*EVIDENCE FOR LONG DNA STRANDS IN THE REPLICATING POOL AFTER T4 INFECTION**

By Fred R. Frankel

DEPARTMENT OF MICROBIOLOGY, SCHOOL OF MEDICINE,
UNIVERSITY OF PENNSYLVANIA, PHILADELPHIA

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T4 DNA extracted from phage-infected bacteria sediments faster and differs in other ways from the DNA found in phage particles.[1, 2] The rapid sedimentation could result from an increased molecular length, from association with other molecules, DNA or otherwise, from an altered conformation, or from any combination of these. Many properties of the DNA suggest increased length, but other possibilities have not been excluded.

If replicating DNA contains any single strands of exceptional length, the rapid sedimentation must signify, at least in part, native molecules of exceptional length. More important, the generation of such strands would furnish a clue to mechanisms of replication or recombination or both. Initial attempts to demonstrate long strands failed.[2, 3] We report here that part of the replicating DNA sediments in alkali at a rate appropriate to single strands of two or more times the normal length of T4 DNA.

Methods.—Lysates containing tritium-labeled DNA were prepared from cultures of *Escherichia coli* B3 (thymine-requiring) grown at 36°C to 2×10^8 cells/ml in a Tris-glucose-ammonium medium[4] containing 5 μg/ml of thymidine. The cells were transferred to 0.1 vol of an adsorption medium[5] containing 2 μg/ml of thymidine, and the suspension was aerated for 10–20 min at 36°C. Five particles per bacterium of phage T4 D (from R. S. Edgar) and 10 μg/ml of L-tryptophan were added. After 4 min, the infected cells were diluted to 2×10^8/ml in warm Tris-glucose-ammonium medium containing 2 μg/ml of thymidine. Four min later, 20–80 μc/ml of H^3-thymidine was added. Samples of the culture were lysed at various times thereafter by first chilling in ice and then adding an equal volume of an ice-cold solution containing 2 mg/ml of lysozyme in 0.1 *M* KCN and 0.05 *M* ethylenediaminetetraacetate (EDTA), pH 8. After 20 min, sodium lauryl sarcosinate[6] was added to 3%. Five min later the tube contents were gently mixed by rotating the tube for 10 min at 5°C. This lysis procedure is a modification of that used by Bode and Kaiser.[7] The procedure that we used previously[2] seldom yields denatured DNA that sediments faster than single strands from phage particles, possibly because of nuclease action during a 65°C incubation step. Both procedures yield native DNA that sediments rapidly.

Phage particles labeled with radiophosphorus were prepared in the usual way by growth in synthetic medium containing P^{32}-phosphate. DNA used as a marker in sedimentation measurements contained 0.2 c/gm P. Phage particles used to infect cultures in which parental DNA was to be analyzed contained 5 c/gm P.

The DNA in lysates of infected cultures was denatured by adding NaOH to 0.3 *N* and mixing gently at 5°C for 10 min. The uncorrected pH of this solution was 12.75 (Leeds and Northrup pH meter; radiometer electrode-type GK 2021 C; manufacturer's correction for sodium ion concentration at this pH is + 0.36). P^{32}-labeled phage particles were then added and gently mixed with the alkaline lysate. This results in dissolution of the phage particles and the release of denatured DNA,[8] henceforth designated reference or marker DNA. Samples of this mixture were layered onto 4.5-ml or 25-ml linear gradients prepared from 5% and 20% sucrose solutions containing 0.9 *M* NaCl, 0.1 *M* NaOH, and 0.01 *M* EDTA, and the tubes were spun at 15×10^8 rpm^2-hr (4.5 ml) or

22×10^8 rpm^2-hr (25 ml) at 15°C. Increasing the NaOH concentration sixfold had no striking effect on the sedimentation patterns obtained. The tube contents were analyzed in the way already described.[2]

The hybridization technique used was that of Richards.[9]

Results.—Labeled parental phage: Shortly after T4 infection, the DNA originating from parental phage particles can be recovered as normal phage DNA molecules in high yield, but at later times the recovery is somewhat reduced.[10, 1] When the parental DNA was examined by sedimentation after alkaline denaturation (Fig. 1), the sample taken early after infection (4 min) resembled the marker DNA. At later times, however, the proportion of normal molecules decreased, and a faster-sedimenting component appeared.

A sample taken at 14 minutes after infection was denatured and sedimented through a 25-ml alkaline sucrose gradient (see inset, Fig. 2). Fractions of this DNA were then mixed with a peak fraction of marker DNA which had also been denatured and sedimented in an alkaline gradient. Figure 2 shows the results of recentrifugation of these mixtures. Fractions 5 and 6 showed higher sedimentation coefficients than the marker. The peak parental fraction was indistinguishable from the marker. There is clearly a marked heterogeneity of the strands.

The rapid sedimentation of the parental label probably does not signify cosedimentation with host DNA or cytoplasmic components. When a culture was infected with a P^{32}-labeled mutant of T4 (T4*am*A453, gene 32)[11] that is unable to synthesize DNA but carries out other early functions,[12] no fast com-

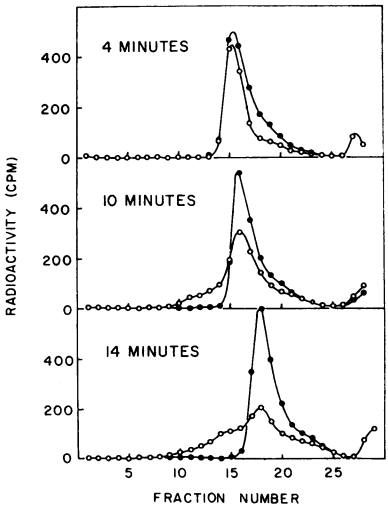

FIG. 1.—Sedimentation of precursor DNA containing P^{32} of parental origin (*open circles*) and admixed H^3-labeled marker DNA (*filled circles*) in alkaline sucrose. Lysates were prepared at times indicated. Sedimentation from right to left.

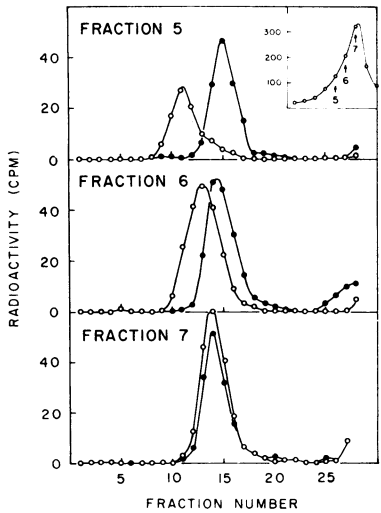

FIG. 2.—Recentrifugation of precursor DNA fractions in alkaline sucrose. *Inset:* Sedimentation in alkaline sucrose of a lysate prepared from a culture infected 14 min earlier with P^{32}-labeled phage. The fractions indicated by arrows were mixed with H^3-labeled reference DNA (also recovered from an alkaline sucrose gradient) and recentrifuged with the results shown. *Open circles*, P^{32}-labeled DNA; *filled circles*, H^3-labeled DNA.

ponent appeared at 15 minutes. However, if cells were mixedly infected with this P^{32}-labeled mutant and unlabeled T4 or T4*am*B22 (gene 43),[13] the fast component did appear.

Newly synthesized DNA: We previously reported that DNA continuously labeled after infection exhibits a gradually increasing sedimentation coefficient for about 15 minutes, when phage particles begin to appear.[2] Such DNA was examined after alkaline denaturation, with the results shown in Figure 3. At seven minutes after infection, the denatured strands sedimented slowly. With increasing time after infection the sedimentation coefficient of the DNA gradually increased, reaching the rate characteristic of the marker at about 9 or 10 minutes after infection, and then exceeding it at 12 minutes. By 17 minutes, DNA resembling the marker had begun to accumulate.

The DNA strands from a culture labeled after infection with H^3-thymidine and lysed at 14 minutes were isolated from a preparative alkaline sucrose gradient (Fig. 4*A*). Fractions of denatured marker DNA were also isolated (Fig. 4*B*). The results of centrifugation of several mixtures of these fractions are shown in Figure 5. A slowly sedimenting fraction (Fig. 5*A*) and a rapidly sedimenting fraction (Fig. 5*B*) of the newly synthesized DNA both retained their characteristic rate of sedimentation when compared with the peak fraction of the marker. However, a rapidly sedimenting fraction of the marker (containing less than 4% of the DNA) did not show rapid sedimentation after recentrifugation (Fig. 5*C*). It appears that the distribution of marker DNA in an alkaline gradient mainly reflects diffusion and mechanical mixing, whereas the distribution of the newly synthesized DNA reflects a heterogeneity of sedimentation coefficients.

When infected cells were labeled from the fourth to the seventh minute after infection, and then transferred to unlabeled medium until 15 minutes, the labeled DNA that moved slowly at 7 minutes was converted, by 15 minutes, into a mixture of normal and rapidly sedimenting strands. When infected cells were labeled at 15 minutes for 30 seconds or less, most of the labeled DNA sedimented heterogeneously and more slowly than the marker; however, a small fraction still sedimented faster. Thus, although pulse-labeled DNA adds directly to a rapidly sedimenting native structure in the replicating pool,[2] it does so mainly, but not entirely, in the form of short strands.

The effect of chloramphenicol and certain phage mutations: When chloramphenicol was added to a culture at 10 minutes after infection, and the culture was lysed at 30 minutes and analyzed after alkali treatment, the bulk of the newly synthesized DNA was found to sediment faster than the marker (Fig. 6, bottom). A similar result was obtained in the absence of chloramphenicol if the infecting phage was T4*am*B17 (gene 23) or T4*ts*L 147 (gene 22), mutants unable to synthesize head protein under restrictive conditions.[11, 14]

As the figure shows, different results were obtained if chloramphenicol was added at earlier times. Under these conditions the component that accumulated in greatest amount had a sedimentation coefficient somewhat lower than marker.

Test for cross-linking between DNA strands: If the polynucleotide chains of a DNA molecule are cross-linked, the molecular weight cannot halve on denaturation, and the structure will sediment faster than the free single strands. To

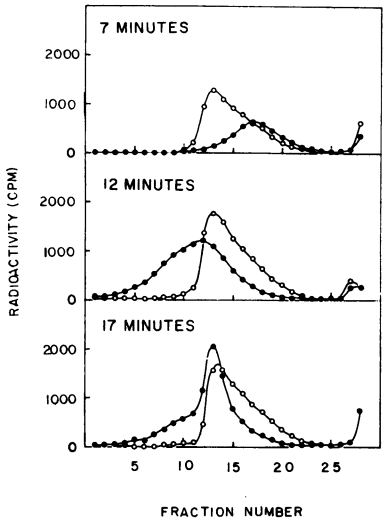

FIG. 3.—Sedimentation of H^3-labeled newly synthesized DNA (*filled circles*) and admixed P^{32}-labeled reference DNA (*open circles*) in alkaline sucrose. The cultures were continuously labeled after infection and samples were lysed at the times indicated.

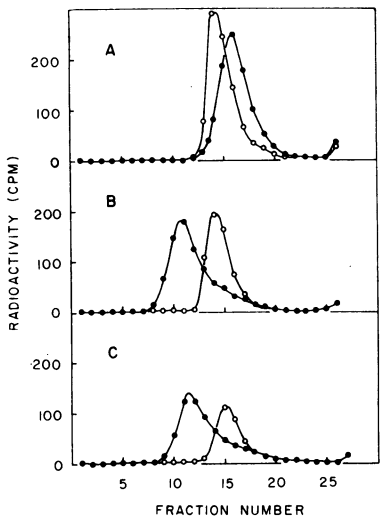

FIG. 5.—Recentrifugation of fractions of H^3-labeled newly synthesized DNA (*filled circles*) and fractions of P^{32}-labeled marker (*open circles*) in alkaline sucrose. (*A*) Fraction 9 of Fig. 4*A* and fraction 6 of Fig. 4*B*; (*B*) fraction 5 of Fig. 4*A* and fraction 6 of Fig. 4*B*; (*C*) fraction 5 of Fig. 4*A* and fraction 4 of Fig. 4*B*.

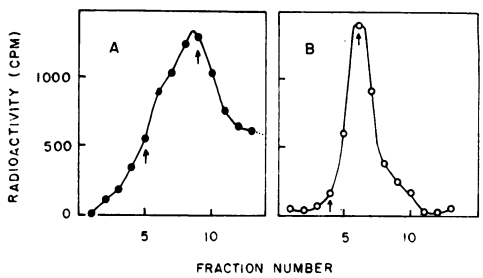

FIG. 4.—Preparative alkaline sucrose gradients. (*A*) DNA from a culture labeled after infection with H^3-thymidine and lysed at 14 min. (*B*) P^{32}-labeled phage particles treated with alkali.

determine whether interstrand linkages are present in the rapidly sedimenting T4 DNA, the possibility of reversible denaturation was examined.[15]

A lysate was prepared at 20 minutes after infection with T4*am*B17, denatured with 0.3 *N* NaOH, and marker added. One sample was analyzed by sedimentation and found to contain predominately fast-sedimenting DNA. A second sample was carefully adjusted to pH 7.5 with 0.5 *M* KH_2PO_4, layered onto a solution of CsCl, and sedimented to equilibrium. The rapidly sedimenting strands and marker both formed single bands indicating a buoyant density 0.015 gm/ml greater than that of native marker. Thus, the rapidly sedimenting DNA does not show reversible denaturation, and we conclude that its high rate of sedimentation does not result from interstrand linkages.

Association with protein also can result in rapid sedimentation of DNA. Therefore, rapidly sedimenting strands were isolated from a lysate prepared at 20 minutes after infection with T4*am*B17, neutralized, and incubated at 36°C for

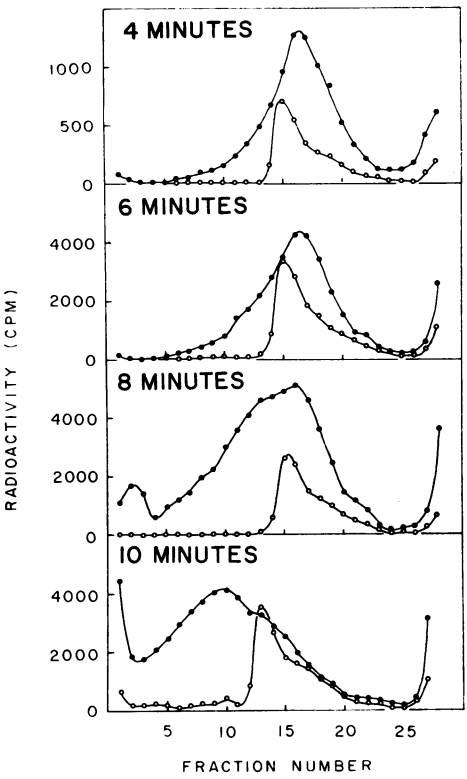

FIG. 6.—Sedimentation in alkaline sucrose of H^3-labeled DNA synthesized in the presence of chloramphenicol (100 μg/ml) (*filled circles*), and admixed P^{32}-labeled marker DNA (*open circles*). Chloramphenicol was added at the times indicated. Lysates were prepared at 30 min after infection.

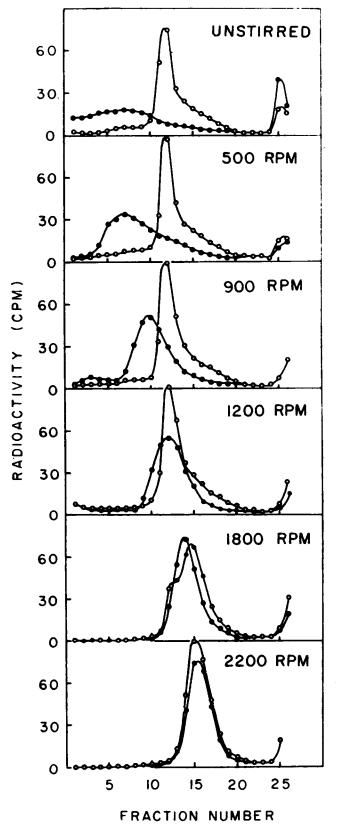

FIG. 7.—The effect of shear on single strands of precursor DNA. A lysate was prepared at 30 min from an infected culture to which chloramphenicol (100 μg/ml) had been added at 10 min and H^3-thymidine at 11 min. After addition of alkali and P^{32}-labeled phage particles, the mixture was stirred at 25° for 5 min each at the indicated speeds.

30 minutes with 1 mg per milliliter of pronase. Sedimentation after treatment with detergent and alkali showed that the enzyme had little effect.

T4 specificity of the rapidly sedimenting DNA: We have found that denatured DNA obtained from uninfected cultures of *E. coli* according to our procedure sediments in alkali about 1.6 times faster than denatured marker DNA. At ten minutes after infection, little of the prelabeled bacterial DNA sediments faster than the marker. Although bacterial DNA synthesis ceases after phage infection,[16] it seemed possible that some incorporation of labeled precursors into fast-sedimenting host DNA strands might occur. Therefore, rapidly sedimenting strands were isolated from cells infected with either T4 or T4*am*B17 and the labeled DNA was tested for base-sequence homology with *E. coli* DNA and T4 DNA. It was found to resemble authentic T4 DNA (Table 1).

TABLE 1. *Specific hybridization tests of mixtures containing precursor DNA and reference DNA.*

DNA test mixture	DNA on filter	Competing DNA	Per Cent Bound Precursor DNA	Per Cent Bound Reference DNA
	—	—	0.1	0.2
T4 precursor DNA	T4 DNA	—	51	58
(93*S*) plus reference DNA	T4 DNA	T4 DNA	21	23
	T4 DNA	*E. coli* DNA	58	60
	E. coli DNA	—	0.4	0.7
	—	—	0.2	0.0
T4*am*B17 precursor	T4 DNA	—	54	64
DNA (80*S* to 113*S*) plus	T4 DNA	T4 DNA	22	26
reference DNA	T4 DNA	*E. coli* DNA	59	62
	E. coli DNA	—	0.3	0.9

The *DNA test mixture* was prepared by mixing an alkaline sucrose fraction containing H^3-labeled precursor DNA isolated at 15 min after infection with P^{32}-labeled reference DNA. After neutralization, the mixture was sonicated for 4 × 10 sec in ice and heated at 100°C for 10 min; aliquots were added to the various membrane filters in vials and incubated for 20 hr at 63°C to 65°C. *DNA on filter:* The filters received 5 μg of heat-denatured T4 DNA or *E. coli* DNA. *Competing DNA* was added at 50 μg/ml to the test mixtures before neutralization.

Fragility under shear: Hershey, Burgi, and Ingraham[17] found a direct relationship between the length of DNA molecules and their fragility, and Davison and Freifelder[18] presented some evidence that the same may be true for single-stranded DNA. We have examined the behavior under shear of an alkaline solution containing rapidly sedimenting DNA and marker.

As shown in Figure 7, the effect of shear on the denatured marker was similar to its effect on native DNA. No change in the sedimentation pattern of the DNA was observed until a critical speed of stirring was exceeded (1800 rpm). At this point, the marker gave rise to a slower-sedimenting species with concomitant loss of the original molecules. The rapidly sedimenting DNA in the mixture showed very different behavior. With each increment in stirring speed, its sedimentation rate decreased. However, strands with the sedimentation rate of the marker were not produced until a stirring speed just below the critical speed for the marker was reached. After being stirred at the critical speed, the DNA sedimented more slowly than the original marker strands but faster than their breakage product. This is the behavior expected of molecules that are longer and more heterogeneous in length than marker DNA, if fragility depends on length.

Native, rapidly sedimenting DNA contains rapidly sedimenting strands: The experiments described up to this point have been concerned with the nature of alkali-denatured DNA in unfractionated lysates of infected cells. The rapidly sedimenting strands described above may arise from a different fraction of the phage-specific DNA pool than the native, rapidly sedimenting DNA that we described previously.[2] Therefore, native rapidly sedimenting DNA was isolated from cultures at 15 minutes after infection with T4 or T4*am*B17. Re-analysis of selected fractions on neutral sucrose gradients showed that the isolated DNA's had high sedimentation rates (in one experiment, 6, 4, and 3.2 times faster than the marker) that depended on their original positions in the preparative gradients (fractions 6, 9, and 11, respectively). Analysis on alkaline sucrose gradients

showed the fractions to be similar to one another in that 40–60 per cent of the DNA sedimented faster than marker DNA. We have been unable to increase the proportion of faster-sedimenting strands either by more rapid re-analysis or by gentler manipulations. Evidently, the rapidly sedimenting native DNA contains both slowly and rapidly sedimenting strands.

Discussion.—DNA extracted from bacteria infected with phage T4 contains a considerable fraction of strands that sediment more rapidly in alkali than reference DNA strands. These strands incorporate isotopic labels both from the parental phage particle and from the culture medium.

Phage particles treated with alkali released DNA whose major component sedimented homogeneously and reproducibly. We took this material to be intact single strands of T4 DNA, and used it as our reference DNA. Occasionally, a small amount of faster-sedimenting material was seen in these preparations[18] (see Fig. 7). The latter may result from cross-links preventing separation of the DNA strands[19] or from incomplete dissociation of protein from the DNA.

We found no indication that the rapid sedimentation of alkali-treated DNA extracted from infected bacteria was due to cross-linking, bound protein, or cosedimentation with host materials. In addition, we showed that the rapid sedimentation could not result from synthesis of long strands of host DNA.

This appears to leave two alternatives: that the rapidly sedimenting strands are longer than strands from phage DNA, or that they are altered conformations of phage DNA strands. The only alternate stable conformation that a single polynucleotide chain might be expected to achieve is one in which the ends are joined to form a circle. A unique circular molecule should exhibit a unique sedimentation coefficient. However, heterogeneous sedimentation was observed in the studies reported here, even under conditions in which the alkali concentration of the sedimentation medium was varied from 0.1 N to 0.6 N. Therefore there is no reason to suspect circular strands at present.

The effect of shear on the rapidly sedimenting strands was like that seen for long double-helical DNA molecules. Very low rates of shear caused the sedimentation rate of the DNA to decrease without generating strands of any characteristic length; this occurred without breakage of marker strands in the mixture. We therefore conclude that the rapidly sedimenting strands extracted from infected cells represent unusually long molecules of phage-specific DNA. These strands sediment up to 1.5 times faster than reference strands and thus appear to be more than twice the length of the reference DNA.[20] This is not to say that all molecules in the pool have this property, or that their rapid sedimentation in the native state may not be due in part to other structures. Rapidly sedimenting strands have previously been observed in cells infected with phage lambda.[21]

Addition of chloramphenicol at a late time after infection, or infection by phage mutants unable to synthesize head protein, resulted in accumulation of rapidly sedimenting strands. These strands showed sedimentation rates appropriate to polynucleotide chains about four times the length of the reference DNA. An analogous effect of chloramphenicol[2] and infection with the phage mutants[22] was observed on the native DNA in the replicating pool. The production of nor-

mal phage DNA molecules appears to be a late step in maturation dependent on head formation, as already suggested by others.[23, 24]

Long strands of DNA could arise by recombination between molecules of ordinary size bearing terminal repetitions of nucleotide sequences. Formation of long strands in this way has been suggested by Streisinger and co-workers.[25] Alternatively, these molecules could be synthesized directly. A mechanism that could generate molecules longer than the template upon which they are replicated has been suggested for chromosome transfer during conjugation.[26] The short strands synthesized both early and late after infection may represent synthesis at new growing points since they are preferentially labeled by a very short pulse of H^3-thymidine.

Very early addition of chloramphenicol to an infected culture resulted in the accumulation of strands that sedimented somewhat more slowly than reference strands. This might result from failure of the appearance of enzymes concerned with the joining of DNA fragments[27] or from abortive DNA replication.

Summary.—Evidence is presented that some DNA in the replicating pool of T4-infected cells contains strands that are longer than those found in the mature phage particle.

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