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Evidence That Both Growing DNA Chains at a Replication Fork Are Synthesized Discontinuously†

Rolf Sternglanz,* Helen F. Wang, and James J. Donegan

ABSTRACT: *Escherichia coli, Bacillus subtilis,* and T7-infected *E. coli* have been labeled with short pulses of [^{3}H]thymidine, and the labeled DNA has been examined by sedimentation in alkaline sucrose. In all three systems, the great majority of the DNA labeled by a short pulse is found in the form of small DNA chains of 10S, the so-called Okazaki pieces. The *B. subtilis* and T7 nascent DNA fragments hybridize with equal efficiency to the separated strands of *B. subtilis* and T7 DNA, respectively. The results suggest that both growing DNA chains at a given replication fork are synthesized discontinuously in the case of *E. coli, B. subtilis,* and T7. We have found that the method used to terminate the pulse affects the size distribution of the labeled DNA; some methods allow joining of nascent DNA fragments after termination of the pulse. Previous reports of discontinuous DNA synthesis on only one growing DNA chain and continuous synthesis on the other DNA chain are probably due to preferential joining of Okazaki pieces on the DNA chain growing in the overall 5′ → 3′ direction.

The well-known work of Okazaki and co-workers has shown that short pulses of [^{3}H]thymidine are incorporated into small DNA chains, the so-called Okazaki pieces, in a variety of bacterial and bacteriophage systems (Okazaki et al., 1968a,b). Pulse-chase experiments have demonstrated that the Okazaki pieces are the precursors of long DNA chains; the latter are indistinguishable in length from the bulk of the DNA. Thus the idea has arisen that one or both of the two growing DNA strands at a replication fork are synthesized discontinuously, and later joined together by DNA ligase.

The question of whether one or both growing DNA chains at a given replication fork are synthesized discontinuously is still not settled. Okazaki et al. (1968a,b) reported that label from a very short pulse appears almost exclusively in small pieces in *E. coli* and T4, implying that both strands are made discontinuously. In three bacteriophage systems, T4, λ, and SPP1, Okazaki pieces hybridize equally to both of the separated DNA strands (Sugimoto et al., 1969; Ginsberg and Hurwitz, 1970; Polsinelli et al., 1969). However, since DNA is now known to replicate bidirectionally in many systems, hybridization data alone cannot be definitive in settling the question.

There have been reports in the case of *E. coli* (Iyer and Lark, 1970; Louarn and Bird, 1974) and *B. subtilis* (Okazaki et al., 1970; Kainuma and Okazaki, 1970) that approximately half the label in a short pulse is found in large DNA chains. DNA hybridization studies as well as other methods have led these workers to conclude that only one DNA strand at a replication fork is synthesized discontinuously in the form of Okazaki pieces, while the other strand is synthesized continuously.

In this paper we report on pulse-labeling studies of *E. coli, B. subtilis,* and T7-infected cells. In all cases, the great majority of the DNA labeled by a short pulse under our conditions is in the form of small DNA fragments, i.e., Okazaki pieces. We have found that the method used to terminate the pulse affects the size distribution of labeled DNA and that some methods allow joining of nascent DNA fragments after termination of the pulse. Previous reports of discontinuous DNA synthesis on only one DNA chain and continuous synthesis on the other DNA chain are probably due to preferential joining of Okazaki pieces on the DNA chain growing in the overall 5′ → 3′ direction.

Materials and Methods

Bacterial Strains. E. coli strains included strain B, 15 TAU *thy*$^-$ *arg*$^-$ *ura*$^-$, and LC 434 F$^-$ *thy*$^-$ *leu*$^-$ B_1^- λ λ*imm 434* (Louarn and Bird, 1974). The *B. subtilis* strain was 168 *thy*$^-$ *trp*$^-$ (Wilson et al., 1966).

Growth, Pulse Labeling, and Lysis of E. coli. Cells were grown in M9 medium supplemented with 0.4% glucose and 0.1% casamino acids. The medium for *E. coli* 15 TAU was supplemented with 2 μg/ml thymine, 20 μg/ml uracil, 50 μg/ml arginine, and 80 μg/ml tryptophan. In some cases [^{14}C]thymine was added to the medium to uniformly label the DNA (0.05 μCi/ml final concentration). The medium for *E. coli* LC 434 was supplemented with 2 μg/ml thymine, 25 μg/ml leucine, and 1 μg/ml thiamine. Cells were grown at 37 °C to 1.5 × 10^8 cells/ml, switched to 25 °C, and grown to 2.5 × 10^8 cells/ml, diluted 1:1 with fresh medium and again grown at 25 °C to 2.5 × 10^8 cells/ml. Five-milliliter aliquots of the culture were pulse labeled at 25 °C by the addition of 50 μl of 1 mCi/ml [^{3}H]thymidine (7 Ci/mmol). Pulses were terminated with 20 ml of −10 °C acetone. The cells were pelleted,

† From the Department of Biochemistry, State University of New York, Stony Brook, New York 11794. *Received August 18, 1975.* Supported by grants from the National Institutes of Health (AI 11519) and the American Cancer Society (NP 142). Part of this work was presented at the ICN-UCLA Symposium on DNA Replication, Squaw Valley, California, March 1975.

washed in 6 ml of cold 0.02 M KCN, 0.02 M EDTA, pH 7.5, and re-pelleted. They were then resuspended in 0.1 ml of 0.02 M EDTA, pH 7.5, and lysed by the addition of 0.5 ml of 0.3 M NaOH, 0.02 M EDTA, and 50 μl of 10% sarkosyl (Geigy Industrial Chemicals). The lysates were placed in boiling water for 2 min and cooled quickly in ice-water.

Growth, Pulse Labeling, and Lysis of B. subtilis. B. subtilis 168 *thy*$^-$ *trp*$^-$ was grown in a minimal medium described previously (Wang and Sternglanz, 1974). In some cases, [^{14}C]thymine was added to the medium to uniformly label the DNA (0.05 μCi/ml final concentration). Cells were grown at 37 °C to 1.5 × 10^8 cells/ml, switched to 25 °C, and grown to 2 × 10^8 cells/ml, diluted 1:1 with fresh medium and grown at 25 °C to 3 × 10^8 cells/ml. Ten-milliliter portions of the culture were pulse labeled at 25 °C by the addition of 0.1 ml of 1 mCi/ml [^{3}H]thymidine (7 Ci/mmol). Pulses were terminated with 40 ml of −10 °C acetone. The cells were pelleted, washed in 12 ml of cold 0.02 M KCN, 0.02 M EDTA, pH 7.5, and re-pelleted. They were then resuspended in 0.1 ml of 0.02 M EDTA, 20% sucrose. Ten microliters of 1 mg/ml lysozyme was added and the mixture incubated at 37 °C for 10 min. The cells were lysed by the addition of alkali and sarkosyl exactly as described above for *E. coli*.

Growth, Pulse Labeling, and Lysis of T7-Infected Cells. E. coli B was grown as described above. Five-milliliter portions of the culture at 2.5 × 10^8 cells/ml were infected at 25 °C with T7 at moi[1] = 5. Twenty minutes after infection the cells were pulse labeled for 7 s and lysed exactly as described above for un-infected *E. coli*.

Alkaline Sucrose Gradient Sedimentation. The lysates described above were layered on top of linear 5–20% (w/v) alkaline sucrose gradients containing 0.3 M NaOH, 0.5 M NaCl, 0.01 M EDTA. At the bottom of each sucrose gradient (10.8 ml) was a 1-ml CsCl shelf (ρ = 1.74 g/cm^3; 5.8 g of CsCl dissolved in 4.2 ml of 0.3 M NaOH, 0.5 M NaCl, 0.01 M EDTA). Sedimentation was in a Beckman L2-65B ultracentrifuge for 16 h at 28 000 rpm using an SW41 rotor and cellulose nitrate tubes. Centrifuge tubes were fractionated from the top and the acid-precipitable radioactivity in each fraction determined as described previously (Wang and Sternglanz, 1974), except that 17 equal fractions were collected from each tube. Also, the bottom of each tube was washed with 1 ml of 0.3 M NaOH, 0.5 M NaCl, 0.01 M EDTA, and then TCA-precipitated. This was called the 18th fraction of each gradient.

Some gradients contained ^{32}P-labeled denatured DNA sedimentation markers. SV40 DNA converted to the linear form by the Eco R1 restriction endonuclease (Mulder and Delius, 1972), a gift of C. Mulder, and T7 DNA, a gift of F. W. Studier, have sedimentation coefficients in alkali of 16S and 37S, respectively.

DNA Hybridization. The procedure for DNA hybridization was essentially as described by Denhardt (1966). T7 DNA *l* and *r* strands were separated according to Summers and Szybalski (1968). The *B. subtilis* DNA separated strands were a gift of R. Rudner (Rudner et al., 1968).

Results

Pulse Labeling of E. coli. In order to re-examine the question of whether one or both growing DNA strands at a replication fork are synthesized discontinuously in bacteria, we wanted a DNA pulse-labeling procedure designed to minimize nuclease or ligase action after termination of the pulse. Also, it seemed desirable to label cells at the same temperature as had been used for growth of the cells, rather than to shift the temperature just before the pulse. Our procedure is summarized below; details are given in Materials and Methods. Cells are grown exponentially at 37 °C, diluted with fresh medium, and then grown at 25 °C for about two generations before pulse labeling with [^{3}H]thymidine. The pulse is terminated with −10 °C acetone, and the cells are washed in 0.02 M KCN, 0.02 M EDTA, and finally lysed with alkali and sarkosyl. The lysates are immediately sedimented through alkaline sucrose gradients.

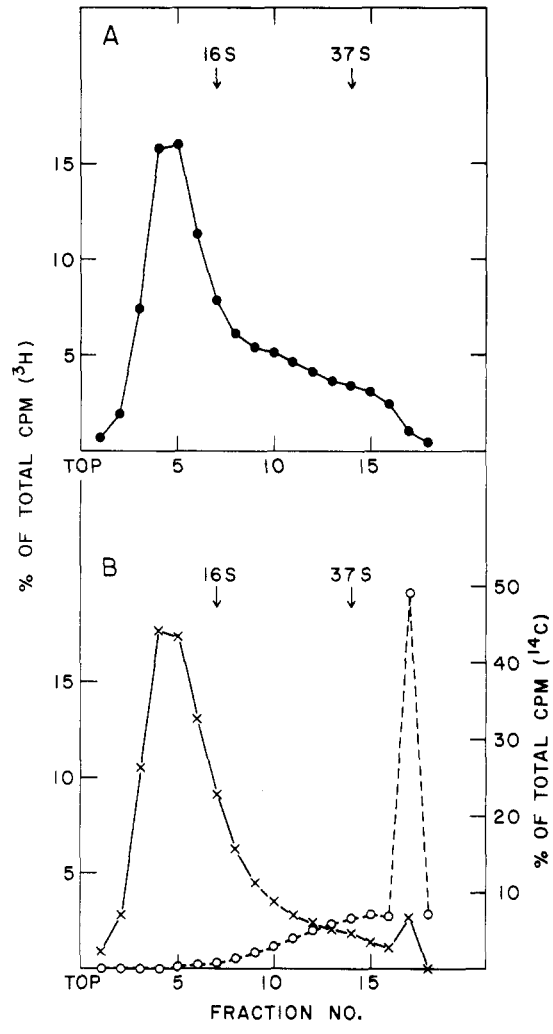

FIGURE 1: Alkaline sucrose gradient sedimentation of pulse-labeled DNA from *E. coli* B and *E. coli* 15 TAU. Cells were pulse-labeled with [^{3}H]thymidine at 25 °C and the pulse terminated with −10 °C acetone as described in Materials and Methods. (A) *E. coli* B, 15-s pulse (●—●), 7693 total cpm. (B) *E. coli* 15 TAU, 13-s pulse, ^{3}H (X—X), 12 678 total cpm; ^{14}C (O- - -O), 11 478 total cpm.

Figure 1 presents the results of pulse labeling two *E. coli* strains, B and 15 TAU, for 15 and 13 s, respectively, using the procedure described above. It can be seen that the great majority of the pulse-labeled DNA sediments at the standard position for Okazaki pieces, about 10S. Specifically, the fraction of the total radioactivity in Okazaki pieces (<20S, in fractions 2–8) is 66% for *E. coli* B and 75% for *E. coli* 15 TAU. For both strains, the fraction of the total radioactivity in large DNA (>40S, at the shelf in fractions 16–18) is only 4%. In the experiment using strain 15 TAU (Figure 1B), the DNA was uniformly labeled with [^{14}C]thymine. It can be seen that most of the uniformly labeled DNA is at the shelf and virtually none is at the position of Okazaki pieces. This shows that our lysis

[1] Abbreviation used: moi, multiplicity of infection.

Table I: Pulse Labeling of *Escherichia coli* LC 434 under Various Conditions.[a]

Expt	Growth Conditions[b]	Stop Solution	Pulse Time (s)	Total cpm	% of Total cpm in Fractions 2-8 (Okazaki Pieces)	% of Total cpm in Fractions 16-18 (Large DNA)
1	Medium A, 25 °C	Acetone	15	5 108	71	8
	Medium A, 25 °C	Pyridine	15	2 931	66	14
2	Medium A, 25 °C	Acetone[c]	15	5 343	67	10
	Medium A, 25 °C	Pyridine	15	5 870	59	20
3	Medium B′, 37→20 °C	Acetone	8	486	79	5
	Medium B′, 37→20 °C	Pyridine	8	274	80	16
4	Medium A, 37→20 °C	Acetone	12	1 449	70	15
	Medium A, 37→20 °C	Pyridine	12	1 484	64	19
	Medium A, 37→20 °C	Pyridine	30	18 275	50	27
	Medium B, 37→20 °C	Acetone	12	1 629	68	12
	Medium B, 37→20 °C	Pyridine[d]	12	1 012	65	20
	Medium B, 37→20 °C	Pyridine[e]	30	10 084	50	24

[a] Cells were grown, pulse labeled, and lysed as summarized in the table, and described in Materials and Methods. The lysates were sedimented through alkaline sucrose gradients, and the fraction of the total radioactivity in Okazaki pieces and in large DNA determined. See Figures 1 and 2 for typical sucrose gradients. [b] Medium A is M9 supplemented with 2 μg/ml thymine and 0.1% casamino acids. Medium B′ is supplemented with 4 μg/ml thymine and 0.1% casamino acids. Medium B is supplemented with 4 μg/ml thymine and 0.5% casamino acids (Louarn and Bird, 1974). In all cases, 25 μg/ml leucine and 1 μg/ml thiamine were also present. [c] Figure 2A. [d] Figure 2B. [e] Figure 2C.

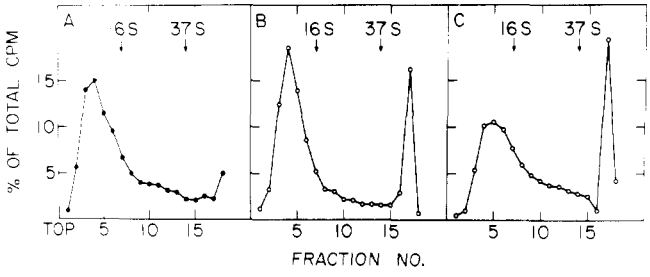

FIGURE 2: Alkaline sucrose gradient sedimentation of pulse-labeled DNA from *E. coli* LC 434. (A) Cells pulse labeled for 15 s at 25 °C and the pulse terminated with −10 °C acetone as described in Materials and Methods, 5343 total cpm. (B and C) Cells grown at 37 °C, pulse-labeled at 20 °C, and the pulse terminated with 10% pyridine, 1 mM KCN as described by Louarn and Bird (1974). (B) 12-s pulse, 1012 total cpm. (C) 30-s pulse, 10 084 total cpm.

procedure does not fragment the DNA. The results presented in Figure 1 are in agreement with Okazaki's work on *E. coli* (Okazaki et al., 1968a,b) and clearly suggest that both strands are synthesized discontinuously at a given replication fork.

On the other hand, a recent paper by Louarn and Bird (1974) concluded that *E. coli* DNA is synthesized discontinuously on only one strand, at least for *pol* A^+ strains. We have examined pulse-labeled DNA from the strain used by Louarn and Bird, *E. coli* K12, strain LC 434 (a gift of R. Bird). Figure 2A shows the labeling pattern for LC 434 pulsed for 15 s under our usual conditions. The result is similar to that seen for *E. coli* B and *E. coli* 15 TAU; 67% of the radioactivity is in Okazaki pieces (fractions 2-8) and only 10% is in large DNA (fractions 16-18). Louarn and Bird reported a different labeling pattern for strain LC 434. They found that a large fraction of the DNA labeled during a 30-s pulse at 20 °C sedimented as intermediate size and large DNA; even with a 5-s pulse at 20 °C at least 40% of the DNA sedimented with $S > 15$ in their hands. Their procedure differed from ours in three major ways: (1) their supplemented M9 growth medium was slightly richer than ours (generation time was 30 min rather than 35 min at 37 °C); (2) they grew the cells at 37 °C, shifted them to 20 °C for 15 min, and then pulsed at 20 °C; and (3) they terminated pulses with 10% pyridine, 1 mM KCN, and washed the cells in the same solution.

When we use the procedure of Louarn and Bird to label strain LC 434 we obtain results similar to theirs. Figures 2B and 2C show 12- and 30-s pulses for strain LC 434 labeled using their conditions. A significant amount of radioactivity is seen in large DNA (fractions 16–18) for both pulses. In order to determine whether the growth medium, the temperature, or the solution used to terminate the pulse (hereafter called the stop solution) was causing the difference in the labeling pattern (contrast Figure 2A with Figures 2B and 2C), we systematically varied the labeling procedure. Table I summarizes the results of four experiments. The quantities to be compared are the percent of the total radioactivity found in Okazaki pieces (<20S) and in large DNA (>40S) under different conditions. Table I clearly shows that the stop solution is an important variable. Although the fraction of labeled DNA found in Okazaki pieces does not depend on the stop solution, the fraction of the pulse-labeled DNA sedimenting to the shelf is always greater in pulses terminated with pyridine than in comparable pulses terminated with acetone. On the other hand, the growth medium does not seem to be a significant variable (experiment 4, Table I; compare medium A with medium B). Comparison of experiments 1 and 2 (cells grown and pulsed at 25 °C) with experiments 3 and 4 (cells grown at 37 °C and pulsed at 20 °C) suggests that shifting the temperature from 37 to 20 °C before pulse labeling also leads to a somewhat greater fraction of large DNA at the shelf.

In conclusion, the data of Figure 2 and Table I show that strain LC 434 gives results similar to those with other *E. coli* strains when pulse-labeled under our conditions; most of the radioactivity is found in Okazaki pieces (<20S). As argued in detail in the Discussion, we believe that the acetone stop solution and the KCN–EDTA washing of the cells inhibit joining of nascent DNA fragments after termination of the pulse. In contrast, pyridine and KCN do allow some joining to occur.

Pulse Labeling of Bacillus subtilis and Hybridization of

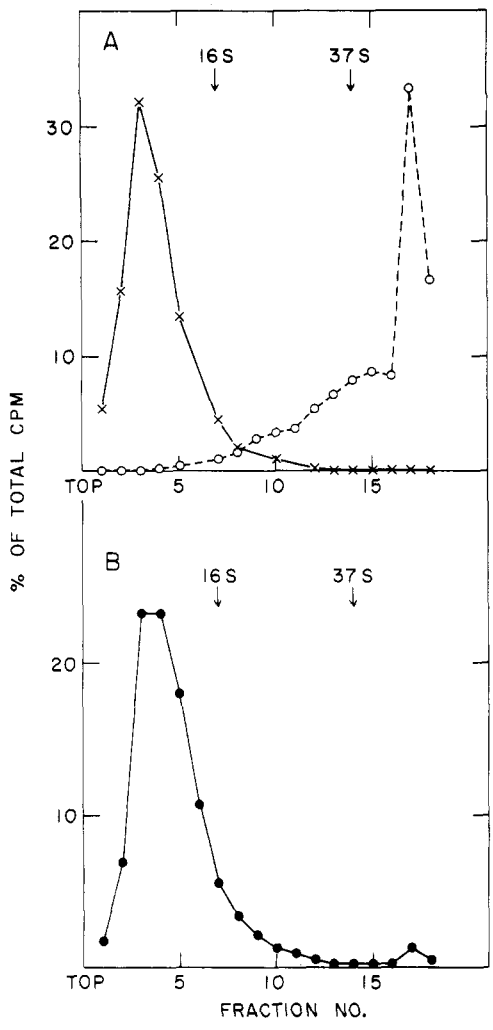

FIGURE 3: Alkaline sucrose gradient sedimentation of pulse-labeled DNA from *B. subtilis* 168 *thy*$^-$ *trp*$^-$. Cells were pulse labeled at 25 °C and the pulse terminated with −10 °C acetone as described in Materials and Methods. (A) 8-s pulse, ^{3}H (X—X), 1655 total cpm; ^{14}C (O- - -O), 8748 total cpm. (B) 15-s pulse (●—●), 6980 total cpm.

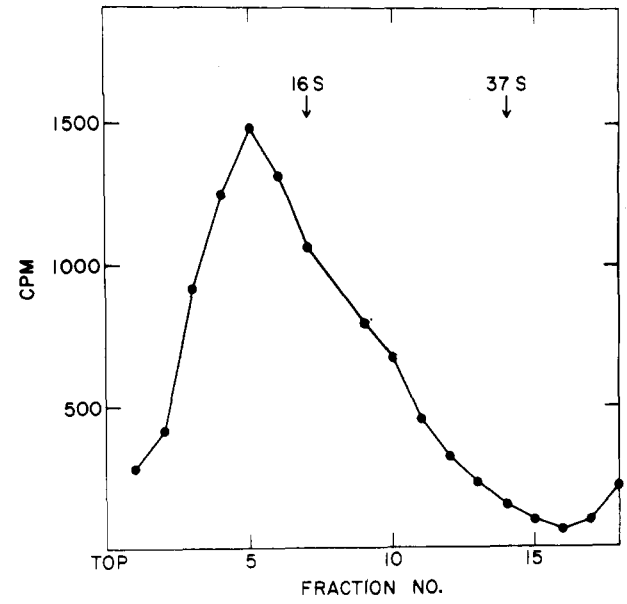

FIGURE 4: Alkaline sucrose gradient sedimentation of pulse-labeled DNA from T7-infected *E. coli* B. Cells were grown at 25 °C to 2.5 × 10^8 cells/ml, infected with T7 at moi = 5, and incubation continued at 25 °C. Twenty minutes after infection the cells were pulse labeled for 7 s, the pulse was terminated with −10 °C acetone, and the cells were lysed as described in Materials and Methods. Six individual sucrose gradients, each containing the lysate from 5 ml of T7-infected cells, were fractionated into one set of tubes. An aliquot of each tube was precipitated with 15% trichloroacetic acid to determine the radioactivity. The rest of the labeled DNA was used for the hybridization experiment shown in Table III (experiment 1).

Table II: Hybridization of *Bacillus subtilis* Nascent DNA with the Separated Strands of *Bacillus subtilis* DNA.[a]

Expt	% Hybridization to L Strand	% Hybridization to H Strand
1	18	18
2	29	30

[a] Both experiments involved a 15-s pulse at 25 °C. The distribution of radioactivity was very similar to that seen for the 15-s pulse in Figure 3. Fractions from the peak of radioactivity in the sucrose gradient were pooled and used for hybridization (Denhardt, 1966). Experiment 1 involved the use of 80 ml of cells concentrated to 5 ml before pulse labeling as described previously (Wang and Sternglanz, 1974). Experiment 2 used unconcentrated cells. Percent hybridization = [(cpm remaining on filter)/(cpm added to filter)] × 100%.

the Nascent DNA with Bacillus subtilis DNA Separated Strands. Okazaki and co-workers have reported that, when *B. subtilis* is labeled with short pulses of [^{3}H]thymidine, some of the radioactivity is found in 10S fragments and the rest is found in large DNA (Okazaki et al., 1970; Kainuma and Okazaki, 1970). When they hybridized the labeled DNA with the separated strands of *B. subtilis* DNA, they found that the 10S pieces hybridized preferentially to the L strand and the large DNA hybridized preferentially to the H strand. Okazaki and co-workers, therefore, concluded that *B. subtilis* DNA is replicated discontinuously off the L template strand and continuously off the H template strand.

However, in our hands the same results are obtained with *B. subtilis* as with *E. coli*. Figure 3 shows the results of 8- and 15-s pulses of *B. subtilis* labeled using the conditions described in this paper (25 °C growth and labeling, acetone stop solution, KCN-EDTA washing of the cells). It can be seen that the great majority of the radioactivity incorporated during a short pulse labels Okazaki pieces and very little radioactivity is found in large DNA. On the other hand, the majority of the uniformly labeled DNA sediments to the shelf (Figure 3A). The results suggest that both growing DNA chains at a replication fork can be replicated discontinuously in *B. subtilis*.

If both DNA chains are replicated discontinuously, then the Okazaki pieces should hybridize to both of the separated strands of *B. subtilis* DNA. Table II shows that indeed the Okazaki pieces do hybridize with equal efficiency to both the L and H strands of *B. subtilis* DNA.

Pulse Labeling of T7-Infected E. coli and Hybridization of the Nascent DNA with T7 DNA Separated Strands. When T7-infected cells are pulse labeled for 7 s at 20 min after infection, the labeling pattern depicted in Figure 4 is observed. The main peak of radioactivity again is at the position of Okazaki pieces, at about 10S, although the peak is broader than is seen in the case of uninfected *E. coli* or *B. subtilis*. Very little radioactivity is seen at fraction 14, the position of unit length T7 DNA single strands.

We have used the labeled DNA from the 7-s pulse shown

Table III: Hybridization of T7 Nascent DNA with the Separated Strands of T7 DNA.[a]

Expt	Pooled Fractions	% Hybridization to *l* strand	% Hybridization to *r* strand
1[b]	3-5	30	28
	6-8	27	22
2	2-4	31	28
	5	37	33
	6-8	28	28

[a] Both experiments involved a 7-s pulse of T7-infected cells 20 min after infection. Cells were lysed and sedimented in alkaline sucrose gradients. Figure 4 shows the distribution of radioactivity for experiment 1. A similar distribution was seen in experiment 2. In both cases, the indicated fractions from the sucrose gradients were pooled and used for hybridization. Percent hybridization = [(cpm remaining on filter)/(cpm added to filter)] × 100%. [b] Figure 4.

in Figure 4 for hybridization to the separated strands of T7 DNA. Fractions 3-5 from the slower sedimenting half of the radioactive DNA peak were pooled for hybridization studies, as were fractions 6-8 from the rapidly sedimenting half of the peak. Experiment 1 in Table III shows that there is approximately equal efficiency of hybridization to the *l* and *r* strands of T7 DNA for both pooled fractions 3-5 and pooled fractions 6-8. Table III also shows another hybridization experiment involving DNA labeled in a 7-s pulse. Radioactive DNA from a sucrose gradient similar to the one shown in Figure 4 was divided into three parts: (1) the slower sedimenting DNA (fractions 2-4); (2) the peak fraction (No. 5); and (3) the more rapidly sedimenting DNA fragments (fractions 6-8). Again the labeled DNA hybridizes equally with the *l* and *r* strands. In summary, the pulse-labeling pattern seen in Figure 4 as well as the hybridization results in Table III suggest that T7 DNA is replicated discontinuously on both growing DNA chains of a replication fork.

Discussion

We have re-examined the question of whether one or both growing DNA chains at a replication fork are synthesized discontinuously. Under our conditions, the majority of the radioactivity incorporated into DNA during a short pulse is found in Okazaki pieces. The fraction of pulse-labeled DNA in short pieces ($S < 20$) is about 70% for *E. coli* (Figures 1 and 2A) and more than 90% for *B. subtilis* (Figure 3). On the other hand, most of the uniformly labeled *E. coli* or *B. subtilis* DNA sediments to the shelf (fractions 16-18 of the sucrose gradients) under these conditions.

If one of the two growing DNA strands at a replication fork is replicated continuously, then one-half of the radioactivity incorporated into DNA during a short pulse should be in large DNA. Presumably the DNA will be as large as the bulk of the DNA. Our evidence is clearly inconsistent with such a model. Even workers who have suggested that one strand is replicated continuously never find as much as 50% of the pulse label in large DNA (Iyer and Lark, 1970; Louarn and Bird, 1974; Okazaki et al., 1970; Kainuma and Okazaki, 1970). Instead they find a substantial amount of labeled DNA sedimenting at intermediate positions, larger than Okazaki pieces but smaller than bulk DNA. Since the fraction of radioactivity in intermediate-size pieces plus that in large DNA (i.e., all the DNA larger than 20S) often approach 50%, both of these size classes have previously been attributed to the strand made continuously.

In the case of *E. coli*, we find approximately 20-30% of the labeled DNA in the intermediate size class (greater than 20S but less than 40S; fractions 9-15 of Figures 1 and 2A). This DNA could either be due to: (1) joining of 10S Okazaki pieces either before or after termination of the pulse, or (2) a rapid rate of DNA polymerization relative to initiation of Okazaki pieces on the strand growing in the overall 5′ → 3′ direction. The latter case would lead to some chains longer than a single Okazaki piece, a situation known to occur during in vitro DNA replication in the cellophane disc system (Olivera and Bonhoeffer, 1972). However, in our view this still should be considered discontinuous synthesis.

Our results show that the method of stopping the pulse and washing the cells affects the fraction of labeled DNA which is larger than Okazaki pieces ($S > 20$). Previous workers who observed about half the pulse-labeled DNA in pieces larger than 20S (and who therefore concluded that one strand is synthesized continuously) all used pyridine and/or KCN to stop the pulse and wash the cells (Iyer and Lark, 1970; Louarn and Bird, 1974; Okazaki et al., 1970; Kainuma and Okazaki, 1970). Comparison of the two methods shows that, while the fraction of labeled DNA found in Okazaki pieces is not significantly different in the two cases, the fraction of labeled DNA found at the shelf is always greater in the case of pyridine-KCN (Figure 2 and Table I). Our results suggest that the pyridine-KCN stop solution allows some joining of intermediate-size pieces (which we consider to be products of discontinuous synthesis as argued above), and that acetone and/or KCN-EDTA used in our procedure prevent much of this joining.

Louarn and Bird (1974) concluded that one strand is synthesized continuously in *E. coli*, and they ingeniously demonstrated that it is the strand growing in the overall 5′ → 3′ direction. They pulse-labeled an *E. coli* strain lysogenic for phage λ and hybridized the labeled DNA to the separated strands of λ DNA. Using a *pol A*$^+$ strain, they found that a short pulse labeled both short and large DNA fragments; the former hybridized preferentially to the *l* strand and the latter to the *r* strand of λ DNA. Knowing the orientation and position of the integrated λ DNA in the *E. coli* chromosome, they concluded that *E. coli* DNA is replicated discontinuously on the strand growing in the overall 3′ → 5′ direction and is replicated continuously on the strand growing in the overall 5′ → 3′ direction. However, in the case of a *pol A*$^-$ strain, they found mainly small DNA after a short pulse, and concluded that both strands are replicated discontinuously.

Our results (Figure 2 and Table I) using the same *pol A*$^+$ strain as Louarn and Bird used lead us to reinterpret their results. We conclude that both strands are replicated discontinuously, and that there is preferential joining of intermediate size nascent DNA fragments on the strand growing in the overall 5′ → 3′ direction under their experimental conditions. Preferential joining of nascent DNA fragments of the strand growing in the overall 5′ → 3′ direction might be expected since these pieces have a greater probability of being immediately adjacent to each other than do pieces on the 3′ → 5′ side of the fork; the latter are initially separated by a large gap.

Additional support for this point of view comes from the results of Louarn and Bird with the *pol A*$^-$ strain. Since the only known defect of *pol A1* mutants in DNA replication is in the joining of Okazaki pieces (Kuempel and Veomett, 1970),

it seems reasonable to conclude that the difference between *pol* A^+ and *pol* A^- strains found by Louarn and Bird (1974) is simply due to the difference in the ability of the two strains to join Okazaki pieces. Okazaki and co-workers have recently come to a similar conclusion in the case of phage P2 (Kainuma-Kuroda and Okazaki, 1975; Kurosawa and Okazaki, 1975).

In the case of T7, we also find that the DNA labeled during a short pulse sediments as 10S fragments, but the size distribution is broader than in the case of un-infected *E. coli* (Figure 4). T7 DNA is known to replicate in the form of unit length linear molecules. Replication is bidirectional, from an origin 17% from the left end of the molecule (Dressler et al., 1972). Concatemers are formed later in infection (Schlegel and Thomas, 1972) but there is no direct evidence that concatemers themselves are replicative forms. If only unit length molecules replicate and if the origin is 17% from the left end, then if one strand is replicated continuously we would expect an asymmetry in the hybridization of the labeled DNA to the separated *l* and *r* strands. Knowing the polarity of the strands relative to the genetic left end of the molecule (Summers and Szybalski, 1968), we would expect the smaller labeled DNA to hybridize preferentially to the *l* strand and the larger labeled DNA to hybridize preferentially to the *r* strand. Table III shows that all size classes hybridize with equal efficiency to both the *l* and *r* strands. Thus, the data of Figure 4 and Table III are consistent with discontinuous replication of both strands of T7 DNA.

We therefore conclude that both growing DNA strands at a replication fork are replicated discontinuously in the case of *E. coli*, *B. subtilis*, and T7. Our conclusion is based on two assumptions: (1) [^{3}H]thymidine labels both growing DNA chains with approximately equal efficiency; and (2) there is no unusual fragility of the newly replicated DNA.

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