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NOTES

End Structure and Mechanism of Packaging of Bacteriophage T4 DNA

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We analyzed by restriction enzyme digestion the end structure of T4 phage DNA by comparing mature, concatemeric, first-packaged, and incompletely packaged DNAs. The structure of mature DNA was also studied using 3′ end labeling with terminal transferase. Our data support the hypothesis that T4 DNA packaging is not initiated at specific packaging initiation sequences on the concatemeric precursor (*cos* or *pac* site mechanisms) but by a different packaging mechanism.

Bacteriophage DNA replication generally involves synthesis of concatemers which are cut during packaging to form the mature chromosome. The ends of the mature DNA are known to be formed by two general packaging mechanisms. (i) Unique ends are created by phage terminases which cleave at specific DNA sequences (e.g., *cos* sites) during head filling (e.g., phages lambda and T7 [17]). (ii) Ends are formed starting from a unique sequence (*pac* site) by a processive mechanism which cleaves off successive headfuls of DNA, leading to a preferred starting sequence and a limited number of other termination sequences (e.g., phages P22 [7, 8, 22], T1 [18], P1 [2], and 5006M [5]). Although it is thought that phage T4 DNA is packaged by cutting which is random with respect to DNA sequence (i.e., by a third mechanism) (3, 4, 13, 15, 16, 21), a *pac* site mechanism with more than one initiating sequence, or where one *pac* site is used infrequently (highly processive filling along a long concatemer), might produce apparently random end sequences along the T4 concatemer. We reasoned that direct biochemical evidence bearing on the mechanism of T4 packaging might come from analysis of the DNA sequences in gene 49-defective, partially filled heads, and in first-filled heads in a *cs*20-*ts*21 infection following temperature shift where packaging can be synchronized (6). *pac* site sequences, if they exist, might be revealed in a comparison of mature and concatemeric DNA. Such sequences would also be predicted to be enriched in the synchronously packaged first-formed heads and in the gene 49 heads, where packaging is incomplete because of a structural defect in the DNA so that only packaging into the first of a processive series of heads could be initiated (4, 11, 12, 14). In addition, direct labeling of the T4 ends might reveal whether the ends of T4 DNA are free and whether specific termination sequences exist. Our experiments with these three approaches support the notion that the T4 3′-OH DNA ends are free and are indeed randomly distributed over the genome.

Isolation of gene 49 partially filled heads 49(*ts*C9)-t(*am*A3). *Escherichia coli* B^E (2 l) in M9s medium was grown to a concentration of 2×10^8/ml and infected at 41°C with the above packaging-defective gene 49 mutant (t[*am*A3] prevents lysis) for 35 min. At the same time *E. coli* B^E in 30 ml of M9 medium (without amino acids) at 2×10^8/ml was infected at 41°C in the presence of ^{14}C-amino acids (1.33 μCi/ml) from 10 to 25 min. The cultures were then mixed and centrifuged for 15 min at 5,500 rpm. Bacteria were suspended in 20 ml of 50 mM PO_4 (pH 7)–370 mM NaCl–1 mM EDTA–10% polyethylene glycol. Then egg white lysozyme (final concentration, 100 μg/ml) and a few drops of chloroform were added to lyse infected bacteria on ice for 12 min. Next, 220 μl of 1 M $MgSO_4$, 400 μl of 10 mg of DNase I per ml, 200 μl of 10 mg of RNase per ml, and 2.2 ml of 1% Nonidet P-40 were added, mixed gently, and incubated at 37°C for 5 min. Bacterial debris was then removed by centrifuging for 5 min at 3,000 rpm. The supernatant containing phage and partially filled heads were fractionated on a linear 10 to 30% sucrose gradient prepared in 50 mM PO_4 (pH 7)–370 mM NaCl–0.1% Nonidet P-40–1 mM $MgSO_4$. Centrifugation was in a Beckman SW27 rotor at 1,800 rpm for 45 min at 10°C. After centrifugation, the sucrose gradient was fractionated, and the radioactivity of each fraction was determined. Two radioactive peaks containing heads were found, pooled, and subjected to equilibrium centrifugation in a linear 1.29- to 1.55-g/cm^3 CsCl gradient by centrifugation in an SW27 rotor at 19,000 rpm for 17 h at 4°C. After centrifugation to equilibrium, four ^{14}C peaks were found which corresponded to densities as follows: δ_1, 1.46 g/cm^3; δ_2, 1.43 g/cm^3; δ_3, 1.34 g/cm^3; δ_4, 1.3 g/cm^3. Particles in position δ_3 were partially filled heads as determined by electron microscopy. We estimated from the density that these particles contained about 25 to 30% of a normal complement of DNA as previously reported (11, 12).

Isolation of phage 20(*cs*N33)-21(*ts*N12)-t(*am*A3) (6). *E. coli* B^E (300 ml) in H broth was grown at 37°C to a concentration of 2×10^8/ml. The culture was transferred to 22°C and after 3 min was infected with 20*cs*-21*ts* at a multiplicity of 1. After 90 min of incubation the infected bacteria were shifted to 42°C. Then 250 ml of culture was poured on ice at 5 min after the shift (three phage per cell), and the remainder (50 ml) was poured after an additional 35 min at 42°C. Both portions of the culture (250 and 50 ml) were centrifuged at 6,000 rpm for

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10 min in a Sorvall SS.34 rotor. The pellets were suspended in 0.3 ml of 50 mM PO_4 (pH 7)–70 mM NaCl–1 mM $MgSO_4$ with 150 μg of DNase I per ml–chloroform. After incubation at room temperature for 1 h, debris was removed by centrifugation at 3,000 rpm for 5 min in an SS.34 rotor. The phage-containing supernatant was then centrifuged on a 4.2-ml-step CsCl gradient (1.13 to 1.55 g/cm^3) in a Beckman SW 50.1 rotor at 40,000 rpm for 25 min (10°C), and the phage band isolated.

Isolation of T4$^+$, 49(*ts*C9)-t(*am*A3) and 24(*ts*L90)-49(*ts*C9)-t(*am*A3) phage and concatemeric DNAs. Phages were grown on strain B40 Su^+_1 (*ts* mutants at 25°C) and purified first by low-speed centrifugation and then on a CsCl step gradient, generally followed by CsCl equilibrium centrifugation. Concatemeric DNA was isolated from *E. coli* P301 (pm$^-$) infected with 24(*ts*L90)-49(*ts*C9)-t(*am*A3) at 41°C for 95 min. The bacteria were pelleted and frozen in a dry ice-ethanol bath. The pellets were treated with 20 μl of 0.5 M EDTA–20 μl of 10 mg of egg white lysozome per ml–390 μl of H_2O at 4°C for 10 min. Then 25 μl of proteinase K (1 mg/ml) and 25 μl of 10% sodium dodecyl sulfate were added, and the suspension was incubated for 15 min at 65°C. The suspension was then extracted with phenol and chloroform several times, dialyzed, digested thoroughly with RNase, and reextracted twice with phenol and chloroform. Phage DNAs were prepared by phenol and chloroform extractions at 4°C and then dialyzed against TE buffer (10 mM Tris hydrochloride (pH 7.8) and 1 mM EDTA). DNA concentrations were determined spectrophotometrically, with an A_{260} of 1 taken as a concentration of 50 μg of DNA per ml. In some cases after twice extracting phage DNAs with phenol, the samples were treated with proteinase K, and once again extracted with phenol-chloroform-ether. For treatment of phage DNAs with proteinase K, the DNA sample in 10 mM NaCl–50 mM Tris chloride (pH 8.0)–10 mM EDTA–0.5% sodium dodecyl sulfate was incubated at 65°C for 1 h with proteinase K (final concentration, 50 μg/ml). Restriction enzymes were from Boehringer Mannheim Biochemicals. Terminal deoxynucleotidyl transferase (TdT) (calf thymus gland) was from P-L Biochemicals, Inc.

End labeling of T4 and *Pst*I-digested pBR322 DNAs by TdT. A 250-μl sample of T4 DNA (130 μg/ml) (or 8 μg of pBR322 DNA digested with *Pst*I) was incubated for 1 h at 37°C with 54 U of TdT in a reaction mixture containing 8 μCi of [α-^{32}P]ddATP (1,500 Ci/mmol; Amersham Corp.), or 60 μCi of [α-^{32}P]dATP (600 Ci/mmol; New England Nuclear Corp.), 140 mM sodium cacodylate (pH 7.6), 0.2 mM dithiothreitol, and 1 mM $CoCl_2$ or 10 mM $MgCl_2$. Reaction was terminated by incubation at 65°C for 10 min. The sample was dialyzed for 18 h at 4°C against several changes ot TE buffer (pH 7.8) until most of the unincorporated radioactivity was removed. Then labeled whole T4 DNA was purified through 4.5 ml of 1 M NaCl in TE buffer by centrifugation for 3 h at 40,000 rpm at 20°C in an SW50.1 rotor. T4 DNA found at the bottom of the tube was dissolved in 100 μl of TE buffer and digested with *Taq*I. Dialyzed pBR322 DNA was electrophoresed without further purification.

Restriction enzyme digestion has provided a simple and sensitive means of studying the packaging-related end structure of P1, 5006M, P22, T1, and λ phage DNAs by comparison of the mature and concatemeric DNAs (1, 2, 5, 7, 8, 18). While T4$^+$ DNA which contains glucosylated 5-hydroxymethylcytosine residues is not cleaved by most restriction enzymes because of the presence of this modified base (9), *Eco*RV, *Taq*I, and *Aha*III are able to provide suitable, specific cleavage patterns (10). When mature and concatemeric T4 DNAs were compared by *Taq*I and *Eco*RV digestion, there was no evidence for specific packaging-related DNA fragments in the mature DNA at equimolar or submolar concentrations, as is seen with DNAs of the phages listed above (Fig. 1). Furthermore, restriction enzyme digestion can provide analysis of sequences packaged from the intracellular concatemer into full and partially filled heads. *Eco*RV yielded, from DNA isolated from T4$^+$ and 49*ts* phages, as well as from 49*ts* partially filled heads, a set of more than 17 specific fragments present in nonequimolar amounts (Fig. 2). The distribution and the number of fragments appeared, however, to be identical in the 49*ts* phage and the 49*ts* partially filled-head DNAs. (We attribute some differences between T4$^+$ and 49*ts* (and other mutant DNAs) with *Eco*RV [Fig. 2] and *Taq*I [Fig. 3] digestion to differences in DNA sequence among strains.) Cleaving the DNAs from T4 phage, full heads (49t-25°C), and partially filled heads (49*ts*-41°C) with *Taq*I produced in all samples more than 20 fragments (Fig. 3). Once more the distribution and intensities of particular bands were the same in the DNAs isolated from full heads (49*ts*-25°C) and heads incompletely filled with DNA (49*ts*-41°C).

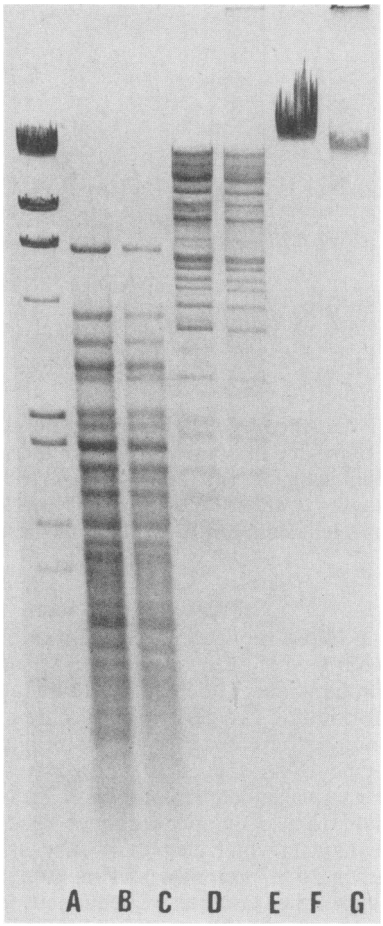

FIG. 1. (G) Concatemeric DNA (2.25 μg) or (F) phage DNA (2.25 μg) of 24*ts*-49*ts*-t was each digested with (E and D) *Eco*RV (2.25 μg) or (C and B) *Taq*I (4.5 μg of DNA), respectively. (A) λ *Hin*dIII, φX-174 RF *Hae*III molecular weight markers. Digestions were for 16 h at 37°C (*Eco*RV) or 65°C (*Taq*I) as described in the legends to Fig. 2 and 3, except that the *Eco*RV digestion was carried out at pH 9.0. Electrophoresis on a 0.8% agarose gel was as described in the legend to Fig. 2.

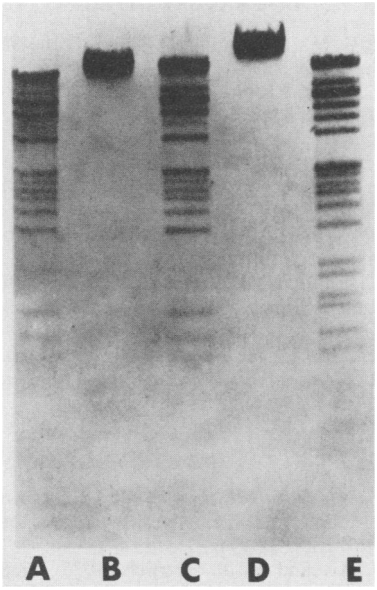

FIG. 2. Digestion of T4 DNAs with *Eco*RV. (A and B) DNA isolated from 49*ts* partially filled heads grown under nonpermissive conditions (41°C). (B) No enzyme added. (A) *Eco*RV added. (C and D) DNA isolated from 49*ts* phage grown under permissive conditions (25°C). (D) No enzyme added. (C) *Eco*RV added. (E) DNA isolated from T4 phage with *Eco*RV added. Digestion of 0.5 μg of DNA in 50 mM NaCl–4 mM Tris (pH 7.8)–10 mM $MgCl_2$–4 mM β-mercaptoethanol (25% glycerol) with 26 U of *Eco*RV at 37°C overnight produced DNA fragments ranging in size from 25 to 2 kilobases. The samples (27 μl) in 25% glycerol–0.2% bromophenol blue were electrophoresed through a 0.7% (wt/vol) agarose gel for 3.5 h at 70 V. Ethidium bromide (1 μg/ml) was incorporated both into the gel and the running buffer, and the gel was photographed under UV light with Polaroid type 665 P/N film through a Wratten 23A filter.

We also examined DNA packaging by using a 20*cs*-21*ts* double mutant. When 20*cs*-21*ts*-infected bacteria are shifted from 22 to 42°C, there is a rapid and synchronous packaging into accumulated proheads to produce viable phage at the same time as further prohead assembly is blocked (6). After 5 min at 42°C an increase of three phage per cell is obtained, whereas at 40 min the full yield is produced. DNAs isolated from 20*cs*-21*ts* at an early stage of packaging (three phage per bacteria) and a late stage of packaging (16 phage per cell) were compared. Both DNAs were digested with *Taq*I, and again no differences in the number of fragments or their intensities were observed (Fig. 3). These observations of the DNA digestion patterns therefore suggest that the distribution of sequences at an early (20*cs*-21*ts* when only three phage per cell are produced) or incomplete (49*ts*-41°C heads filled with 25 to 30% of a full complement of DNA) stage of packaging of the DNA is no different from the distribution in a fully randomized late $T4^+$ phage population. Therefore, restriction enzyme digestion provides no evidence for specific initiating DNA sequences for packaging in T4 phage as judged by comparison of DNA sequences in first or incompletely filled heads with mature and concatemeric T4 DNA.

The mechanism of T4 DNA packaging as it relates to the sequence specificity of the DNA ends might also be studied by direct end group analysis. So far, nothing is known about 3′- and 5′-terminal residues of T4 DNA. In fact, it is possible that the ends could be blocked, since it has been suggested from genetic experiments that the T4 DNA ends might be protected by a terminal-linked protein from exonuclease V attack (20). In this paper we present evidence for 3′ end labeling of T4 DNA in the presence of [α-^{32}P]ddATP or dATP and TdT. In our experiments we used two conditions for the TdT reaction (Fig. 4). (i) The reaction was very efficient when the sodium cacodylate buffer contained 1 mM Co^{2+}. Under these conditions the labeling at nicks in DNA (internal 3′ ends) is also expected to be very efficient (19). Isolation of nick-free high-molecular-weight T4 DNA even when precautions were taken to eliminate endonuclease action and mechanical sharing presented considerable technical difficulties, since the 3′ end labeling (Fig. 4A) showed that the T4 DNA used in our studies was labeled more or less randomly over all the *Taq*I fragments, presumably in nicks, or less likely, in gaps. (ii) When Co^{2+} was replaced by Mg^{2+} (10 mM) in sodium cacodylate buffer, the reaction was very much reduced. Only 1,400 cpm were found incorporated into mature T4 DNA compared with 300,000 cpm in the presence of 1 mM Co^{2+} (the same amount and batch of DNA was used in both reactions). Nevertheless, 3′ DNA ends can be labeled under these conditions, as judged by labeling of the *Pst*I fragment of pBR322 DNA (Fig. 4E to G). In the reaction buffer containing Mg^{2+}, TdT is expected to catalyze predominantly the addition of ddAMP or dAMP to the 3′-terminal

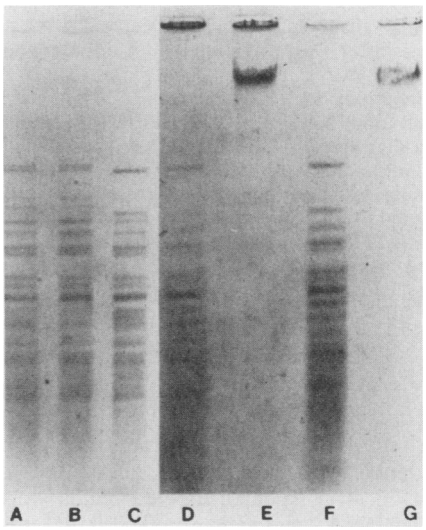

FIG. 3. Digestion of T4 DNAs with *Taq*I. (A) DNA isolated from 49*ts* partially filled heads grown under nonpermissive conditions (41°C) with *Taq*I added. (B) DNA isolated from 49*ts* phage grown under permissive conditions (25°C) with *Taq*I added. (C) DNA isolated from T4 phage with *Taq*I added. (D and E) DNA isolated from 20*cs*-21*ts* grown at 42°C for 5 min (three phage per bacteria). (D) Hydrolysis. (E) No enzyme added. (F and G) DNA isolated from 20*cs*-21*ts* grown at 42°C for 40 min. (F) Hydrolysis. (G) No enzyme added. (A to C) 0.4 to 0.45 μg of DNA in 50 mM NaCl–3 mM Tris (pH 7.8)–19 mM $MgCl_2$–8 mM β-mercaptoethanol were digested with 10 U of *Taq*I for 45 min at 65°C. (D to G) DNA (0.4 to 1.2 μg) in 30 mM NaCl–9 mM Tris (7.4)–11 mM $MgCl_2$–8 mM β-mercaptoethanol was digested with 12 U of *Taq*I for 5 h at 65°C. Reactions were terminated by addition of 100 mM EDTA (pH 7.3) to a final concentration of 20 mM. The lengths of the *Taq*I fragments correspond to a range of molecular weights from about 9.5 to 0.3 kilobases (λ DNA-*Hin*dIII/φX-174 RFDNA-*Hin*cII digest). The samples (30 to 35 μl) in 10% glycerol–0.2% bromophenol blue were applied to an 0.8% agarose gel. The gels were stained and photographed as described in the legend to Fig. 1.

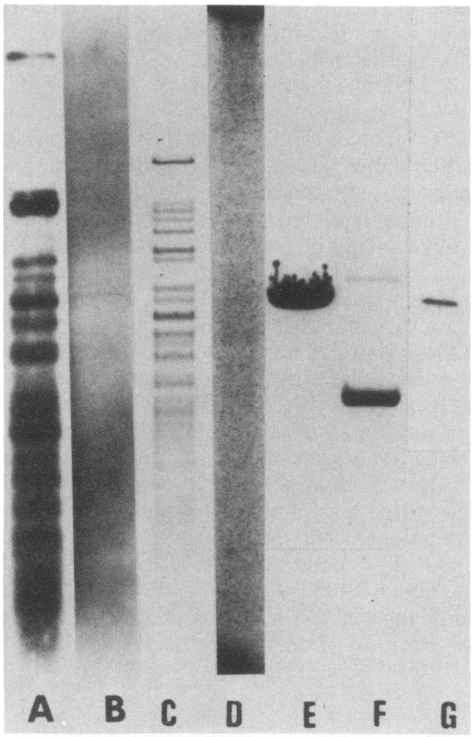

FIG. 4. Analysis of TdT-3′-end-labeled T4 DNA by agarose gel electrophoresis after *Taq*I digestion. (A) Autoradiography of *Taq*I-digested T4 DNA, ddATP 3′ end labeled in presence of 1 mM $CoCl_2$. (B) Autoradiography of *Taq*I-digested T4 DNA, ddATP 3′ end labeled substituting 10 mM $MgCl_2$ for $CoCl_2$. (C) Ethidium bromide-stained *Taq*I-digested T4 DNA, dATP 3′ end labeled in 10 mM $MgCl_2$. (D) Autoradiography of lane C. (E) *Pst*I-digested pBR322 DNA, ethidium bromide-stained agarose gel. (F) pBR322 DNA, ethidium bromide-stained agarose gel. (G) Autoradiography of *Pst*I-digested pBR322 DNA, dATP 3′ end labeled in 10 mM $MgCl_2$. *Taq*I digestion and electrophoresis on a 0.8% agarose gel were as described in the legends to Fig. 2 and 3. Autoradiography on the dried gel was at −70°C with an intensifying screen.

OH of the DNA molecule, whereas addition to nicks should be minimal (19). In contrast to the DNA into which label had been apparently randomly incorporated into nicks over the entire sequence with labeling of all of the *Taq*I DNA fragments (Fig. 4A), specific labeled bands were not seen when the T4 DNA labeled in the TdT-Mg^{2+} reaction was digested with *Taq*I (Fig. 4B and D). The smear of radioactivity is consistent with the expectation that the label is indeed largely concentrated in the 3′ OH ends and suggests, furthermore, that no unique or preferred nucleotide sequences are found at the 3′ termini of T4 DNA.

Overall, our experiments therefore support the inference from genetic and electron microscopic experiments that the T4 DNA ends are formed not by a sequence-specific mechanism but by a different mechanism such as initiation at intermediate recombinational sites (16). However, it is also possible that more sensitive in vivo or in vitro studies would reveal limited end sequence specificity in the T4 DNA packaging mechanism.

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