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Model for DNA Packaging into Bacteriophage T4 Heads

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The mechanism of DNA packaging into bacteriophage T4 heads in vivo was investigated by glucosylation of hydroxymethlycytosine residues in a conditionally glucose-deficient host. Cytoplasmic DNA associated with partially packaged ts49 heads can be fully glucosylated, whereas DNA already packaged into these heads is shown to be resistant to glucosylation. After temperature shift and completion of arrested packaging into the reversible temperature-sensitive ts49 head, the structure of the DNA in the mature ts49 phage was investigated by restriction enzyme digestion, autoradiography, and other techniques. Such mature DNA appears to be fully glucosylated along part of its length and nonglucosylated on the remainder. Its structure suggests that the DNA is run into the head linearly and unidirectionally from one mature end and that there is little sequence specificity in that portion of the T4 DNA which first enters the capsid. This technique should be useful in investigation of the three-dimensional structure of first- and last-packaged DNA within the head; preliminary studies including autoradiography of osmotically shocked phage suggest that the DNA which first enters the head is deposited toward the center of the capsid and that the end of the DNA which first enters the head exits first upon injection. In conjunction with studies of the structure of condensed DNA, the positions and functions of T4 capsid proteins in DNA packaging, and the order of T4 packaging functions [Earnshaw and Harrison, Nature (London) **268**:598-602, 1977; Hsiao and Black, Proc. Natl. Acad. Sci. U.S.A. **74**:3652-3656, 1977; Müller-Salamin et al., J. Virol. **24**:121-134, 1977; Richards et al., J. Mol. Biol. **78**:255-259, 1973], the features described above suggest the following model: the first DNA end is fixed to the proximal apex of the head at p20 and the DNA is then pumped into the head enzymatically by proteins (p20 + p17) which induce torsion in the DNA molecule.

The structure of condensed DNA within viruses and the mechanism of its condensation and decondensation are of interest for understanding higher-order DNA structural organization and intracellular mobilization. A plausible model for the general organization of DNA within bacteriophage heads has come from electron micrographs of highly condensed DNA suddenly released from capsids (31) and X-ray analysis of isometric phage heads in solution (8). These investigations have suggested that DNA within the phage head is tightly wound as a coaxial spool (see Fig. 10i, which was taken from reference 8).

How DNA is tightly packaged into the phage head from the metabolically active and extended DNA within the cell and how it can subsequently be rapidly delivered (within seconds in the case of phage T4) from the head upon injection into the infected cell are not understood. It had been proposed that the DNA is packaged into the head by a pulling mechanism arising from cleavage or exit of core proteins from the prehead (4, 20), by expansion of the prehead (13, 32), or in a spontaneous process involving charge neutralization by small molecules and/or attachment to capsid inner-surface proteins (12). Any proposed mechanism should attempt to account for the energetics and the reversibility of DNA condensation.

In this paper a model is advanced for DNA condensation which appears to be consistent with structural analyses of condensed DNA (8, 31), with experiments reported here on the dynamics of DNA packaging into the phage T4 head and the structure of this DNA, with structural protein locations in the bacteriophage T4 capsid and their functions in DNA packaging (14, 28), and with T4 functions known to be required for packaging and their order of function (14; C. L. Hsiao and L. W. Black, Virology, in press).

MATERIALS AND METHODS

Bacteria and bacteriophage. *Escherichia coli* DF2000 (phosphoglucose isomerase$^-$, glucose-6-P-de-

hydrogenase$^-$) is unable to synthesize or metabolize glucose (10). *E. coli* CR63su_I^+ r_6^- $r_{2,4}^-$ is permissive for amber mutants and non-glucosylated phage T4; *E. coli* B40su_I^+ r_6^+ $r_{2,4}^+$ is permissive for amber mutants and nonpermissive for non-glucosylated phage T4. Phage T4 49(*tsC*9)-t(*amA*3) was used for labeling; t(*amA*3) prevents lysis of infected nonpermissive cells before addition of chloroform (15). 24(*tsL*90)-49(*tsC*9)-t(*amA*3) and 20(*csN*33)-21(*tsN*12)-t(*amA*3) were prepared from the single mutants. Other techniques were as previously described (5, 14).

Radioactive labeling procedures. (i) Method 1. *E. coli* DF2000 (3×10^8 cells per ml) growing in tryptone broth–M9 salts–0.5% glycerol at 42°C were infected with T4 49(*tsC*9)-t(*amA*3) at a multiplicity of 6. At 21 min after the infection, chloramphenicol was added to a final concentration of 100 μg/ml, and at 25 min D-[6-^{3}H]glucose (6 to 14 Ci/mmol; Amersham/Searle) was added to a concentration of 10 to 100 μCi/ml in various experiments, and the bacteria were immediately shifted to 20°C. At 100 min the bacteria were centrifuged from solution, and the phage were purified on CsCl band gradients.

(ii) Method 2. The procedure was the same as method 1, except that beginning one generation before infection, *E. coli* DF2000 were grown in Tris–glycerol–amino acids medium containing 5 μg of PO_4 per ml, (1 μCi of $^{32}PO_4$ per μg of PO_4). Infection was in the same $^{32}PO_4$ medium.

(iii) Method 3. The procedure was identical to method 2, except that $^{32}PO_4$ was added to a specific activity of 40 μCi/μg of PO_4.

(iv) Method 4. To complete glucosylation for the experiments shown in Fig. 3 and 5, the labeling procedures above were used, except that D-[*U*-^{14}C]glucose was added at 25 min. At 31 min, nonradioactive glucose was added to 0.4%, and the *ts*49-t-infected cultures were immediately shifted to 25°C until 110 min.

Nuclease digestions with exonuclease VII. Exonuclease VII degrades single-stranded DNA progressively from the 3′ and 5′ ends and is active on glucosylated T4 DNA (6). DNA was phenol extracted from purified *ts*49-t phages labeled by method 2. Alkaline sucrose gradients showed one slightly skewed peak in the thoroughly dialyzed DNA, suggesting relatively few single-strand breaks (data not shown). DNA was digested with exonuclease VII in EDTA after heat denaturation as described previously (6). Acid-soluble counts were determined with time at 37°C by addition of 50-μl portions to a solution containing 100 μl of water, 50 μl of bovine serum albumin (1 mg/ml), 50 μl of salmon sperm DNA (1 mg/ml), 50 μl of saturated $NaPP_i$, and 150 μl of 10% trichloroacetic acid. After centrifugation for 15 min at 12,000 rpm, the supernatant was counted.

Restriction enzyme digestion. In the experiments shown, commercial *Eco*RI enzymes from Boehringer Mannheim Corp. and Bethesda Research Laboratories were used.

Blending experiments and autoradiography. In the standard procedure, *E. coli* CR63r_6^- $r_{2,4}^-$ growing in H-broth plus M9 salts were infected with double-labeled *ts*49-t phage (method 3) at 5 min after addition of chloramphenicol to a concentration of 100 μg/ml. After adsorption at 37°C for 4 min, T4 antiserum was added for 2 min, and the infected bacteria were centrifuged from solution and suspended in an equal volume of 1 mM $MgSO_4$–0.1 mM $CaCl_2$–0.125% Antifoam A. One-half of the suspended bacteria was blended in a Sorvall Omnimixer microchamber at top speed for 4 min in an ice bath, and the blended and unblended portions were then centrifuged from solution for 5 min at 4,500 rpm. The pellets were digested with lysozyme-DNase solution in the presence of chloroform and counted. The recovery of infected bacteria was only slightly reduced by the blending (70 to 100% recovery), and parallel experiments showed that the blending procedure removed about 80% of the counts from late-labeled ^{14}C-amino acid-containing purified T4$^+$. In the same procedure, about 70% of the [^{3}H] thymidine counts in T4$^+$ were resistant to blending. The relative amount of ^{3}H and ^{32}P removed into the supernatant from the *ts*49-t labeled phages increased with increasing time of storage, with proportionally more ^{3}H found in the supernatant. The ^{32}P- and ^{3}H-labeled phage, mixed with 1% of the initial titer of nonradioactive T4rII phage, were stored at 4 to 6°C in H-broth. Autoradiography was carried out by standard methods by using Ilford L4 emulsion spread over rotary Pt-Pd-shadowed and then lightly carbon-shadowed Kleinschmidt spread DNA (3, 18). In the osmotic shock procedure, ^{3}H-labeled phage (method 1) were dialyzed versus 2 M ammonium acetate–0.05% isopropanol and rapidly spread on a water hypophase (18). DNA for the Kleinschmidt procedure was purified from ^{3}H-labeled phage (method 1) by phenol extraction followed by dialysis. In some cases the DNA was stained with 0.05% uranyl acetate before the metal casting. Metal and carbon cast grids were spread with emulsion to serve as controls of grain formation during storage. Photographic development was generally for 3 min in D19 developer, and grids were examined in Siemens 1A microscopes.

RESULTS

A number of distinct, maturable bacteriophage T4 head intermediates accumulate in vivo in mutant-infected cells: (i) *ts*24 preheads empty of DNA and containing early, uncleaved precursor forms of the capsid proteins (1); (ii) *cs*20 heads empty of DNA and containing fully cleaved capsid proteins (14); and (iii) *ts*49 heads which contain a partial complement of DNA and fully cleaved capsid proteins (12, 17, 19, 24). Filling of *ts*49 heads appears to be arrested by failure to remove recombinational intermediates from the concatameric DNA (17). Maturation of these head precursors is blocked by reversible temperature-sensitive mutations at different stages of development along the head assembly pathway.

Wild-type phage T4 DNA is normally glucosylated on all its hydroxymethylcytosine residues. Such glucosylation is dispensible; however, nonglucosylated T4 phage (T* phage) are unable to grow on many hosts due to restriction systems directed against non-glucosylated DNA. Gluco-

sylation of T4 DNA is dependent not only upon phage-induced α- and β-glucosyltransferases but also upon host enzyme synthesis of UDP glucose (30). Because glucosylation of T4 DNA follows replication and DNA packaging into heads is not coupled to replication (14, 38), these features of phage T4 development provide a means of analyzing the dynamics of DNA packaging into T4 heads in vivo. As is shown in the following experiments, packaged DNA appears to be resistant to glucosylation, whereas cytoplasmic DNA can undergo very rapid glucosylation after replication. A host conditionally deficient in UDP glucose synthesis (10) therefore provides a means of distinguishing between packaged and unpackaged DNA in head intermediates by specifically labeling the latter.

When bacteriophage T4 *cs*20-*ts*21-t empty heads which accumulate in vivo at low temperature are converted into mature phage by raising the temperature (*ts*21 prevents maturation of preheads at 42°C [14]), DNA which is packaged can be fully glucosylated when glucose is added to the glucose-deficient infected cells at the moment of temperature shift (Fig. 1). The proportion of phage which escape a host restriction system directed against nonglucosylated phage T4 DNA falls off exponentially from 100% with increasing time of glucose addition after temperature shift-up (Fig. 1, line b); the resistant fraction at various times of glucose addition is comparable to the fraction of the potential phage which fail to mature at those times if the temperature is once more lowered to 20°C, arguing that packaging into this fraction of the phage has not yet been activated (Fig. 1, line c). These kinetics suggest that glucosylation of the T4 DNA pool can be very rapid once glucose is provided to the glucose-deficient host.

Although their rate of maturation is physiologically comparable to that of the *cs*20 heads, partially filled *ts*49-t heads, when matured in chloramphenicol by temperature shift-down, do not package DNA which can be similarly fully glucosylated by the same growth criterion (Fig. 2). This is not a consequence of the distinct, very-fast-sedimenting *ts*49 DNA structure, which is known not to depend upon aberrant *ts*49 head formation (17). First, when chloramphenicol is added to the *ts*49-t-infected cells before late proteins are synthesized but after onset of T4 DNA synthesis, removal of chloramphenicol allows phage to be formed which can be fully glucosylated (data not shown). Second, maturation in chloramphenicol upon temperature shift-down of *ts*49-t heads (partially filled) and *ts*24-*ts*49-t heads maturation blocked in the prehead stage [1]) produces phages which

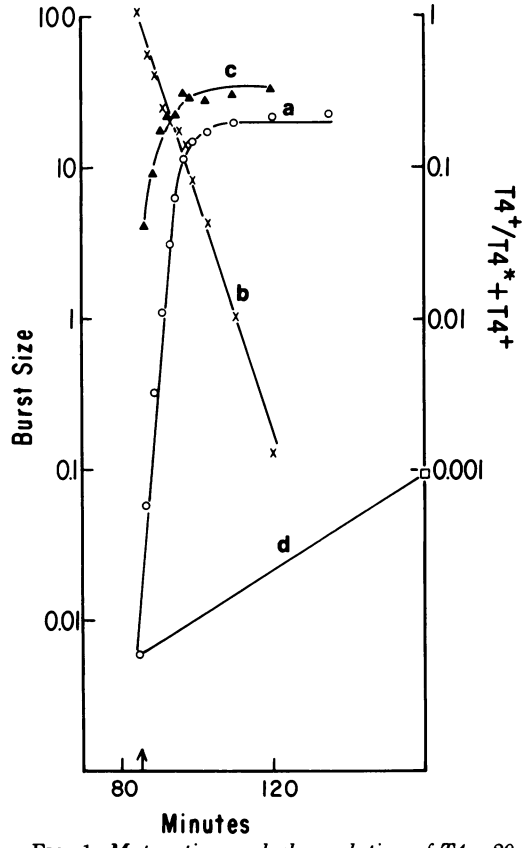

FIG. 1. *Maturation and glucosylation of T4 cs20-ts21-t empty heads. E. coli DF2000 growing in tryptone broth–M9 salts–0.5% glycerol at 20°C were infected with T4 cs20-ts21-t at a multiplicity of 2; nonadsorbed phage were inactivated with T4 antiserum at 3.5 min. At 85 min the infected culture was transferred to 42°C, and samples were removed at the indicated times by 100× dilution into chloroform-containing medium to determine the total yield at various times after temperature shift (line a) or medium at 42°C containing 0.4% glucose. The proportion of glucosylated phage ($T4^+/T4^* + T4^+$) in the final yield at 120 min with the different times of glucose addition (×) was determined by the plating difference on E. coli B40su_I^+ r_6^+ $r_{2,4}^+$ and CR63su_I^+ r_6^- $r_{2,4}^-$ (line b). Portions of the culture were also shifted back to 20°C at the indicated times, and the final yields were determined at 160 min (line c). The yield of the infected culture maintained at 20°C throughout the experiment is given by line d.*

are distinctively differently glucosylated. The kinetics of phage formation in the *ts*49-t-and *ts*24-*ts*49-t-infected cells are overlapping (Fig. 2, lines a and b); however, the *ts*24-*ts*49-t phage formed are immune to restriction when packaging is allowed to proceed in the presence of glucose, whereas only 10% of the *ts*49-t phage can escape restriction (Fig. 2, lines A and B).

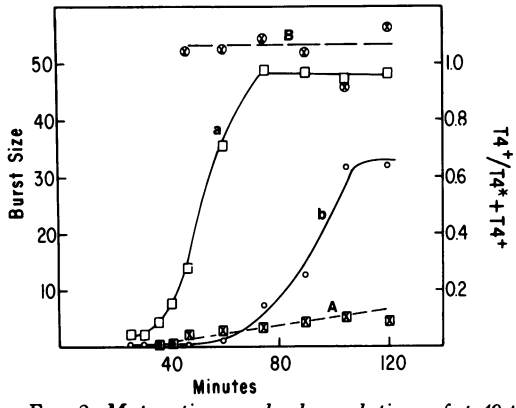

FIG. 2. *Maturation and glucosylation of ts49-t partially filled heads and of ts24-ts49-t preheads. E. coli DF2000 growing in tryptone broth–M9 salts–0.5% glycerol at 42°C were infected with ts49-t (line a) or ts24-ts49-t (line b) phage at a multiplicity of 5. At 4 min unadsorbed phages were inactivated with T4 antiserum. At 21 min chloramphenicol was added to a final concentration of 100 μg/ml, and at 26 min the infected bacteria were diluted into medium at 25°C containing 0.4% glucose and 100 μg of chloramphenicol per ml. Samples were removed by dilution into chloroform-containing buffer at the indicated times for the total phage yield. The proportion of glucosylated phage ($T4^+/T4^* + T4^+$) was also determined at the various times as described in the legend to Fig. 1 for ts49-t (line A) and ts24-ts49-t (line B). The burst size of the ts24-ts49-t-infected cells is given by the ordinate divided by 10.*

The proportion of *ts*49-t phage able to grow on the restricting host does not rise significantly at later times, arguing that the 10% restriction value is characteristic of the partially glucosylated DNA. The data strongly suggest that in the *ts*49-t-infected cells, DNA is under-glucosylated not as a consequence of the *ts*49 DNA structure, but as a result of its partial packaging into heads.

Analysis of the structure of mature *ts*49-t DNA partially glucosylated in this way (abbreviated $49T^{*p}$ DNA) suggests that DNA molecules are packaged which are essentially fully glucosylated in one portion and non-glucosylated in another portion. To produce this DNA, glucose (in some cases ^3H or ^{14}C labeled) was provided to the glucose-deficient host infected with *ts*49-t phage after chloramphenicol inhibition of new head precursor protein synthesis and depletion of the protein precursor pool into partially filled heads; in some experiments DNA was also uniformly labeled with ^{32}P (see above). When radioactive glucose was added in this way, essentially all the radioactivity in the purified *ts*49-t phage particles was found to be in the phenol-extracted DNA and was acid precipitable (Fig. 3 and data not shown). In addition, paper chromatography of acid hydrolysates of such phenol-extracted $49T^{*p}$ DNA showed that, when [^{14}C]glucose was provided to the *ts*49-t-infected cells, essentially 100% of the radioactivity in the DNA hydrolysate chromatographed with standard D-glucose (data not shown). When labeling with high levels of [^3H]glucose was carried out in five trials, analysis of the mature *ts*49-t phage DNA suggested approximately 40 to 50% occupation of hydroxymethylcytosine residues with [^3H]glucose. In a more accurate determination, $T4^+$ and *ts*49-t were grown in *E. coli* DF2000 with uniform ^{32}P labeling. Low-specific-activity [^3H]glucose was present throughout the wild-type infection, but was added at the same specific activity to the *ts*49-t-infected cells only after chloramphenicol addition and temperature shift-down (method 3). The ratio of [^3H]glucose to ^{32}P in the purified *ts*49-t and $T4^+$ phages showed that the *ts*49-t phage contained 45% of the glucose-supplied ^3H radioactivity of wild type.

Nuclease digestion of $49T^{*p}$ DNA provides a means of further analyzing its structure. $T4^+$ DNA is known to be resistant to digestion by the site-specific *Eco*RI restriction endonuclease, but T* DNA is digested to specific fragments, suggesting that glucosylation protects $T4^+$ DNA from the restriction endonuclease (16). *Eco*RI digests of T*, $49T^{*p}$, and $T4^+$ DNAs were analyzed by neutral sucrose gradient centrifugation and by agarose gel electrophoresis. Upon neutral sucrose gradient centrifugation analysis, T* DNA appears to be highly fragmented by *Eco*RI

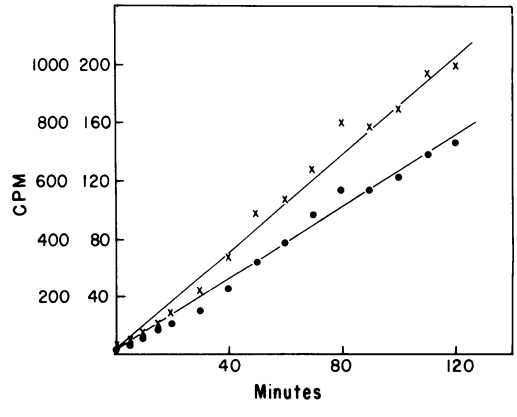

FIG. 3. *Exonuclease VII digestion of $49T^{*p}$ DNA labeled with ^{32}P and [^3H]glucose. The DNA (ca. 0.6 μg), uniformly labeled with ^{32}P and partially labeled with [^3H]glucose in a ts49-t infection of E. coli DF2000 (method 2), was treated (after heat denaturation) with ca. 0.6 U of exonuclease VII, and trichloroacetic acid-soluble counts were measured as a function of time as described in the text. Symbols: ○, ^{32}P; ×, ^3H (0 to 1,000 cpm).*

digestion (Fig. 4a and b), and T4$^+$ DNA sedimentation is apparently unaffected (Fig. 4e and f), whereas 49T^{*p} DNA sedimentation rate is significantly reduced by *Eco*RI digestion (Fig. 4c and d, and g and h). It appears that after exhaustive *Eco*RI digestion of 49T^{*p} DNA a large and relatively homogeneous resistant DNA remains which has a sedimentation coefficient 0.84 times that of undigested DNA (in three analyses); from this it can be calculated that the *Eco*RI-resistant DNA has an average molecular weight of about 7×10^7, as compared with 1.2×10^8 for native T4 DNA (36). It can also be noted that there is relatively little overlap between the 49T^{*p} and *Eco*RI-digested 49T^{*p} DNA peaks, suggesting that few whole molecules escape digestion (Fig. 4c and d, and g and h).

Similarly, electrophoresis of *Eco*RI digests of T4$^+$, T*, and 49T^{*p} DNAs on agarose gels shows that T* DNA is wholly degraded into specific fragments (Fig. 5a), T4$^+$ DNA is not apparently degraded (Fig. 5e), and 49T^{*p} DNA gives specific fragments and an *Eco*RI-resistant high-molecular-weight DNA (Fig. 5c and d). Agarose gel electrophoresis of *Eco*RI digests of independently prepared radioactive T*, T4$^+$, and 49T^{*p} DNAs indicates a very similar pattern of fragments (Fig. 6). In addition, autoradiography of the dried agarose gel demonstrates a significant difference in distribution of radioactivity in 49T^{*p} DNA labeled with ^{32}P uniformly and with [^{14}C]glucose and 49T^{*p} DNA labeled with [^{14}C]glucose. Both DNAs give rise to the same *Eco*RI-specific digestion fragments which are seen in the T* DNA (Fig. 6a, b, and d); however, [^{14}C]glucose radioactivity is found only in the *Eco*RI-resistant large-molecular-weight DNA (Fig. 6D), whereas ^{32}P is found in both the high-molecular-weight DNA and *Eco*RI-specific fragments (Fig. 6B). Radioactivity in the *Eco*RI low-molecular-weight fragments (Fig. 6B) penetrates into a second film (^{14}C radioactivity does not penetrate the first film) with an intensity also

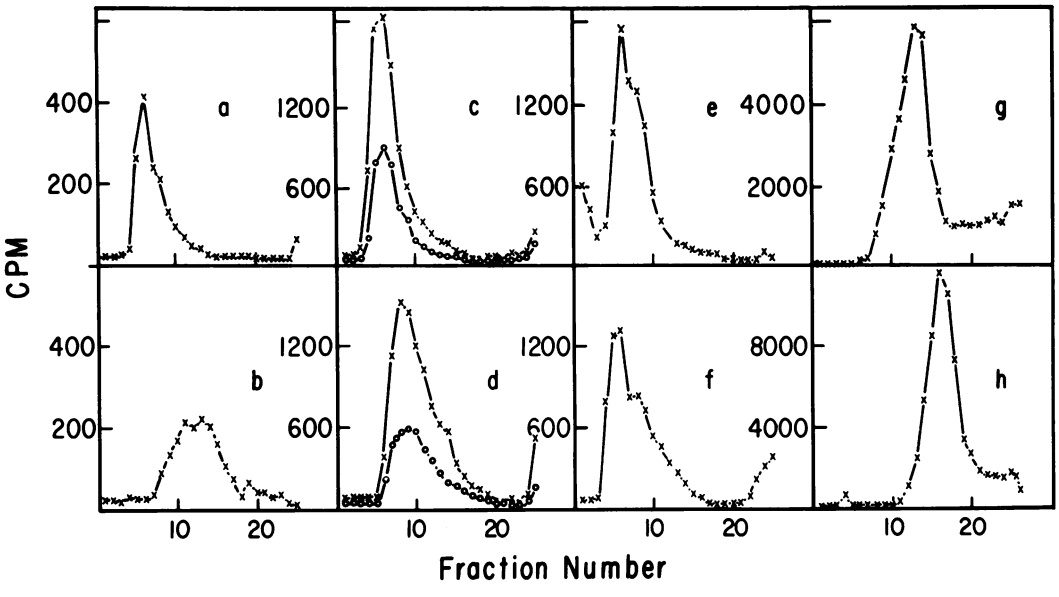

FIG. 4. *Sedimentation analysis on neutral sucrose gradients of glucosylated, non-glucosylated, and partially glucosylated DNAs after EcoRI restriction enzyme digestion. The following samples were centrifuged: (a) T* DNA labeled with ^{32}P; (b) T* DNA labeled with ^{32}P + EcoRI; (c) 49T^{*p} DNA labeled with ^{32}P (○) and [^{14}C]glucose (×); (d) 49T^{*p} DNA labeled with ^{32}P (○) and [^{14}C]glucose (×) + EcoRI; (e) T4$^+$ DNA labeled with ^{32}P; (f) T4$^+$ DNA labeled with ^{32}P + EcoRI; (g) 49T^{*p} DNA labeled with [^{3}H]glucose; and (h) 49T^{*p} DNA labeled with [^{3}H]glucose + EcoRI. T* DNA was prepared by growth of phage T4 in E. coli DF2000 in the absence of glucose; 49T^{*p} DNA was prepared by growth of phage ts49-t in E. coli DF2000 in the absence of glucose until radioactive glucose was added after chloramphenicol addition and temperature shift (methods 1 and 4). T4$^+$ DNA was prepared from phage grown in E. coli B^E. Samples were incubated at 37°C for 2.5 h in 100 mM Tris, pH 7.6–6 mM $MgSO_4$–6 mM dithiothreitol–50 to 100 mM NaCl in the presence or absence of 10 U of EcoRI. After the incubation, Sarkosyl was added to 1%, and the samples were heated at 60°C for 5 min before being centrifuged in an SW50.1 rotor at 40,000 rpm for 2.25 h at 11°C in neutral 10 to 30% sucrose (10 mM Tris, 1 mM EDTA, 1 M NaCl, pH 7.6). A total of 11 drop fractions were collected from the bottom of the tube and counted on Whatman GFC filters after precipitation with trichloroacetic acid. (Samples g and h were treated with 21 U of EcoRI for 5 h and centrifuged for 2 h at 4°C.)*

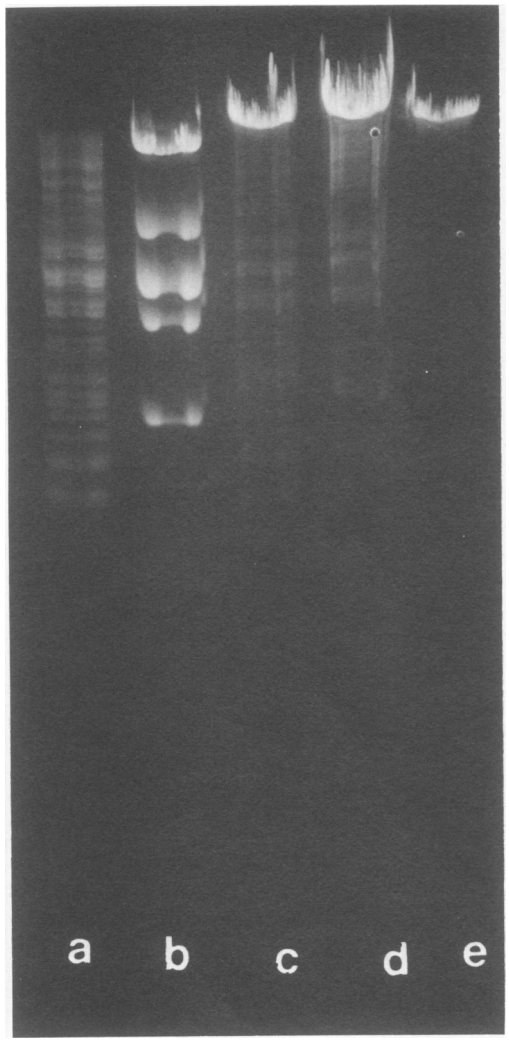

FIG. 5. *Agarose gel electrophoresis of glucosylated, non-glucosylated, and partially glucosylated DNAs after EcoRI restriction enzyme digestion. The following samples were digested with a constant amount of EcoRI restriction enzyme (ca. 5 U) for 4.7 h at 37°C as described in the legend to Fig. 4: (a) T* DNA, 3 μg; (b) lambda DNA, 3 μg; (c) 49T*^{p} DNA, 3 μg; (d) 49T*^{p} DNA, 9 μg; and (e) T4^{+} DNA, 3 μg. Nonradioactive DNAs were prepared on E. coli DF2000 as described in the legend to Fig. 4 and in the text. After heating in 1% sodium dodecyl sulfate at 60°C for 5 min, samples were subjected to electrophoresis on a 1.4% Seakem agarose gel, stained with ethidium bromide, and photographed with UV light. Degradation of λ DNA and electrophoresis of the fragments on the same gel served as a control of the EcoRI enzyme and the completion of the digestion and as a size reference for the T4 fragments. Lambda fragments are 13.7 × 10^{6}, 4.68 × 10^{6}, 3.7 × 10^{6}, 3.56 × 10^{6}, 3.03 × 10^{6}, and 2.09 × 10^{6} daltons (16). (The smallest fragment is poorly visible in this gel.)*

suggesting that most, if not all, of the radioactivity is due to ^{32}P (data not shown). Therefore, autoradiography strongly suggests that 49T*^{p} DNA is not uniformly under-glucosylated and that the glucose distribution is nonrandom. Rather, 49T*^{p} DNA appears to be fully glucosylated in part along its length, with the glucose concentrated in one block which protects it from *Eco*RI digestion. In addition, because T* and 49T*^{p} DNAs yield the same spectrum of *Eco*RI fragments (compare Fig. 5a, c, and d and 6A and B; unpublished data), it would appear that no subset of the T4 *Eco*RI-specific sequences is preferentially protected from digestion by initial packing into the *ts*49 partially packaged head.

It would be of interest to know how the glucosylated region is located with respect to the mature ends of the 49T*^{p} DNA molecules. A number of observations suggest the glucose is located toward a single end of the 49T*^{p} DNA molecules. (i) Restriction enzyme digestion suggests that the glucose is located together in a block on the DNA and that the average molecular weight of the resistant DNA (Fig. 4) is about 60% of that of wild type. Because it is estimated that there is 50% wild-type glucosylation of these molecules, the average molecular weight of the resistant fraction is a maximum most consistent with location at an end. (ii) Exonuclease VII digestion of 49T*^{p} DNA labeled with ^{32}P uniformly and with [^{3}H]glucose releases [^{3}H]glucose and ^{32}P linearly and without lag from the DNA in a ratio which is close to the ratio of the two isotopes in the substrate (Fig. 3). If the 49T*^{p} molecules are labeled to the extent of about 50% in one-half portion only, then the release is most consistent with labeling at one end, but not at both ends (^{3}H release expected to be higher than ^{32}P release) or the middle (^{3}H release slower than ^{32}P release and accelerating). (iii) Autoradiographic analysis of Kleinschmidt spread-isolated 49T*^{p} DNA labeled with [^{3}H]glucose also supports the same distribution, in that DNA molecules appear to have grains located toward one but not the other end of the molecule in a preliminary statistical analysis of such molecules (Fig. 7 and 8).

The data so far presented strongly suggest that *ts*49-t phage particles can be formed with DNA which is fully glucosylated toward one end (the last packaged) and nonglucosylated toward the other end (first packaged). It would be of interest to study the three-dimensional structure of such DNA within the phage head, because the relative orientation of first- and last-packaged DNA would help determine the structure of the linear molecule within the head and the mechanism of its packaging. It is also significant

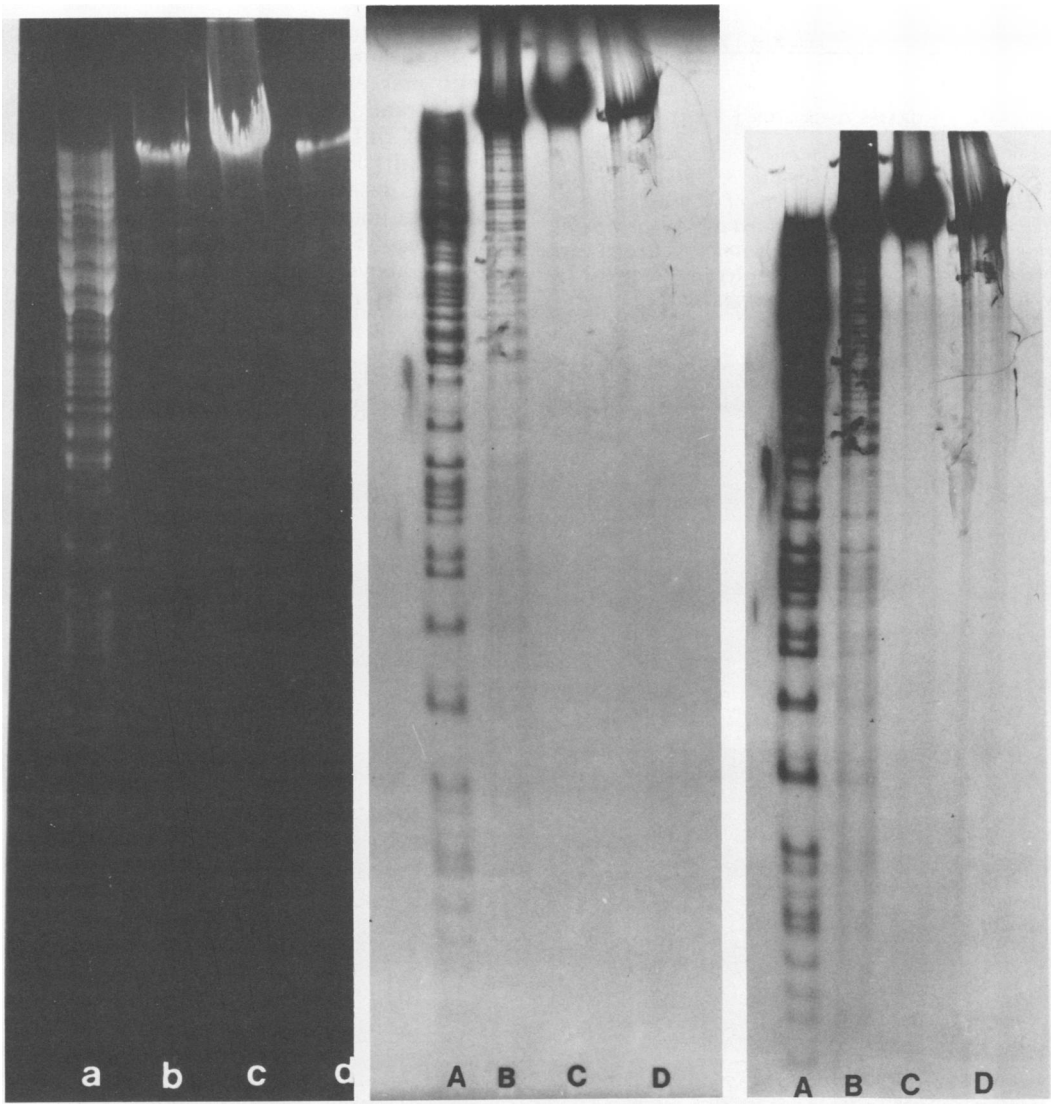

FIG. 6. *Ethidium bromide-stained and autoradiographic patterns on agarose gels of restriction fragments of glucosylated, non-glucosylated, and partially glucosylated radioactive DNAs after EcoRI digestion. The following DNA samples were digested with ca. 20 U of EcoRI restriction nuclease for 12 h at 37°C as in the legend to Fig. 4: (a) T*, 2.3 μg, 10^4 cpm of ^{32}P; (b) 49T*^{P}, 0.55 μg, 10^4 cpm of ^{32}P and 1.5×10^5 cpm of [^{14}C]glucose; (c) T4$^+$, 2.8 μg, 1.4×10^4 cpm of ^{32}P; (d) 49T*^{P}, 0.2 μg, 5.5×10^4 cpm of [^{14}C]glucose. The radioactive DNAs were prepared on E. coli DF2000 as previously described (Fig. 4; method 4). After incubation the samples were heated in 1% sodium dodecyl sulfate at 60°C for 5 min and run on a 1.4% Seakem agarose gel. The gel was stained in ethidium bromide, photographed under UV light (a through d), and then dried for autoradiography with Kodak SB-5 film (A through D).*

to know whether the first DNA end to enter the head is the first or the last end of the DNA to exit from the head upon injection into the host cell. Because it appears that the 49T*^{P} phage DNA is glucosylated toward the last- but not the first-packaged end of the molecule, a direct approach to this question is through interrupted injection experiments, in which ^{32}P decay can sever the DNA duplex within the head at random, allowing entry into the infected cell of that portion of the DNA up to the double-strand break. Interruption of transfer of the chromosome of a uniquely terminated phage by ^{32}P decay has been clearly demonstrated (25). Sim-

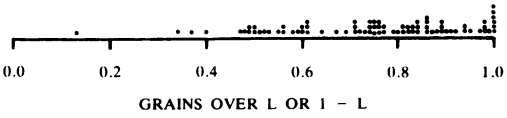

FIG. 7. *Grain distribution over Kleinschmidt spread 49T*^{*P} *DNA (e.g., see Fig. 8, top) labeled with [*^{3}*H]glucose (method 1). A total of 79 grains distributed on 26 molecules were located with respect to the ends of molecules with a planimeter. Grains are located over the length of the molecules (L or 1 – L), because the ends cannot be distinguished.*

ilarly in the case of bacteriophage T4, ^{32}P decay has been shown to prevent rescue of linked genetic markers, suggesting that ^{32}P-induced double-strand breaks may also prevent full transfer of T4 DNA (34). When *ts*49-t phage are labeled with [^{3}H]glucose and high-specific-activity ^{32}P, ^{32}P decay occurs, causing inactivation of labeled but not of control mixed nonradioactive T4rII phage (Fig. 9). Blending of infected bacteria (see above) allows comparison of the ratio of ^{32}P to ^{3}H in the blended and nonblended

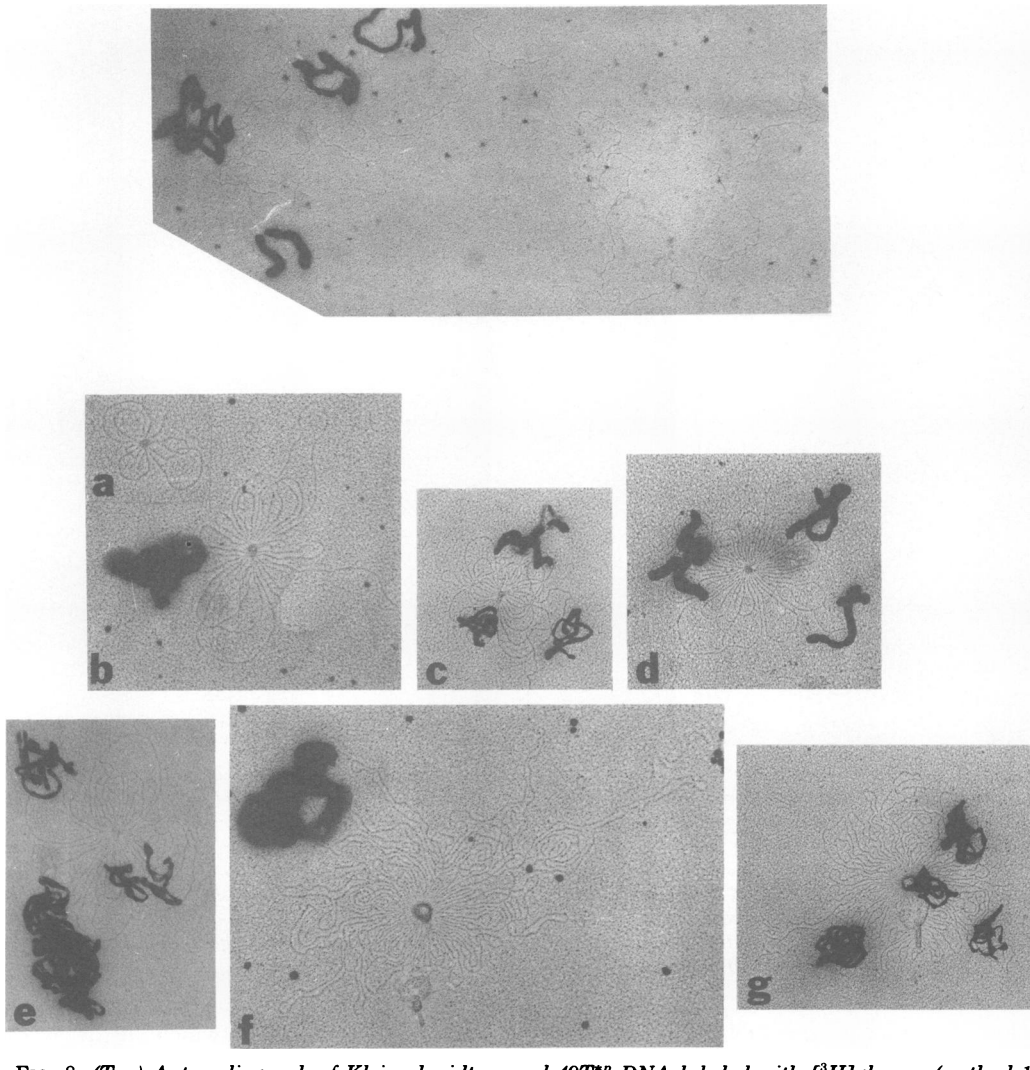

FIG. 8. *(Top) Autoradiograph of Kleinschmidt spread 49T*^{*P} *DNA labeled with [*^{3}*H]glucose (method 1). Exposure time, 2.5 months. (Bottom) Autoradiographs of ts49-t phage labeled with [*^{3}*H]glucose (method 1) osmotically shocked by spreading from 2 M ammonium acetate–0.05% isopropanol onto a water hypophase. Rosettes are arranged (a through g) in approximate order of increasing amount of DNA apparently released from the central condensate; neighboring phage ghosts are apparent in (f) and (g). Magnifications (×16,400) are equal. Exposure times: (a) 0.75, (b) 0.75, (c) 4.5, (d) 2.5, (e) 4.5, (f) 0.75, and (g) 4.5 months.*

bacterial pellets. Blending should remove from the bacteria the nontransferred portion of the T4 chromosome which would stay in the phage coat. When analyzed in this fashion, T4 phage which have accumulated lethal double-strand breaks show a small but consistent rise in the ratio of ^{32}P to ^{3}H in the blended pellet when compared with the unblended bacterial pellet, which increases with time (Fig. 9). The results, which were similar in a number of independent phage preparations, suggest that the [^{3}H]glucose-labeled protion of the chromosome (last to enter the head) is preferentially excluded from injection into the host (last to leave the head).

Although the resolution is probably not sufficient for a determination within the intact head, autoradiography allows analysis of the distribution of radioactivity in the DNA as it is released suddenly from the head by osmotic shock. When subjected to osmotic shock, [^{3}H]glucose-labeled *ts*49-t phage release their DNA in a variety of patterns, but most commonly in rosette patterns around the broken capsid or around a central ball or condensate of DNA which has apparently shot out of an adjacent phage ghost (Fig. 8) (18). These latter condensates may be comparable to structures visualized previously by high-resolution electron microscopy (31). The apparently circular symmetry of most DNA rosettes strongly suggests that the DNA is not naturally released through the tail and these rosette structures are comparable to the classical pictures of osmotically shocked T-even bacteriophage heads releasing DNA (18). However, the structural interpretation given here is speculative, because rosette DNA structures can also be formed artifactually during spreading. Nevertheless, the structures were produced only by osmotic shock as described previously (18) and were not seen with isolated phenol-extracted T4 DNA. The most probable interpretation, given the structure shown in Fig. 10i, is that outer rosette loops come from outer shells of DNA in the condensed structure. Furthermore, some DNA condensates appear to have released a smaller amount of the total DNA (compare Fig. 5a through g) and these should have released only the outermost DNA shells, because it is improbable that inner shells could protrude through the outer ones in the tightly packaged residual condensate (Fig. 10i). Therefore, DNA which is only slightly released from the condensate is particularly significant with respect to grain distribution. Visualization of large numbers of shocked *ts*49-t phage leads to the impression that grains are located most frequently on outer rosette loops and also on the loops which have emerged from only partially decondensed DNA. The impression is that the [^{3}H]glucose-labeled DNA (last packaged) is arranged in the outer DNA shells and that, therefore, the last-packaged DNA is found in the periphery rather than the center of the capsid.

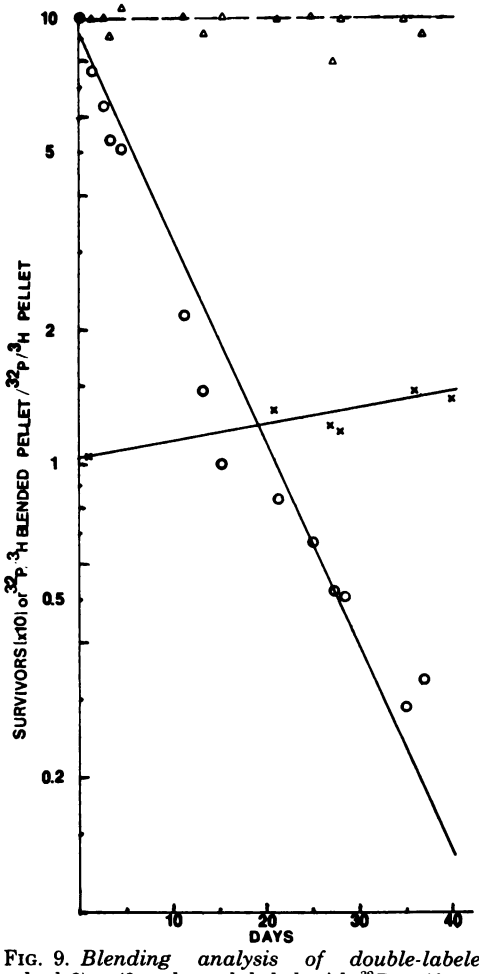

FIG. 9. *Blending analysis of double-labeled (method 3) ts49-t phage labeled with ^{32}P uniformly and with [^{3}H]glucose. Symbols: △, Mixed nonradioactive rII phage titer; ○, ts49-t phage titer; ×, ratio of $^{32}P/^{3}H$ in blended bacterial pellet to $^{32}P/^{3}H$ in the nonblended bacterial pellet (see text).*

DISCUSSION

In this paper it is shown that replicated phage T4 DNA in the cytoplasmic DNA pool can be rapidly and fully glucosylated and then packaged into certain head intermediates when glucose is provided to an infected host unable to synthesize glucose. However, partially packaged DNA within *ts*49 heads appears to be resistant

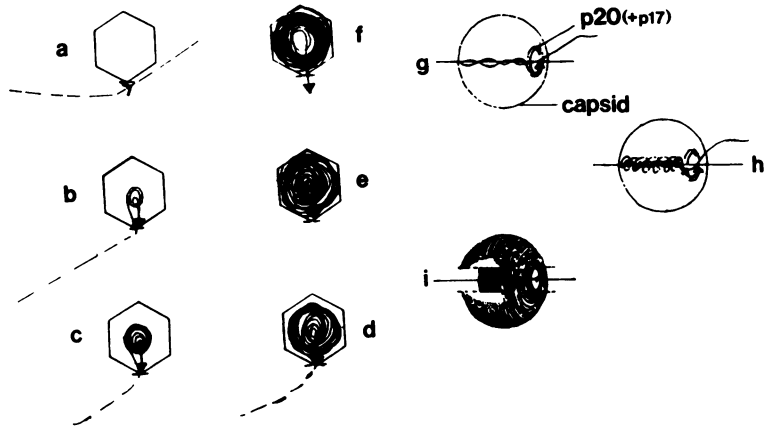

FIG. 10. *Schematic illustration of the suggested mechanism of in vivo DNA condensation and decondensation into the T4 capsid (a through f), a model (g and h) postulated from this mechanism (a through f), and the determined general structure of condensed viral DNA (i) (8, 31). See text. ↓ indicates a mature DNA end.*

to glucosylation. Therefore, packaged and cytoplasmic T4 DNA associated in vivo with a head intermediate blocked in completion of DNA packaging by a reversible temperature-sensitive mutation can be structurally distinguished. In this way, first- and last-packaged DNA in the mature *ts*49 head can be differentiated. Resistance to glucosylation of packaged DNA within the *ts*49 head intermediate is reasonable, whether or not its condensed structure might be expected to inhibit glucosylation, in view of the observed exclusion of cytoplasmic DNA-binding proteins from the mature head. In addition, those DNA-binding proteins found associated with the DNA in the mature head (T4 internal proteins) have been shown to enter the head separately and before the DNA (2, 14, 33). Evidently there is a barrier to entry of cytoplasmic proteins into the head with the DNA.

49T*^{p} DNA appears to be essentially fully glucosylated in a block toward one end of the mature T4 DNA molecule, and such molecules appear relatively homogeneous in extent of glucosylation (Fig. 3 through 7). Previous analyses of the structure of partially packaged *ts*49 DNA isolated from defective heads also suggested quite a homogeneous length of about 50% that of mature T4 DNA (19); the reason for an arrest in packaging toward 50% completion in this mutant is not clear. The 49T*^{p} DNA structure suggests that DNA is run into the head in a unidirectional sense from one mature end, in agreement with electron microscopic observations suggesting that partially packaged *ts*49 heads are associated in vivo with ends of DNA duplexes (17, 38). That there is full glucosylation along one block of the mature 49T*^{p} DNA and little if any along the remainder of the DNA also suggests that the DNA is actually run into the head linearly from an end in the concatameric DNA which is created by the packaging mechanism. This argues against the possibility that DNA might condense into the head in bunches from multiple nucleation sites for condensation along its length after creation of an end. *Eco*RI digestion of 49T*^{p} DNA suggests that many sequences along the T4 genome initiate entry into the head, because *Eco*RI fragments found in 49T*^{p} DNA represent all of the sequences found in T* *Eco*RI digests, with little apparent difference in frequency (Fig. 5 and 6). This is to be expected from the circular permutation and terminal redundancy of the T4 DNA, wide distribution of terminal heterozygotes, and distribution of petite phage DNA segments over the entire genome (26).

The experiments reported suggest that the end of the DNA which first enters the head also leaves first upon injection and that the DNA which first enters the head is deposited toward the center of the mature phage head rather than on the capsid surface (Fig. 8 and 9). The suggested order of entry and exit of the DNA ends and the suggestion that the first DNA to enter the head is deposited toward the center of the head rather than on the capsid wall are consistent, given the structure of condensed DNA (Fig. 10i) (8, 31). It is improbable that DNA could be removed from the head upon injection by being first peeled off the outer DNA-capsid interface or by rotation of the DNA within the head. Therefore, it is to be expected that the DNA should be pulled back out of the center of the head (Fig. 10f). These findings are illustrated schematically in Fig. 10a through f. The organization of the nascently condensing DNA mole-

cule is not that expected if charge neutralization and binding to the capsid surface play major roles in condensation. In this case, the DNA first to enter would be expected to distribute along the capsid wall and also would probably first assume positions of maximum curvature where the energy requirement for condensation is lowest (8), so that DNA would condense from the outside in. Because it appears that, on the contrary, DNA is condensed from the inside out, these experiments suggest that an active process is drawing the DNA into the center of the phage head. In fact, the observations are consistent with the ingenious core cleavage model of Laemmli et al., in which proteolytic digestion of the core at the center of the head could drive the DNA into the center (19, 20). However, it appears from our recent experiments that DNA packaging into the head follows proteolysis of core and capsid proteins, so that coupling of packaging to the active process of core removal seems unlikely (14). Nevertheless, it is possible that after their formation, acidic peptides (not the basic internal proteins, because they are dispensible [2]) could pull the DNA toward the center of the head. The order of entry and exit of the T4 DNA ends suggested here is believed to be the opposite for bacteriophage lambda (7, 9, 35, 37). Therefore, presumably also the first λ DNA to condense should be located on the inner surface of the capsid. Both modes of packaging could operate in the different phages. However, in view of this conflict and the uncertainties possible in interpreting Fig. 8 and 9, the method given here is being applied to provide a more definitive answer for phage T4 by DNA-protein (capsid and tail) cross-linking and other methods (7). It should be noted, however, in support of this packaging mode, that thin sections of maturing phage T4 and P22 heads reveal that partially packaged DNA is apparently first condensed in the center of the capsid (21–23). Such structures revealed by thin sections could be artifactual, but if the DNA and the inner surface of the capsid repel each other, the T4 packaging mode suggested here might help explain rapid ejection of DNA from the head upon infection. Part of the force leading to ejection from the head could result form DNA packing into the head against repulsion from the capsid wall, as well as from DNA-DNA interaction, and binding of the last DNA to leave the head to the capsid wall would not have to be overcome in injection.

Many features of phage T4 DNA packaging in vivo suggest that an active process is packing DNA into the head. A plausible enzymatic model for phage T4 DNA packaging is suggested by a number of recent findings: experiments showing that T4 capsid protein p20 is actively involved in DNA condensation in conjunction with protein p17 (14; Hsiao and Black, in press); the fact that condensation is apparently not dependent upon replication, proteolytic cleavages, or capsid expansion (14, 38; Hsiao and Black, in press); the completion of the structural assignment of minor phage T4 proteins to positions in the capsid (28); and the observation that p20 appears to be a strong DNA-binding protein (unpublished data).

Of the two apparently absolutely essential proteins of the T4 head, p23 forms the capsid and p20 is located at the proximal apex (5, 14, 28). In fact, p20 which can be extracted from the capsid could form a ringlike structure at the base of the head (Fig. 10g) (28). p20 is also the product of a gene which is involved first in prehead assembly initiation and second in DNA packaging, the latter in concert with p17. Since the prehead is assembled on the membrane at the p20 vertex in interaction with p40, DNA packaging could be separated from prehead formation on the assembly pathway. p20 interaction with p17 and initiation of DNA packaging would follow maturation of the prehead and its detachment from the membrane. The gene *cs*20 mutation which blocks DNA packaging appears to function after replication, proteolytic cleavages, and capsid expansion, but before completion of the T4 neck (14; Hsiao and Black, in press). The fact that the *cs*20 mutation can be suppressed by a compensating temperature-sensitive mutation in gene 17 argues for a close association between the two gene products in packaging (14; Hsiao and Black, in press). Also, the finding that certain gene 17 mutants lead to partially filled heads argues that p17 is likely to be more actively involved in head filling than simply in creating the mature DNA ends (27, 38, 39). It is of significance that gene 17 mutations can confer resistance to quinacrine inhibition of T4 DNA packaging (29).

It is possible to speculate that p20 and p17 which are located at the proximal apex of the head are part of a pump which actively drives DNA into the head. Such a pump could work as suggested in Fig. 10g and h. Introduction of DNA through a ringlike structure at the proximal vertex with the first end to enter fixed to the ring and unable to turn could allow for driving the DNA into the head by introduction and relaxation of superhelical turns. ATP (or an alternative energy source) would be utilized to introduce torsion into the double-helical molecule enzymatically by p17 adding superhelical turns as is the case with DNA gyrase (11). Su-

perhelical twists would then be relaxed by entry of the DNA into the head. A macroscopic model of this sort suggests that such a process could drive the DNA into the head. Such a model would help explain the sealing of single-stranded breaks in the late T4 DNA—in this case the process would pause until the nick was sealed—and the observed inhibition of T4 DNA packaging by T4 DNA ligase mutations (14; Hsiao and Black, in press). In addition, the apparent expulsion of DNA-binding proteins from the DNA entering the mature head might be explained if these are displaced by interaction with p17 or by their attachment to single-stranded regions, because the DNA might be frozen into a double-helical form by this mechanism. This highly speculative model, which appears consistent with the reported experiments defining the general mechanism of packaging, is being examined by biochemical analysis of p20, p17, and their functions.

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