*A PREFERRED ORIGIN FOR THE REPLICATION OF LAMBDA DNA**

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It has been suggested that replication of DNA in general should start at genetically specific molecular sites.[1] Unique origins of replication exist in *Bacillus subtilis*[2] and *Escherichia coli*.[3]

In the present communication, I report preliminary results concerning the origin of replication in the DNA of phage lambda.

Lambda DNA extracted from phage particles has regions of dissimilar nucleotide composition.[4] When sheared to small fragments and banded in a density gradient of Cs_2SO_4-$HgCl_2$, the DNA is resolved into two major peaks.[5] The denser fragments, rich in adenine and thymine (AT), come from a continuous right-hand section of the molecule representing 56 per cent of its length. The less dense fragments, rich in guanine and cytosine (GC), originate from the remaining left-hand section. A given sample of lambda DNA subjected to analysis of the sort indicated may be characterized by the ratio

$$R = \frac{\text{DNA content of denser band}}{\text{DNA content of less-dense band}}$$

which measures 1.27 for DNA extracted from phage particles.

If DNA synthesis starts in the AT-rich section, replicating DNA should give a ratio R that exceeds 1.27 by an amount related to, among other things, the distance between the point of origin and the boundary between sections. However, if replication starts at random sites, R should be the same for replicating and nonreplicating DNA. The experimental approach suggested by these considerations is similar in principle to the genetic method used by Sueoka and Yoshikawa[2] to locate the origin of replication in *B. subtilis*.

The following experiments showed that lambda DNA synthesized during the first few minutes after infection (when it is reasonable to assume that most of the DNA is replicating) contains an excess of AT-rich sections.

Materials and Methods.—Bacterial and phage strains: A clear-plaque (c_I) mutant of lambda was used to infect *E. coli* HF4704 T^-, Hcr^- (provided by Dr. R. L. Sinsheimer). DNA synthesis is arrested in this host by treatment with mitomycin C and restored by infection with phage ϕX174[6] or lambda.[7]

Culture medium: Experimental cultures were grown in a medium containing, per liter, 0.05 mole of tris(hydroxymethyl)aminomethane (Trizma-Base, Sigma Chemical Co.); 1.0 ml of 0.66 *M* KH_2PO_4; 1.0 ml of 1.0 *M* $MgSO_4$; 1.0 ml of 1.0% $CaCl_2$; 0.1 ml of 0.5% $FeSO \cdot 6H_2O$; 0.8 gm NH_4Cl; 0.2 gm Na_2SO_4; 1.0 gm KCl; 10 ml of 40% glycerol; and 20 ml of 10% "vitamin-free" casein hydrolysate (Nutritional Biochemicals Corp.). The pH of the medium was adjusted with HCl to 7.4. Thymidine was added as required.

Experimental cultures: Bacterial cultures were grown with aeration at 37°C in the specified medium supplemented with 10 μg/ml of thymidine. When they reached 2×10^8 cells/ml, they were treated with 50 μg/ml of mitomycin C for 10 min in the dark without aeration. Then the cells were collected on a membrane filter (B-4, Schleicher & Schuell Co.), washed with medium devoid of thymidine, and resuspended in culture medium

without thymidine (2×10^9 cells/ml). They were infected by addition of 6–7 phage particles per cell and incubation at 37°C for 15 min in the dark without aeration. Then the infected cells were diluted fivefold with medium containing 4 μg/ml of H^3-thymidine (specific activity 3 μc/μg), aerated, and grown at 37°C in the dark. Under these conditions, 91% of the phage particles were adsorbed and 1.4 phage progeny were produced, as assessed by plaque titration of culture samples treated with $CHCl_3$ at 50 min. The poor phage yield of mitomycin C-treated cells is unexplained, but is deemed irrelevant to the results presented.

Extraction of DNA: Samples taken from the culture at various times "after infection" (literally, times after dilution with radioactive medium) were placed in tubes containing KCN to make a final concentration of 0.01 *M* and cooled in an ice bath. DNA was extracted according to Smith.[8] Tris(hydroxymethyl)aminomethane and sodium ethylenediaminetetraacetate (pH 8) were added to each sample to make final concentrations of 0.1 *M* and 0.01 *M*, respectively. The suspensions were then warmed to 37°C, and lyozyme (Mann Research Laboratories) was added to make a final concentration of 0.5–1 mg/ml. After 5–10 min, sodium lauryl sarcosinate (Sarkosyl NL-97, Geigy Industrial Chemicals) was added to make a final concentration of 0.5–1%. After 5–10 min at 37°C, the suspensions were heated at 65°C for 5 min, during which time they cleared. The lysates were extracted twice with phenol at 1–5°C. Then the aqueous phase was dialyzed to remove the phenol and treated for 30 min at 37°C with pancreatic ribonuclease (10 μg/ml; Worthington Biochemical Corp.) that had been heated at 80°C for 10 min.[9] After another phenol extraction, the DNA was dialyzed against 0.01 *M* Na_2SO_4 containing 0.1% ethanol. At least 84% of the labeled DNA was recovered. The same procedure was used for extracting *E. coli* HF4704 DNA. Reference DNA was extracted as described by Frankel[10] from λc_I phage particles that had been purified by banding in CsCl.

Results and Discussion.—Under the conditions described, thymidine uptake began promptly and accelerated for at least an hour in infected cultures (Fig. 1). Little if any DNA synthesis occurred in uninfected cultures of the same sort.

Tritiated lambda DNA extracted from infected bacteria was mixed with P^{32}-labeled DNA extracted from phage particles, sheared, and banded in Cs_2SO_4-$HgCl_2$. Figure 2 shows a typical result for replicating DNA extracted at three minutes after infection. The left band, representing the AT-rich section of the molecule, contains considerably more H^3 than P^{32}. Table 1 shows ratios *R* calculated from the density distributions of replicating DNA extracted at various times after infection. DNA extracted at three or five minutes after infection shows a significant excess of AT-rich DNA fragments. This bias gradually disappears as the infection progresses, probably owing to the accumulation of nonreplicating DNA in the cells. The simultaneous measurements for P^{32}-labeled reference DNA illustrate the error of the method. I conclude that some

TABLE 1. *Density analysis of replicating lambda DNA.*

Time after infection (min)	*R*		Time after infection (min)	*R*	
	H^3	P^{32}		H^3	P^{32}
3	2.30	1.37	40	1.74	1.27
5	2.24	1.39	50	1.61	1.16
10	1.79	1.16	60	1.61	1.31
20	1.76	1.15	70	1.49	1.24
30	1.76	1.26			

H^3-labeled DNA extracted from bacteria at various times after infection was mixed with P^{32}-labeled DNA from phage particles and analyzed as shown in Fig. 2.

The ratio *R* expresses the isotope content of the AT-rich band divided by that of the GC-rich band. For nonreplicating DNA, this ratio measures about 1.63 in terms of H^3-thymine content and about 1.27 in terms of nucleotides labeled with P^{32}.

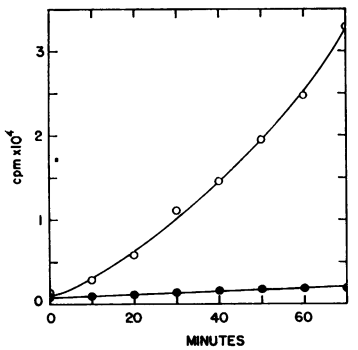

FIG. 1.—DNA synthesis in cultures treated with mitomycin C. A culture of *E. coli* HF4704 was grown and treated with mitomycin C as described in *Materials and Methods*. A portion of the culture was infected with 3.7 phage particles/cell. Both infected and uninfected samples, containing 2.5 × 10^8 cells/ml, were grown with aeration in the dark at 37°C in a medium containing 5 μg/ml of H^3-thymidine (specific activity 1 μc/μg). At various times, samples from each culture were precipitated with 5% trichloroacetic acid in the presence of excess thymidine, and precipitates were collected and washed with 5% trichloroacetic acid on membrane filters (B-6, Schleicher & Schuell Co.). Infected cells, O—O; uninfected cells, ●—●.

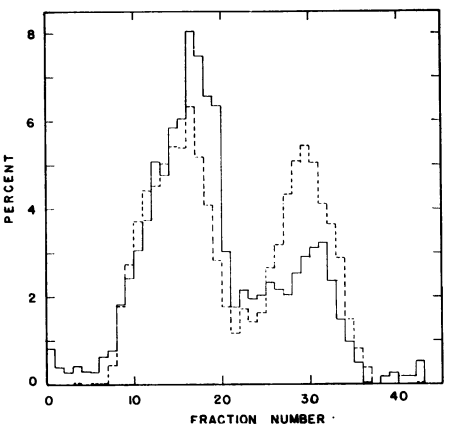

FIG. 2.—Density distribution of mercury complexes of replicating and nonreplicating lambda DNA. H^3-labeled replicating DNA extracted at 3 min after infection was mixed with P^{32}-labeled reference DNA from phage particles. The DNA in the mixture was broken to 3 × 10^6 dalton fragments, mixed with 0.24 mole of $HgCl_2$/mole nucleotide in 43% Cs_2SO_4, and centrifuged in a Spinco type-40 rotor at 36,000 rpm for 48 hr at 4°C.[5] *Solid line:* H^3-labeled DNA; *broken line:* P^{32}-labeled DNA.

or all of the AT-rich section of the molecule is replicated first during the individual replication cycle. Since lambda DNA is potentially circular,[11] replication need not start at a molecular end. My results therefore suggest one or more starting points in or near the AT-rich section of the molecule.

The coincidence of band positions of reference and replicating DNA's in

TABLE 2. *Specificity of hybridization of DNA synthesized in infected cells.*

Time after infection (min)	Per Cent Hybridized with Lambda DNA*		Per Cent Hybridized with *E. coli* DNA†	
	H^3	P^{32}	H^3	P^{32}
5	84.6	81.0	4.3	34.0
10	81.6	76.2	4.2	40.8
20	87.3	82.3	1.6	28.8
30	85.3	81.0	3.3	32.0
40	82.4	77.4	3.7	61.8
50	85.9	78.2	3.2	39.2
60	85.6	78.7	—	—
70	82.6	76.4	—	—

* H^3-labeled replicating lambda DNA extracted at the times indicated was mixed with P^{32}-labeled lambda DNA from phage particles. The mixtures were treated sonically, heated at 100°C for 5 min, cooled quickly, and incubated with unlabeled lambda DNA filters (5 μg/filter) according to Denhardt.[12]

† H^3-labeled replicating lambda DNA was mixed with P^{32}-labeled DNA extracted from *E. coli* HF4704, treated as in asterisk footnote, and added to unlabeled *E. coli* HF4704 DNA filters (100 μg/filter).

Figure 2 makes it unlikely that bacterial DNA is labeled under the conditions of these experiments. This possibility was excluded more directly by the following method. First, tritiated lambda DNA extracted from infected cells at various times was mixed with P^{32}-labeled lambda DNA from phage particles and tested for ability to hybridize with lambda DNA. As shown in Table 2, equal fractions of both kinds of DNA were bound. Second, H^3-labeled replicating lambda DNA was mixed with P^{32}-labeled *E. coli* DNA and tested for ability to hybridize with *E. coli* DNA. This time, P^{32}-labeled DNA hybridized much more efficiently than H^3-labeled DNA (Table 2). These results show that the amount of labeled *E. coli* DNA in the bacterial extracts is negligible.

Summary.—The results presented show that replication of lambda DNA starts preferentially in or near the AT-rich section of the molecule.

Note added in proof: Properties of bacteria lysogenic for phage $\phi 80$ or a λ-$\phi 80$ hybrid, modified by deletions extending for various distances into the prophage, suggest that phage replication starts in the right half of the DNA molecule (Franklin, N. C., W. F. Dove, and C. Yanofsky, *Biochem. Biophys. Res. Commun.*, **18,** 910 (1965); Gratia, J. P., *Biken's J.*, **9,** 77 (1966); Dove, W. F., in *Edmonton Symposium on Molecular Biology of Viruses* (New York: Academic Press, 1967)). Results reported here lead to the same conclusion.

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