**Direction of bacteriophage λ DNA replication in a thymine requiring *Escherichia coli* K-12 strain.
Effect of thymidine concentration**

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ABSTRACT

The direction of replication was established for the first round of bacteriophage λ DNA replication in thymine requiring *E. coli* K-12 cells exposed to different concentrations of thymidine. It was found that a dramatic decrease in the proportion of bidirectionally replicating molecules followed a decrease in the concentration of thymidine. Moreover, the rightward mode of replication appears to be exclusively favored in unidirectionally replicating molecules found at low concentrations of thymidine.

INTRODUCTION

During our studies on the first round of λ DNA replication, we have noticed that although replication commences at a unique region of the λ genome and appears to proceed in a bidirectional fashion, a low but significant proportion of the molecules replicate unidirectionally. Moreover, the rightward mode of replication appears to be favored in all λ variants studied so far (1,2). More recent studies have confirmed these observations and shown that the bias can be reversed in a λ variant in which the origin containing region has been inserted in opposite polarity (3). Since an understanding of the way unidirectionally replicating molecules are generated may throw some light on the initiation of DNA replication, we have investigated conditions that would alter the proportion of bidirectional and unidirectional molecules.

Very recently we have reported the isolation of novel DNA structures found among λ replicating intermediates, which showed a strong bias towards unidirectional replication (4). Since these molecules were found in infected cells that had been previously starved for thymidine, we have investigated the effect of thymidine concentration on the direction of the first round of λ DNA replication. The results presented below suggest that unidirectional replication is strongly favored at low concentrations of thymidine.

MATERIALS AND METHODS

(a) Bacterial and phage strains

E. coli K-12 CR34 (thy, leu, thr) and bacteriophage λ CII₆₇CIII₆₈ were used in this study.

(b) Growth of labeled phages

Radioactive ³H-labeled phages were obtained by growing infected CR34 cells on agar plates in the presence of [methyl-³H]thymidine (Valenzuela, to be published elsewhere). Phages were purified by low and high speed centrifugation and resuspended in 0.01 M Tris, 0.01 M MgSO₄ in D₂O (pH 7.2).

(c) Growth of CR34 cells

CR34 cells were grown at 37°C in M9-maltose medium supplemented with 1% casamino acids and 5 µg/ml thymidine. At OD₅₉₀ = 0.2, cells were pelleted and washed twice in 0.02 M MgSO₄, 0.02 M Tris, pH 7.4, and resuspended into 2 ml of the same medium. Aliquots were poured into 5 ml M9-maltose medium plus casamino acids containing either 0, 0.2, 0.5 or 5 µg/ml thymidine. Cultures were incubated at 37°C and cell viability was checked at various times after transferring to new medium.

(d) Isolation of intracellular λ DNA

CR34 cells were grown at 37° in 20 ml D₂O-maltose medium (5) supplemented with yeast extract (.02%), leucine (10 µg/ml), threonine (10 µg/ml) and thymidine (5 µg/ml). At OD₅₉₀ = 0.60, cells were pelleted, washed twice with 10 ml of 0.01 M Tris, 0.01 M MgSO₄ in D₂O and resuspended in 2 ml of the same solution. After incubation at 37° for 1 hr, cells were infected with ³H-labeled λ phage at moi = 6 and adsorption allowed for 15 min at 37°. The cell suspension was then poured into warmed 20 ml D₂O-glucose medium supplemented with leucine and threonine each at 10 µg/ml and thymidine at either 5, 0.5, 0.2 or 0 µg/ml. After incubating the cells for 5-7 min at 37°, replication was stopped, cells lysed and λ DNA isolated according to a methodology described previously (6).

(e) Electron microscopy and computation of electron microscope data

λ DNA replicative intermediates were photographed at random and the position of branch points established with respect to the denaturation map by the method described by Valenzuela and Imman (6).

(f) Classification of directionality of replication

The method described by Schnös et al. (3) was followed.

RESULTS

(a) Thymidine concentration, cell viability and λ infectivity

First we checked cell growth of *E. coli* CR34, a thymine requiring mutant, that had been transferred from a minimal medium containing 5 $\mu\text{g/ml}$ thymidine to another containing 0, 0.2 or 0.5 $\mu\text{g/ml}$ thymidine. We found that although cell mass increased at all concentrations (data not shown), the number of viable cells decreased considerably after 120 min at thymidine concentrations below 0.5 $\mu\text{g/ml}$ (Fig. 1). After 60 min of transfer, cell cultures at 5, 0.5 and 0.2 $\mu\text{g/ml}$ had doubled, whereas at 120 min the cultures at 0.5 and 0.2 $\mu\text{g/ml}$ had decreased 30% and 44% with respect to the one containing 5 $\mu\text{g/ml}$. When no thymidine was added to the medium the number of viable cells did not double after

Figure 1. Growth of *E. coli* K-12 CR34 at Different Concentrations of Thymidine

Cells were grown at 37°C in M9-maltose medium supplemented with 1% casamino acids and 5 $\mu\text{g/ml}$ thymidine. At $\text{OD}_{590} = 0.2$, cells were washed and resuspended in fresh medium containing 5 (●), 0.5 (○), 0.2 (△) and 0 (□) $\mu\text{g/ml}$ thymidine. At the times indicated, the number of viable cells was determined.

transfer but were reduced to about 32% after a 120 min incubation. These results suggest that the first doubling of CR34 cells is not appreciably affected if cells are transferred to a medium containing at least 0.2 $\mu\text{g/ml}$ thymidine. With this in mind, we investigated bacteriophage λ infectivity in cells which had been grown at 5 $\mu\text{g/ml}$ thymidine, infected with phage λ and then incubated at various thymidine concentrations. We measured the amount of phage produced under these conditions and found that phage yield is about 43%, 37% and 2% for 0.5, 0.2 and 0 $\mu\text{g/ml}$ thymidine respectively, compared to the culture at 5 $\mu\text{g/ml}$.

(b) λ DNA replicative intermediates

E. coli K-12 CR34 was infected with λ cIIcIII and phage replication allowed for 5-7 min at 37°C in a D_2O -glucose medium (5) containing various concentrations of thymidine. Intracellular λ DNA was isolated from cell lysates and purified through two consecutive CsCl equilibrium centrifugations. In all samples there was a clear shift of the parental phage DNA towards the heavy side of the CsCl gradient, indicating that incorporation of heavy isotope had occurred. Fractions from the heavy side of the parental DNA band were inspected in the electron microscope. A high proportion of the circular molecules were simple replicative intermediates, either double branched (theta), or single branched (sigma) structures (1); a low, but significant (1-6%), proportion of circles contained multiply branched structures in which additional bubbles were present on theta or sigma structures. Properties of these multiply branched molecules have been reported in a recent study (4). Table 1 shows the distribution of the types of circular molecules obtained. First, it is apparent that below 0.5 $\mu\text{g/ml}$ thymidine the percentage of replicative intermediates decreases, however even when no thymidine has been added to the medium, there are still low but significant numbers of replicative intermediates present. This indicates that the amount of thymidine remaining in the cells plus that contained in the yeast extract (see Materials and Methods) is sufficient to allow at least some replication. Second, once the thymidine concentration is reduced below 5 $\mu\text{g/ml}$ the proportion of multiply branched molecules increases. From these findings, and a previous study reporting the occurrence of these structures (4), it is likely that the limited supply of thymidine is crucial in the formation of multiply branched molecules.

(c) Direction of replication of λ DNA replicative intermediates

Fractions containing replicative intermediates were prepared for

TABLE 1

Electron Microscope Analysis of Intracellular λ DNA

Thymidine Added to Medium ($\mu\text{g/ml}$)	Number of Molecules	Percent of Circular Structures			
		Circles*	Replicative Intermediates		
			Theta	Sigma	Multiply Branched Intermediates
5	170	39	35	25	1
0.5	176	35	28	31	6
0.2	172	65	15	15	5
0	173	60	25	10	5

*Includes dimers and monomeric circles containing D-loops. Both of these structures account for about 10 to 21% of all circles.

the electron microscope under conditions of high pH partial denaturation. Partially denatured molecules were photographed at random and the position of branch points determined by alignment of the denaturation patterns with that of mature λ cIIcIII DNA. If we assume that the segments occupied by the known ori mutations give a reliable location for the origin of replication, then, according to recent mapping of these positions (7), the origin is situated 13.75μ from the left end of the 17.06μ λ genome. Assuming that our error in aligning denaturation sites is $\pm 0.25 \mu$ (3), then the region 13.5 – 14.0μ can be used as a gate to classify molecules as either unidirectional or bidirectional. Namely, a molecule with a replicating region spanning the gate was defined as bidirectional. If one branch point fell within the gate, the molecule was scored as unidirectional to the right if the other branch point was to the right of the gate and unidirectional to the left if it was to the left. Molecules with replicated regions that did not span the gate region were rejected. This classification is illustrated in Fig. 2. Here, 38 molecules from an experiment in which no thymidine was added to the medium were photographed at random and positions of branch points in each molecule assigned with the aid of the known denaturation map of mature λ DNA. Replicating segments appear as boxes and the vertical dotted lines crossing all molecules indicate the gate region mentioned above. In this collection, molecules 2–21 are defined as unidirectional to the

Figure 2. Diagrammatic Representation of the Position of Branch Points in Replicative Intermediates Isolated from Infected Cells With No Added Thymidine Present in the Medium

Circular λ DNA maps were aligned at the mature ends with the aid of the known denaturation pattern of this DNA. They are represented here in a linear fashion for display purposes only. Boxes represent those segments of the λ DNA that have replicated. In the cases where broken boxes are shown, the free end of a tail segment has been positioned on the λ genome using the best alignment of denatured sites present in both the parental DNA and the tail segment. Broken vertical lines indicate the position of the origin region of λ DNA replication ($13.75 \pm 0.25 \mu$). Molecules were sorted according to the position of their left branch points in order to emphasize the predominant unidirectional type of replication in this collection of molecules. Denatured sites and single stranded regions have been omitted for clarity. Molecules have been normalized so that the circular part of each structure is 17.06μ .

right, molecules 22-38 are bidirectional and molecule 1 is a reject, since its replicated region is outside the gate. When this classification is applied to the collection of replicative intermediates from all our experiments, we find that as the thymidine concentration decreases, the proportion of unidirectional types increases from 12% to 52% (Table 2). The bias favoring unidirectional replication to the right also increases as the thymidine concentration is reduced. At 10 $\mu\text{g/ml}$ thymidine this bias is 2:1 whereas below 0.2 $\mu\text{g/ml}$ thymidine it is complete. The bias observed is not due to the activation of a different origin of replication at lower concentrations of thymidine because the number of rejections found in all experiments (Table 2, last column) falls within the range found in earlier studies of five other λ derivatives (3), implying that unidirectional molecules are generated at the normal origin region of λ DNA replication. Moreover double regression plots of bidirectional molecules show that the extrapolated origin sites lie at $13.5 \pm .9$, 14.5 ± 1.0 , $13.9 \pm .5$, and $13.6 \pm .6 \mu$ for 5, 0.5, 0.2, and 0 $\mu\text{g/ml}$ thymidine respectively, and fall within the range of $13.75 \pm 0.25 \mu$, our best estimate of the origin of λ DNA replication. If bidirectional

TABLE 2

Mode of Replication of λ DNA Replicative Intermediates

Thymidine Added to Medium ($\mu\text{g/ml}$)	Number of Molecules	Mode of Replication (in Percent)			
		Bidirectional	Unidirectional		Rejects
			To Right	To Left	
10 ^a	67	80	8	4	8 ^b
5	44	77	18	5	0
0.5	45	60	38	2	0
0.2	55	53	38	0	9 ^c
0	38	45	52	0	3 ^c

^aData of Schnöls and Inman (1970).

^bOf these 8 rejects, 5 are located to the right of gate, 3 to left.

^cAll of these rejects would be classified as unidirectional to the right if the gate width was enlarged from $\pm 0.25 \mu$ to $\pm 0.50 \mu$ (i.e. twice the estimated error of our alignment procedure).

replicators also showed a similar bias, then rightward growing points should move further from the origin than their leftward counterparts. To this end we calculated the average positions of left and right branch points (when replication had advanced 59% of the λ genome) by making use of double regression plots of our experimental data. Table 3 shows that the ratio of the average distance of right and left branch points from the origin is appreciably higher only at 0 $\mu\text{g/ml}$ thymidine (2.7) compared to that at 10 $\mu\text{g/ml}$ thymidine (1.3). The large uncertainties attached to the ratios (R/L) shown in the table, reflect the large random deviations from perfectly symmetrical bidirectional growing point movement that has previously been documented (1-3).

DISCUSSION

The first round of bacteriophage λ DNA replication occurs predominantly in a bidirectional fashion. However a fraction of the replicative intermediates are unidirectional and in these replication proceeds either to the right or to the left of the origin (1,2). More recently it has

TABLE 3
Bias in Bidirectionally Replicating λ DNA Molecules

Concentration of Thymidine	Number of Molecules	Average Distance of Branch Points from the Origin at 59% Replication (μ) ^a		
		Right (R)	Left (L)	R/L ^b
10	54	5.62	4.38	1.3 (1.0-1.6)
5	34	6.60	3.40	1.9 (1.2-3.3)
0.5	27	4.97	5.01	1.0 (0.7-1.4)
0.2	29	5.57	4.43	1.3 (1.0-1.5)
0	17	7.29	2.71	2.7 (1.9-4.1)

^aRegression lines were used to estimate, at 95% confidence, the best value for the position of left and right branch points when the separation between them was 10 μ .

^bThe numbers in parentheses represent extreme values (if, at the 95% confidence level, the position of the left branch point was between L_{low} and L_{high} and the right branch point between R_{low} and R_{high} , then the extreme value was calculated as $R_{\text{low}}/L_{\text{high}}$ and $R_{\text{high}}/L_{\text{low}}$).

been established that unidirectionality to the right of the origin is preferred amongst the latter type of replicative intermediates (3). In the present investigation we have shown that this bias is significantly increased at low thymidine concentrations. Under this condition the number of bidirectionally replicating molecules decreases and is replaced by molecules which replicate unidirectionally to the right of the origin. One simple view of initiation of bidirectional DNA replication is to assume the existence of two distinct primer sites on opposite strands of the DNA (at or near the origin region) which will ensure the formation of two replication complexes at each side of the origin and thus lead to a bidirectional mode of DNA replication (8). Unidirectional replication would occur if either the formation of one of the replication complexes was impaired or its progression halted. A bias in growing point propagation in a bidirectional replicator would also result if both complexes were not equivalent. The more efficient complex might start earlier, move more quickly or halt less often. According to this model a more efficient rightward moving replication complex could account for the observations reported above, if this difference in efficiency was exaggerated by a low concentration of thymidine. It is unclear, at present, how thymidine concentration could exert such an effect.

Our results are supported by similar findings made by Edlund *et al.* (9) on *E. coli* DNA replication. These investigators, using the same *E. coli* strain as in our studies, found that changing the thymine concentration from 5 $\mu\text{g/ml}$ to 0.5 $\mu\text{g/ml}$ caused a shift in the mode of replication from bidirectional to unidirectional. Furthermore, in a study of plasmid RF100.1, under conditions in which thymine was added after a long thymine starvation period, replicative intermediates showed a unidirectional mode of replication (10) whereas bidirectional replication had been proposed for this plasmid when grown in the absence of such a thymine starvation step (11). It should be stressed that in our system, even when no thymidine is added to the medium, a significant proportion of all replicating intermediates appear to replicate bidirectionally (Table 2). Although there is a large change in the mode of replication such that unidirectional replication to the right is favored, this change is not complete. It is also interesting to note that, at low concentrations of thymidine, multiply branched replicative structures can be isolated (4). It is possible that both the change in directionality and occurrence of multiple branches may be a consequence of the same

factor that affects λ DNA replication when the concentration of available thymidine is reduced. The possibility that SOS induced functions (12) may be playing a role in this process is currently under investigation.

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