO₄ equilibrium density gradient and its application for the study of T-even phage DNA: glucosylation and replication. *Virology* 22:111-124.

13. Frankel, F. R. 1966. Studies on the nature of replicating DNA in T4 infected *Escherichia coli*. J. Mol. Biol. 18:127-143.
14. Frankel, F. R. 1968. Evidence for long DNA strands in the replicating pool after T4 infection. Proc. Nat. Acad. Sci. U.S.A. 59:131-138.
15. Frankel, F. R. 1968. DNA replication after T4 infection. Cold Spring Harbor Symp. Quant. Biol. 33:485-493.
16. Hickson, F. T., T. F. Roth, and D. R. Helinski. 1967. Circular DNA forms of a bacterial sex factor. Proc. Nat. Acad. Sci. U.S.A. 58:1731-1738.
17. Hosoda, J., and C. Levinthal. 1968. Protein synthesis by *E. coli* infected with bacteriophage T4D. Virology 34:709-727.
18. Hosoda, J., and E. Mathews. 1968. DNA replication *in vivo* by a temperature sensitive polynucleotide ligase mutant of T4. Proc. Nat. Acad. Sci. U.S.A. 61:997-1004.
19. Hosoda, J., and E. Mathews. 1971. DNA replication *in vivo* by polynucleotide ligase defective mutants of T4. II. Effect of chloramphenicol and mutations in other genes. J. Mol. Biol. 55:155-179.
20. Huberman, Joel A. 1968. Visualization of replicating mammalian and T4 bacteriophage DNA. Cold Spring Harbor Symp. Quant. Biol. 33:509-524.
21. King, J. 1968. Assembly of the tail of bacteriophage T4. J. Mol. Biol. 32:231-262.
22. 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.
23. Kutter, E. M., and J. S. Wiberg. 1968. The degradation of cytosine-containing bacterial and phage DNA after infection of *E. coli* B with bacteriophage T4D and with mutants defective in gene 46, 47 and 56. J. Mol. Biol. 38:395-414.
24. MacHattie, L. A., D. A. Ritchie, C. A. Thomas, Jr., and C. C. Richardson. 1967. Terminal repetition in permuted T4 bacteriophage DNA molecules. J. Mol. Biol. 23:355-364.
25. Oishi, M. 1968. Studies of DNA replication *in vivo*. III. Accumulation of a single stranded isolation product of DNA replication by conditional mutant strains of T4. Proc. Nat. Acad. Sci. U.S.A. 60:1000-1006.
26. Shah, D. B., and H. Berger. 1971. Replication of gene 46-47 amber mutants of bacteriophage T4D. J. Mol. Biol. 57:17-34.
27. Shalitin, C., and S. Kahana. 1970. Conversion of T4 gene 46 mutant deoxyribonucleic acid into nonviable bacteriophage particles. J. Virol. 6:353-362.
28. Shalitin, C., and Y. Naot. 1971. Role of gene 46 in bacteriophage T4 deoxyribonucleic acid synthesis. J. Virol. 8:142-153.
29. Streisinger, G., R. S. Edgar, and G. H. Denhardt. 1964. Chromosome structure in phage T4. I. Circularity of the linkage map. Proc. Nat. Acad. Sci. U.S.A. 51:775-779.
30. Streisinger, G., J. Emrich, and M. M. Stahl. 1967. Chromosome structure in phage T4. III. Terminal redundancy and length determination. Proc. Nat. Acad. Sci. U.S.A. 57:292-295.
31. Studier, F. W. 1965. Sedimentation studies of the size and shape of DNA. J. Mol. Biol. 11:373-390.
32. Thomas, C. A., Jr., and I. Rubenstein. 1964. The arrangements of nucleotide sequences in T2 and T5 bacteriophage DNA molecules. Biophys. J. 4:93-106.
33. Wiberg, J. S. 1966. Mutants of bacteriophage T4 unable to cause breakdown of host DNA. Proc. Nat. Acad. Sci. U.S.A. 55:614-621.
34. Yamamoto, K. R., B. M. Alberts, R. Benzinger, L. Lawhorne, and G. Treiber. 1970. Rapid bacteriophage sedimentation in the presence of polyethylene glycol and its application to large-scale virus purification. Virology 40:734-744.
35. Young, E. T., and R. L. Sinsheimer. 1967. Vegetative bacteriophage λ DNA. II. Physical characterization and replication. J. Mol. Biol. 30:165-200.