*MECHANISM OF DNA CHAIN GROWTH, III. EQUAL ANNEALING OF T4 NASCENT SHORT DNA CHAINS WITH THE SEPARATED COMPLEMENTARY STRANDS OF THE PHAGE DNA**

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Abstract.—Nascent short DNA chains isolated from T4-infected *E. coli* under a variety of conditions anneal equally to the separated complementary phage DNA strands. The samples examined include: pulse-labeled short chains isolated by alkaline sucrose gradient sedimentation from the T4D (wild type)-infected cells in both the early and late stages of phage DNA synthesis; nascent chains accumulated during ligase inhibition of T4 *ts* B20-infected cells; and the single-stranded nascent short chains isolated from T4D-infected cells by mild procedures involving no denaturation treatment. The results are consistent with the hypothesis that *both* strands of DNA are synthesized discontinuously.

Previous studies performed in this laboratory[1-5] have shown that the nascent portion of chromosomal DNA exists in the cell as short chains which sediment at about 10*S* in alkali. Nascent short chains with similar properties accumulate upon temporary inhibition of the polynucleotide ligase of temperature-sensitive ligase mutants of phage T4.[4, 5] These findings, confirmed in several other laboratories,[6-15] strongly suggest a discontinuous mechanism of DNA replication in which short segments of DNA are synthesized at the replicating point and are subsequently joined by ligase action.

After a sufficiently short pulse of radioactive thymidine, virtually all of the incorporated label is found in the short chains.[1-3, 5, 9, 10, 15] Furthermore, almost all of the label is in the accumulated short chains during ligase inhibition.[4, 5, 12] These facts imply that *both* the daughter strands are synthesized discontinuously. The present study was undertaken to test this point directly by annealing experiments. Pulse-labeled T4 short chains prepared under a variety of conditions annealed equally with the separated complementary strands of the phage DNA, supporting the two-strand-discontinuous mechanism.

Materials and Methods.—The following commercial products were used: H^3- and C^{14}-thymidine (New England Nuclear); poly U, control no. 411754, and poly GU, control no. 335 (Miles); Ficoll, av. mol wt 400,000 (Pharmacia); polyvinylpyrrolidone, av. mol wt 360,000 and bovine serum albumin, Fraction V (Sigma); CsCl (Merck); membrane filter MF 50 (Sartorius). Bacteriophages T4D (wild type) and T4D OS were kindly supplied by Dr. J. Tomizawa. T4 *ts* B20 (gene 30) and *E. coli* B/5 were gifts from Dr. R. S. Edgar.

Preparative separation of complementary strands of T4 DNA: Lysates of T4D OS were prepared by infecting *E. coli* B/5 in Medium B.[3] Lysates of T4D OS labeled with C^{14}-thymidine were prepared as follows: *E. coli* B/5 (1×10^9 cells/ml) was infected at 37° with phages at a multiplicity of 10; 9.5 min later C^{14}-thymidine (54 μC/μmole) was added to 10^{-5} *M* and the shaking continued until lysis was complete. The phages were puri-

fied by low- and high-speed centrifugations and banding in preformed gradients of CsCl.[16] The strands were separated by the procedure described by Cohen and Hurwitz[17] for λ DNA. Poly GU or poly U was used in place of poly G, according to Guha and Szybalski.[18] Unlabeled separated strands were used as immobilized DNA in hybridization experiments after self-annealing in 5 × SSC at 65° for 4 hr.

Preparation of DNA: T4D OS DNA was prepared by phenol extraction. *E. coli* B DNA was prepared by the method of Marmur.[19] To denature DNA, samples in SSC were heated at 100° for 5 min and rapidly cooled. C^{14}-labeled fragmented T4 DNA was prepared by heating C^{14}-labeled T4D OS in 0.5 *N* NaOH at 100° for 7 min. Various samples of nascent short chains were prepared as indicated in Table 1. The methods for phage infection, pulse labeling, temperature shift, DNA extraction, alkaline and neutral sucrose gradient sedimentation, and hydroxylapatite chromatography were described previously.[3, 4]

DNA-DNA hybridization: Hybridization was carried out as described by Denhardt[20] except that in most experiments the "preincubated" membrane filters loaded with unlabeled DNA were transferred to vials containing labeled DNA in 0.7 ml of fresh preincubation medium for annealing at 65°. Labeled DNA for hybridization was usually fragmented by heating in 0.5 *N* NaOH at 100° for 7 min. This treatment was often omitted for the nascent short chains.

Results.—Strand separation and model experiments: Using the method developed by Guha and Szybalski[19] for preparative separation of the intact complementary strands of T4 DNA, unlabeled and C^{14}-labeled T4 DNA were separated into two fractions: "W" and "C" with poly GU, and "L" and "H" with poly U (Fig. 1). To ascertain the purity of the separated strands and to provide standards for the experiments with the nascent short chains, hybridization experiments with the membrane filter technique[20] were performed with the labeled and unlabeled strands.

The results in Figures 2 and 3 reveal the following: (1) The labeled W strand annealed with the unlabeled C strand or a mixture of equal amounts of unlabeled W and C strands (W + C strands), but little with the unlabeled W strand. (2) Similarly, the labeled C strand annealed with the unlabeled W strand or the W + C strands but barely with the unlabeled C strand. (3) The labeled W + C

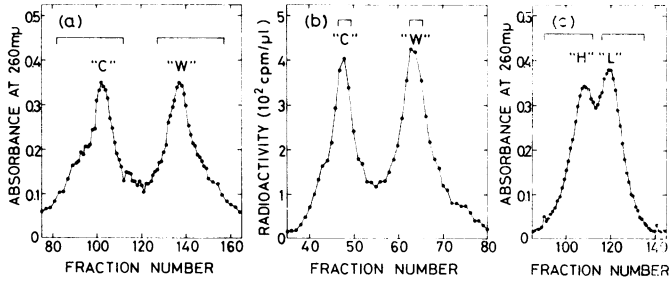

FIG. 1.—Separation of the complementary strands of T4 DNA by preparative CsCl gradient centrifugation in the presence of poly GU or poly U. (*a*) Poly GU-effected fractionation of unlabeled DNA; (*b*) poly GU-effected fractionation of C^{14}-labeled DNA; (*c*) poly U-effected fractionation of unlabeled DNA. The total volume of each gradient was 9.5 ml; 45-, 90-, and 50-μl fractions were collected from the bottom in *a*, *b*, and *c*, respectively. Bracketed fractions were used for annealing experiments.

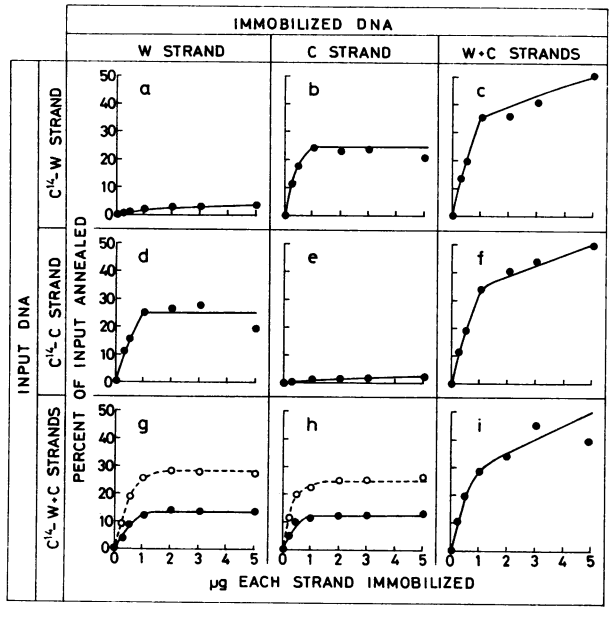

FIG. 2.—Model annealing experiments with labeled and unlabeled W and C strands: annealing of the labeled strand(s) as a function of the amount of unlabeled strand(s) immobilized. 0.16 μg (750 cpm) of each fragmented labeled strand was annealed with the unlabeled strand(s) as indicated. "W + C strands" denotes an equal mixture of the W and C strands. ●: Per cent annealing with respect to the total labeled strand(s) added. ○: Per cent annealing with respect to the labeled strand complementary to the immobilized unlabeled strand.

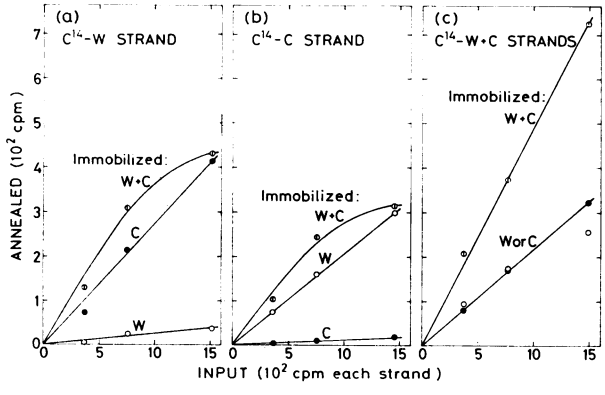

FIG. 3.—Model annealing experiments with labeled and unlabeled W and C strands: the amount of the labeled strand(s) annealed as a function of the amount added. The indicated quantity of fragmented labeled strand(s) (750 cpm/0.16 μg) and 1 μg each of unlabeled strand(s) were used.

strands annealed with the unlabeled W, C, and W + C strands. (4) With variable amounts of the immobilized W or C strand and a fixed amount (0.16 μg of each strand) of the labeled sample, annealing of the labeled complementary strand reached a saturation plateau of 25 per cent[21] at about 1 μg of the immobilized strand (Fig. 2*b*, *d*, *g*, and *h*). (5) Saturation for the immobilized W + C strands, unlike that for the W or C strand alone, exhibited a biphasic pattern (Fig. 2*c*, *f*, and *i*); the amount of the labeled strand annealed increased sharply up to 1 μg of each strand immobilized and gradually beyond this point. (6) At low levels of immobilization, approximately the same amounts of the labeled strand (W or C) were annealed with the complementary strand immobilized alone or that immobilized along with the other strand. At high levels of immobilization, however, greater amounts of the labeled strand were annealed in the presence of both strands on membrane filters than in the presence of the complementary strand alone.[22]

(Compare, in Fig. 2, *b* with *c*, *d* with *f*, and *g* or *h* with *i*.) (7) With a fixed amount of the immobilized DNA, the amount of DNA annealed varied linearly with the amount of input DNA; these results obtained with various combinations of the labeled and unlabeled strands and within a wide range of the input amount (Fig. 3). It is evident from these results that the separated W and C strands are almost free from contamination from each other[23] and that three cases—(*a*) 100 per cent W, (*b*) 100 per cent C, and (*c*) 50 per cent each W and C—can clearly be distinguished by the annealing test.

Furthermore, as seen from Table 1, unequal ratios of the two complementary strands can be assayed. Table 1 also shows that the labeled W strand was hybridized to the unlabeled L strand, and the labeled C strand to the unlabeled H strand. Virtually no reaction was found between the W and H strands and between the C and L strands. These results, consistent with the notion that the W and C strands correspond, respectively, to the H and L strands,[18] assure a reasonable purity of our preparations of the L and H strands.

TABLE 1. *Annealing of mixtures of the C^{14}-labeled W and C strands with the unlabeled W, C, H, and L strands.*

Labeled strands added W:C*	Per cent of the input annealed with 1 μg of: W strand	C strand	H strand	L strand
4:0	3.5	38.5	3.8	36.6
3:1	11.6	30.6	—	—
2:2	21.8	20.7	22.6	18.8
1:3	29.8	10.7	—	—
0:4	40.2	1.0	36.7	1.5

* Total of 925–960 cpm.

Experiments with the isolated nascent short chains: The preparations of the short chains examined are listed in Table 2. These short chains annealed efficiently with unfractionated T4 DNA but barely with *E. coli* DNA; furthermore, annealing was competed for by unlabeled fragmented T4 DNA but not with *E. coli* DNA.

Figure 4 and Table 3 (expt. 1) show that the pulse-labeled short chains isolated by alkaline sucrose gradient sedimentation from wild-type T4-infected cells in the late stages of phage DNA synthesis (preparations 1 and 2) annealed equally with the isolated W and C strands. The results with these short chains are indistinguishable from those obtained with mixtures of equal amounts of the C^{14}-labeled W and C strands. An experiment with the isolated H and L strands gave similar results (Fig. 5). The nascent short chains isolated with alkali from T4D-infected cells in the early stages of phage DNA synthesis (preparations 3 and 4) also annealed equally with the W and C strands (Table 3, expt. 2).

Part of the nascent short chains are extracted in the single-stranded form by a mild procedure involving no treatment for denaturation.[2, 3, 5, 24] Such material sediments slowly in a neutral sucrose gradient and is eluted from hydroxylapatite at a relatively low phosphate concentration. Nascent short chains isolated in the single-stranded form by these procedures (preparations 5 and 6) were also shown to consist of two complementary populations (Table 3, expts. 3 and 4).

TABLE 2. *Preparations of the nascent short chains.*

Preparation no.	Source	Extraction and isolation
1	T4D-infected cells pulse labeled for 30 sec at 20° at 70 min of infection	Extracted by Thomas method; 8–10*S* (peak) fraction from alkaline sucrose gradients
2	T4D-infected cells pulse labeled for 15 sec at 20° at 70 min of infection	Extracted with NaOH–EDTA; 5–11*S* fraction (peak at 8*S*) from alkaline sucrose gradients
3	T4D-infected cells pulse labeled for 15 sec at 20° at 45 min of infection	Extracted with NaOH–EDTA; 6–13*S* fraction (peak at 9*S*) from alkaline sucrose gradients
4	T4D-infected cells pulse labeled for 30 sec at 20° at 45 min of infection	Extracted by Thomas method; 6–13*S* fraction (peak at 9*S*) from alkaline sucrose gradients
5	T4D-infected cells pulse labeled for 30 sec at 20° at 70 min of infection	Extracted by Thomas method; 9–20*S* fraction (peak at 14*S*) from neutral sucrose gradients
6	T4D-infected cells pulse labeled for 30 sec at 20° at 70 min of infection	Extracted by Thomas method; single-strand fraction from hydroxylapatite chromatography
7	T4 *ts* B20-infected cells exposed to 43° for 1 min at 70 min of infection and labeled for 30 sec at 43°	Extracted with NaOH-EDTA; 6–15*S* fraction (peak at 10*S*) from alkaline sucrose gradients
8	T4 *ts* B20 infected cells exposed to 43° for 1 min of infection and labeled for 30 sec at 43°	Extracted with NaOH–EDTA; 7–13*S* fraction (peak at 10*S*) from alkaline sucrose gradients

Phage infection was at 20°.

In experiment 5 of Table 3, the nascent short chains were isolated from cells infected with T4 *ts* B20, a thermosensitive ligase mutant. Pulse labeling was performed one minute after a temperature shift from 20° to 43° during the early or late stages of phage DNA synthesis. The results indicate that short DNA chains accumulated during ligase inhibition also anneal equally with the two complementary strands of T4 DNA.

Discussion.—The present study indicates that both the T4 nascent short DNA

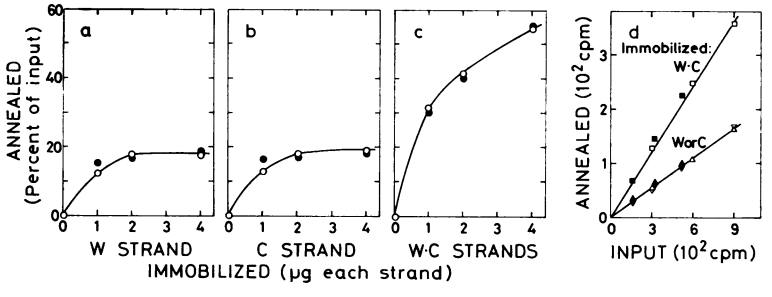

FIG. 4.—Annealing with the W and C strands of the nascent short chains isolated by alkaline sucrose gradient sedimentation from T4D-infected cells (prep 1, Table 2). In *a*, *b*, and *c*, 590 cpm of prep. 1 (○) or 365 cpm of fragmented C^{14}-labeled T4 DNA (●) were annealed with the unlabeled strand(s) as indicated. In *d*, indicated amount of prep. 1 (□,△,▽) or fragmented C^{14}-labeled T4 DNA (■,▲,▼) was annealed with the fixed amount (2 μg each) of unlabeled strand(s). "W·C strands" denotes unfractionated T4 DNA.

TABLE 3. *Annealing of the nascent short chains preparations 2–8 (cf. Table 2) with the W and C strands.*

Expt. no.	Sample tested	Per Cent Input Annealed with: W strand	C strand	Unfractionated T4 DNA 1 μg	2 μg
1	Prep. 2, untreated (2100 cpm)	15.0†	14.0†	25.4	37.9
2	Prep. 3, untreated (2280 cpm)	25.5*	22.9*	48.2	54.9
	Prep. 4, untreated (5220 cpm)	26.9*	24.0*	49.8	63.8
3	Prep. 5, alkali denatured (240 cpm)	21.0†	20.0†	50.2	62.5
	Prep. 5, untreated (240 cpm)	18.7‡	18.4‡	—	—
4	Prep. 6, fragmented (530 cpm)	29.0†	30.0†	57.6	72.0
	Prep. 6, unfragmented (530 cpm)	22.4*	27.4*	48.3	—
5	Prep. 7, untreated (1548 cpm)	20.7*	17.7*	32.5	53.4
	Prep. 8, untreated (2264 pm)	23.4*	17.8*	41.8	49.9

* Plateau values obtained from assays with 1, 2, and 4 μg of the immobilized strands.
† Values obtained with 1 μg of the immobilized strands.
‡ Values obtained with 2 μg of the immobilized strands.

chains found normally and those accumulated upon inhibition of polynucleotide ligase are comprised equally of the complementary strands of T4 DNA. This is true for short chains isolated early or late in phage DNA synthesis. Similar results have been obtained with the replicative λ DNA by Tomizawa and Ogawa.[11] Thus, the interruptions of the phosphodiester bonds in the replicating region reside on both daughter strands as suggested from previous studies.[1–5, 9, 10, 12, 15, 25]

The discontinuity of both daughter strands most probably arises from discontinuous synthesis along the two template strands. This implies that the chain initiation is a frequent event in DNA replication *in vivo* and that synthesis could take place simultaneously at many points along each template strand in a single replicating fork. An alternative possibility is that the discontinuity results from selective *in vivo* endonucleolytic scission of the nascent daughter strands that are synthesized by a *continuous* mechanism in the 3′ to 5′ direction, on the one hand, and in the 5′ to 3′ direction, on the other. This is unlikely

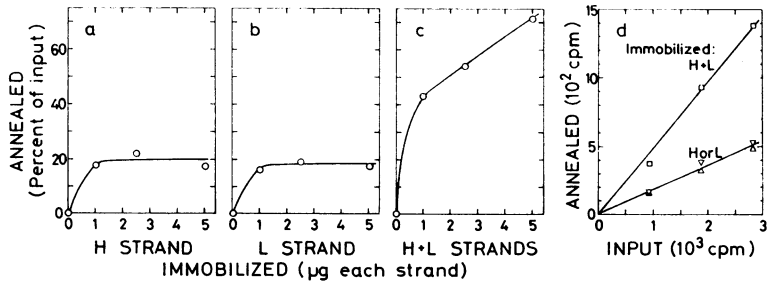

FIG. 5.—Annealing with the L and H strands of the nascent short chains isolated by alkaline sucrose gradient sedimentation from wild-type T4-infected cells (prep. 2, Table 2). In *a*, *b* and *c*, prep. 2 (1700 cpm) fragmented by heating in 0.5 *N* NaOH was annealed with the unlabeled strand(s) as indicated. In *d*, the indicated amount of fragmented prep. 2 was annealed with the fixed amount 2.5 μg each) of the unlabeled strand(s). "H + L strands" denotes equal mixture of the H and L strands.

particularly in view of our recent experiments[26] indicating that the short chains of both strands are products of a 5′ to 3′ synthesis. The T4 short chains complementary to the W and C strands were labeled at their growing ends by a very short pulse and the label in both types of short chains was shown by exonucleolytic degradations to be at the 3′ end. Still another model[27, 28] postulates the formation of hairpin structures along the two template strands in the fork region followed by a specific endonucleolytic cleavage of the hairpin at its apex. This model is also difficult to reconcile with our results at least in its original form, since it predicts fragment formation along only one template strand.

It was suggested previously[5] that part of the nascent short chains may be peeled off from the replicating region during extraction and thus be obtained in the single-stranded form. The peeling off may be caused by winding back of the parental strands in the replicating fork region, which would occur upon removal of cellular elements which are attached to this region and serve to stabilize its structure. This appears plausible particularly if the discontinuous replication along one template strand is ahead of the replication along the other. The fact that the nascent short chains obtained in the single-stranded form by mild extraction anneal equally with the W and C strands lends no positive support to this idea, since one might expect the preferential annealing with one of the two strands provided that replication is uniquely ahead along one of the strands.

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[21] This value varied from experiment to experiment in the range of 20 to 50%. Low values were obtained with the original procedure of Denhardt[20] used in this experiment. The modifi-

cation which is described in *Materials and Methods* and was used in most of the other experiments resulted in the improved annealing efficiency.

[22] The reason for this phenomenon is not known. Similar observations were made with the separated complementary strands of *Bacillus subtilis* (Kainuma and Okazaki, unpublished).

[23] Some of the reaction between the labeled and unlabeled W or C strand may be due to an impurity in the labeled strands rather than in the unlabeled strands, because the degree of the reaction between the labeled and unlabeled preparations of the same strands did not change with different preparations of unlabeled strands. This is most clearly seen in Table 1 by comparing the results with unlabeled W and C strands and those with unlabeled H and L strands.

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[25] Such results which apparently support the two-strand discontinuous mechanism can be obtained even though the replication is discontinuous only on one of the strands if 50 per cent of the population of the replicating forks travel in one direction on the chromosome and the remaining 50 per cent in the opposite direction. This possibility, though it seems remote, has not been excluded in the T4 system. Replication (movement of the replicating point) of λ DNA has been reported to be unidirectional (ref. 11, Markover, S., these PROCEEDINGS, **59**, 1345 (1968), and Lepecq, J. B., and R. L. Baldwin, in *Cold Spring Harbor Symposia on Quantitative Biology*, vol. 33 (1968), p. 609.

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