UNBIASED PARTICIPATION OF T_4 PHAGE DNA STRANDS IN REPLICATION

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Nitrocellulose filters were charged with right or with left strands of T_4 DNA preparatively isolated by the procedure of Guha and Szybalski (1968). Replicative hybrid (of labeled parental DNA) and progeny phage DNA's were annealed, using differentially labeled DNA from mature phage as an internal control.

In T_4 phage infection of E.coli B (with either wild or ligase negative Amber mutant), both parental strands of DNA participate in semiconservative replication. An equal proportion of DNA from each parental strand is transferred also to progeny phage. Newly synthesized progeny DNA is also composed of left and right strands. This is true even at the earliest times of DNA synthesis (4-4:30), that is when less than 10% of the length of the parental molecule has been replicated. These observations apply both to wild and to ligase-negative T_4 phages. Both strands are also produced in E.coli B infected with the ligase negative mutant in the presence of CM added for a period, early after infection which permits, after removal, a considerable yield of phage.

Experimental

Analytical methods have been described (Kozinski, 1968). The preparative separation of T_4 strands was accomplished as described by Guha and Szybalski, (1968). Annealing of DNA on nitrocellulose filters was performed as described by Denhardt, 1966. In this paper we use the terms left and right strands instead of light and heavy, to avoid confusion with 5BU substituted strands.

A. The Fate of Parental Strands in Replicative and Progeny Phage DNA.

Heavy (5BU substituted) E.coli B23 was infected in heavy medium with light ^{32}P labeled phage. Two phages were used in separate infections: (1) Wild (L+) T4BO$\dagger$, (2) ligase-negative (L-) (Fareed and Richardson, 1967), T4 AM H39X. At intervals, each suspension was sampled and the DNA extracted and banded in CsCl. Fractions located between light and heavy reference DNA and containing parental ^{32}P were pooled and supplemented with ^{3}H phage DNA. After dialysis (10-^{3}M EDTA), sonication and denaturation it was used for annealing as described in Table #1. In another experimental permutation,

Table #1

		No CM - 30 min.		No CM - 60 min.		CM 0-30 min.		CM 5-30 min.		CM 10-30 min.	
Phage		L-	L+	L-	L+	L-	L+	L-	L+	L-	L+
I	A	1.01	0.95	1.02	1.05	-	-	1.00	0.95	0.98	0.97
	B	1.00	1.04	1.10	1.04			0.99	1.02	0.99	1.05
II	A	-	-	0.96	1.06	1.03	1.01	0.95	0.99	0.98	1.00
	B			0.97	1.00	1.00	0.98	0.97	1.01	0.95	1.11

Description of Table #1 - Fate of Parental ^{32}P-Labeled Strands Inside Bacteria and Progeny.

Intracellular DNA was extracted at the indicated times, and banded in CsCl. Parental ^{32}P labeled DNA, which banded between ^{3}H light and heavy references, was isolated. Progeny phage were obtained at 120 min. after infection, from suspensions not treated with CM. Those treated with CM were washed free of the antibiotic at 30 min., and incubated further for 90 min. Lysis was performed in the presence of indole, and progeny phage purified in CsCl. (Table #2 provides plating data and parent to progeny transfer values for this particular experiment.) Both intracellular and progeny DNA was supplemented with ^{3}H DNA extracted from mature phage,* sonicated, denatured and transferred to scintillation vials containing three nitrocellulose filters: (1) charged with left strand DNA (L); (2) charged with right strand DNA (R); (3) not charged with DNA. Annealing was performed at 65° for 12 hrs. The counts recovered on filter #3 were subtracted from the counts detected on the remaining filters. Part of the sample was also transferred onto a fiberglass filter, serving for measuring the input. The data are expressed in two ways:

$$A = \frac{\text{reference label } \frac{\text{L-cpm}}{\text{R-cpm}}}{\text{test label } \frac{\text{L-cpm}}{\text{R-cpm}}} \qquad B = \frac{\frac{\text{Input reference label}}{\text{Input test label}}}{\frac{\Sigma\ \text{L+R reference label}}{\Sigma\ \text{L+R test label}}}$$

In the case of equal annealing to both left and right strands by the tested DNA, the figures for both A and B should be equal to 1.0.

*Due to the significant variation in the absorbing capabilities of charged nitrocellulose filters, the use of an internal reference DNA is a necessity for obtaining meaningful results.

A control experiment in which radioactive, L and R strands derived from mature phage were preparatively separated and then annealed to nitrocellulose filters, charged with L or R strands, revealed, on the average, a 5x bias in annealing between strands of opposite sides.

I - Intracellular, Replicative DNA

II - Progeny Phage DNA

CM was added at 0, 5, or 10 minutes after infection. As demonstrated in a previous paper (Kozinski, 1968) CM added between 3-5 minutes to E.coli infected with the ligase-negative (L-) mutant, preserves the integrity of parental DNA

and allows production of large progeny DNA. Moreover, after withdrawal of CM, each of the infected bacteria produces viable phages (a CM rescue phenomenon--Fed. Proc., 1969, and in preparation for press). In this experiment, progeny phage were obtained from bacteria incubated in the presence of CM up to 30 minutes and rendered free of the antibiotic by washing. Phage were purified in CsCl, their DNA extracted, supplemented with reference DNA and used for annealing, the results of which are shown in Table #1.

The outcome of the biological part of this experiment is summarized in Table #2. Note that although the number of infective centers detected, phage produced, parent to progeny transfer and even integrity of transferred DNA (not documented here) differs greatly, depending on the time of addition of CM, there is no bias in replication or transfer to the progeny of the parental strands.

Table #2

	No CM		CM 0-30		CM 5-30		CM 10-30	
Phage	L-	L+	L-	L+	L-	L+	L-	L+
IC/ml.	2.0×10^6	3.2×10^8	3.0×10^5	3.5×10^8	2.8×10^8	3.1×10^8	1.0×10^7	2.8×10^8
Ø/IC	2.00	180.00	1.05	150.00	19.00	165.00	5.00	207.00
% Transf.	0.30	55.00	0.25	40.00	20.00	47.00	5.00	45.00

Description of Table #2 - "CM Rescue" and Parent to Progeny Transfer in L- or L+ Phage.

All platings were done on Cr63. Infective centers (IC) for the samples not treated with CM were plated at 5 min. after infection; those treated with CM were plated at 30 min. after infection. The number of infected bacteria was 3×10^8. The average burst size (Ø/IC) was estimated at 120 min. after infection. In the case of the CM treated bacteria, this included 30 min. incubation in CM. Lysis was performed in the presence of indole. The % of parent to progeny transfer (% Transf.) was estimated on the level of purified progeny phage considering as 100% the number of counts adsorbed at 5 min. after infection. Note that CM added at 5 min. rescues the infective centers of the non-permissive host, and allows up to 20 viable phage to be produced from each infected bacteria. In the case of the wild phage, depending on the addition or no addition of CM, there is no difference in the physico-chemical properties of progeny phage or their DNA. In the case of the ligase-negative mutant however, phage produced from bacteria, not treated with CM, band in CsCl very near to the top of the tube, contains very short fragments of DNA, as revealed from a sucrose gradient, and are dead. The same applies to phage obtained from bacteria treated with CM at the interval between 0-30 or 10-30 min. after infection. Phage produced from bacteria treated with CM between 5-30 min. on the contrary, coband with reference phage in CsCl contains mostly integral DNA (as determined from sucrose gradients), and have a sizable proportion of viable phage. (Thorough physico-chemical characteristics of phage and DNA produced in a "CM rescue" system will be the topic of a separate paper.)

The transfer of DNA from each of the parental strands to progeny phage confirms the data of Carlson (1968) for T_4 and T_7 phages.

B. Newly Synthesized Progeny DNA was labeled either with ^3H thymidine or with ^3H-5BU. Replicative moieties were isolated from CsCl, supplemented with reference ^{32}P DNA and used for annealing. In the density labeling experiment the replicative moiety (^3H-5BU) banded between the light and heavy locations. An exception was the short labeling period between 4 and 4:30 minutes where the ^3H label was incorporated into molecules which essentially retains its parental density having initiated replication and cobanding with the parental density reference. (See Figure #1 for more detailed description.)

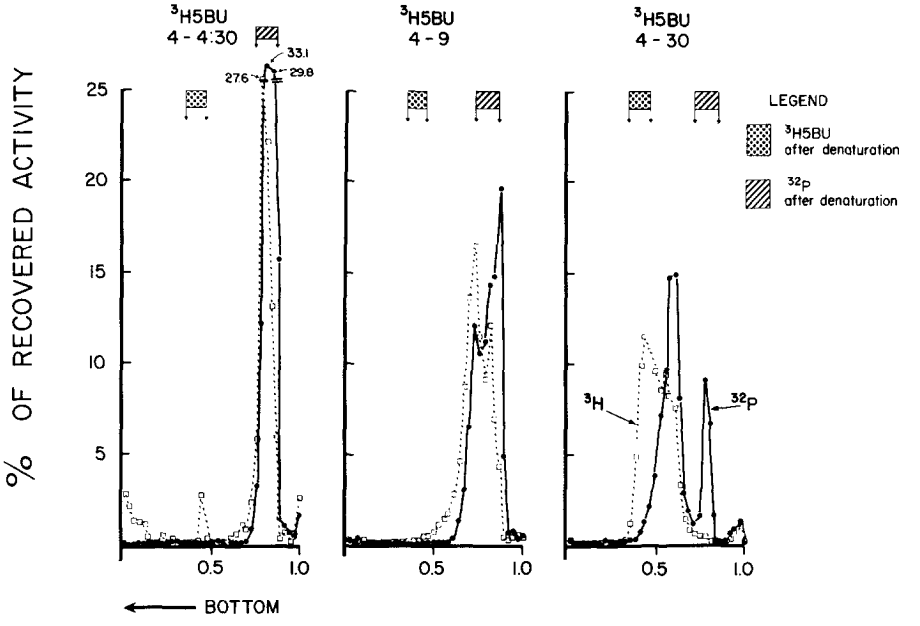

Figure 1. - CsCl Gradient Analysis of Parental (^{32}P Labeled Light) and Progeny (3H 5-BU Labeled) DNA at Variable Times after Infection.

E.coli B23 were infected with a multiplicity of 7 of parental phage, T4 AM H39X, and CM was added at 4 minutes after infection together with 3H: 5-BU, and 5µg/ml FUDR. Samples were taken at 4:30 minutes, 9 minutes, and 30 minutes. The results of analysis shown in this figure were obtained without the addition of non-replicative references. The same samples were also analysed in a system where a significant excess of reference, 3H labeled, light DNA was added. This proved that the peak of ^{32}P located at the light end of the gradient coincided with pure light DNA. After denaturation there is a complete separation of 3H and ^{32}P labeled moieties (progeny from parental).

3H bands within the shaded area of the heavy side while the ^{32}P bands within the shaded area on the light side. Again the addition of a surplus of light 3H reference proved that the parental label at any time after infection remains covalently unassociated with the progeny. (The addition of CM at 4 minutes prevents molecular recombinations--Kozinski, 1968.)

Alkaline sucrose gradient analysis demonstrated that the heavy fragments of DNA, extracted at 4:30, band close to the top of the gradient, at the location of a fractional molecular size smaller than 0.1 phage equivalent units. At 9 and 30 minutes progeny moieties assume a position of integral DNA and fragments of an approximate molecular weight of 10-15 deltons.

This was due to the short size of the heavy fragment and its very small proportional (less than 10%) density contribution. After denaturation the 3H label assumed a heavy location in the gradient. This verifies the lack of covalent attachment of progeny to parental strands at the initiation of replication (Kozinski, Kozinski, and James, 1967). The results of this group of experiments is summarized in Table #3.

Table #3

	No CM Added					CM at 4 min.				CM at 10 min.	
Label (min.)	3-3:15	3-30		3-60		4-4:30		4-30		10-30	
Phage	L+	L-	L+	L-	L+	L-	L+	L-	L+	L-	L+
I A	-	0.98	1.10	1.07	0.91	0.99	-	1.00	1.02	0.94	1.01
I B	-	1.03	1.05	1.01	0.93	1.02	-	1.03	0.99	0.95	1.03
II A	1.00	0.91	0.93	1.06	0.97	1.05	1.09	0.92	1.03	1.11	0.99
II B	1.01	1.04	0.97	0.96	1.04	1.05	1.02	1.00	1.01	0.98	0.95

Description of Table #3 - Nature of Newly Synthesized Progeny Strands: Annealing Experiment.

Labeling of the progeny strands was performed at the time intervals specified in the row labeled "min.". At the end of the indicated time the bacterial suspension was sampled. DNA extracted and banded in CsCl and after extensive dialysis used for annealing in a manner identical to that described in Table #1. The reference used in this case was ^{32}P phage DNA. The data are expressed in an identical manner to Table #1. Group I - the outcome of the experiment where 3H-5BU was added at the intervals indicated to heavy bacteria infected with light parental phage (moi = 7). Group II - the experiment where 3H-TDR was used to label progeny strands; no density label was used during the experiment. In both cases the 3H labeled moieties were isolated from CsCl.

Analysis in alkaline sucrose gradient revealed that DNA labeled during the interval of 4-4:30, both wild and in L- phage, was composed of a fragment of approximately 1/20 phage equivalent units. Those labeled between 4-30 min. were virtually integral in both cases. Otherwise all newly synthesized DNA for L- phage shown in this Table was small, banding close to the top of the tube.

Similarly to the parental label, the progeny label without exception reveals no bias in annealing to left or right T_4 strands.

The obtained results should be of value while considering any hypothesis precluding assymetric replication of T_4 DNA.

The characteristics of the progeny phages produced with or without CM rescue in the non-permissive host will be the topic of a separate paper.

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References

Carlson, K. 1968. Intracellular Fate of Deoxyribonucleic Acid from T7 Bacteriophage. J. of Virol. 2: 1230-1233.

Denhardt, D. 1966. A Membrane-Filter Technique for the Detection of Complementary DNA. Biochem. Biophys. Res. Commun. 23: 641-646.

Fareed, G. C., and C. C. Richardson. 1967. Enzymatic Breakage and Joining of Deoxyribonucleic Acid. II. The Structural Gene for Polynucleotide Ligase in Bacteriophage T4. Proc. Natl. Acad. Sci. U.S. 58: 665-672.

Guha, A., and W. Szybalski. 1968. Fractionation of the Complementary Strands of Coli Phage T4 DNA Based on the Asymmetric Distribution of the Poly U and Poly UG Binding Sites. Virol. 34: 608-616.

Kozinski, A. W. 1968. Molecular Recombination in the Ligase Negative T4 Amber Mutant. Cold Spring Harbor Symposium on Quantitative Biology, in press.

Kozinski, A. W., P. B. Kozinski, and R. James. 1967. Molecular Recombination in T4 Bacteriophage Deoxyribonucleic Acid. I. Tertiary Structure of Early Replicative and Recombining DNA. J. of Virol. 1: 758-770.