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DNA Replication in *Escherichia coli*: Location of Recently Incorporated Thymidine Withi Molecules of High Molecular Weight DNA

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Abstract. More than 50% of the [^{3}H]thymidine incorporated during short pulses into the DNA of *Escherichia coli* 15T$^-$ can be extracted by alkali as high molecular weight DNA. Density gradient centrifugation and digestion with exonuclease I suggest that these large pieces of DNA are composed of newly synthesized DNA attached to pre-existing material at the 3′ end of the molecule.

In vivo, the circular chromosome of *Escherichia coli* is replicated sequentially in one direction.[1] As a result, the two newly formed nascent polynucleotide chains must be extended by addition at the 3′ position of the deoxyribose of one chain and at the 5′ position on the other. All the DNA polymerases have been shown to have an absolute requirement for adding nucleotides to a free 3′-OH group of a pre-existing deoxypolynucleotide primer.[2,3]

The contradiction between the *in vitro* and the *in vivo* replication of DNA has yet to be resolved. One solution involves the *in vivo* synthesis of DNA by polymerase as short pieces which are subsequently joined to a pre-existing deoxypolynucleotide, presumably by the enzyme polynucleotide ligase. The existence of newly synthesized DNA as short pieces has been demonstrated in a wide variety of biological systems[4,5] and several replication mechanisms have been proposed utilizing such a form of replication.[4,6,7] In most of these, replication is envisioned as extending one deoxypolynucleotide chain continuously in a 5′ to 3′ direction while synthesizing the opposite strand via the formation of short pieces and their subsequent joining together. Okazaki and co-workers[8] recently concluded that short pieces are synthesized which are complementary to *both* strands of T_4 DNA and that such pieces are synthesized in a 5′ to 3′ direction. However, the T_4 system is known to require a special polymerase[9] which is necessary for DNA replication, and replication occurs by a multi-fork mechanism[10] different from that observed in bacteria.

For these reasons, we have carried out similar experiments on replication in *E. coli* 15T$^-$. Previous work in this laboratory[11] led to the following observations. (*a*) Newly formed DNA can be extracted by alkali as small (1 micron) and large (3–300 microns) single strand pieces of DNA. (*b*) At any instant, there are about five short pieces per chromosome in cultures in which the chromosome is replicated in 40 min, and about three in cultures in which it is replicated in 80 min. (*c*) The time required to synthesize a short piece, about 2 sec at 37°C. is the same for rapid and slow rates of growth.

Materials and Methods. The growth of the quadruple auxotroph *E. coli* 15T⁻ (555-7) in thymine-, arginine-, methionine-, and tryptophan-supplemented glucose-M9 or aspartate-M9 media has been described in detail recently.[11] The rate of *in vivo* DNA replication and of generating low molecular weight "Okazaki pieces" was studied and the following techniques were described: continuous and pulse labeling of cells with [2-^{14}C]thymine and [^{3}H-methyl]thymidine (New England Nuclear) respectively; extraction of DNA from such radioactive cells with 0.1 N NaOH in 0.01 M EDTA (in the present experiments more than 80% of the acid-insoluble radioactivity was extracted from the cells and recovered from the sucrose gradients); CsCl density centrifugation of DNA; and velocity centrifugation of DNA through alkaline sucrose.

After velocity sedimentation, fractions of about 0.35 ml were collected from two tubes and combined, and 0.1 ml aliquots from each were precipitated with cold 10% trichloroacetic acid for radioactive counting (11). The remaining fractions were used as substrates for exonuclease I digestion.

^{14}C-labeled λ DNA was purified[12] and the vacant regions opposite each of the single-stranded 5′ ends were filled in by an *in vitro* DNA polymerase-mediated reaction using unlabeled deoxytriphosphates and [^{3}H]dTTP.[13] The intactness of these DNA molecules was monitored by velocity sedimentation.

E. coli exonuclease I (fraction VI) was purified by Lehman's procedure.[14] No endonuclease was detected by assay of infectivity of φX-174 DNA incubated with it.[15]

In all experiments, the concentration of exonuclease I had previously been determined to saturate the substrate. The reaction mixture has been described (14). For the bacterial DNA substrate, 13.4 mM $MgCl_2$ was used—twice the amount used in our experiments with λ DNA. The λ DNA was heat-denatured just before use. The bacterial DNA was dialyzed for 10 hr at 2°C against 3.0 liters of glycine buffer, pH 9.4, 0.001 M in EDTA. Subsequent digestion was at 30°C. In all experiments described, activity was assayed as the amount of ^{3}H or ^{14}C precipitable on membrane filters by 10% trichloroacetic acid after exonuclease digestion. In one experiment not presented, the degree of hydrolysis was measured as the amount of label both soluble and insoluble in cold trichloroacetic acid. Essentially similar rates of digestion were obtained.

Results. Fig. 1 presents the sedimentation profiles of alkali-extracted DNA. Glucose cultures of 15T⁻ labeled with [^{14}C]thymine at 37°C were placed at 20°C and pulsed with [^{3}H]thymidine for different lengths of time (pulses of 0.1 and 0.5 min are shown). The DNA was extracted with 0.1 N NaOH and sedimented through alkaline sucrose gradients. There is a peak of low molecular weight [^{3}H]DNA (meniscus to arrow in Fig. 1) as well as larger material which is heterogeneous with respect to molecular weight. In all our experiments, the amount of high molecular weight ^{3}H-labeled material has been greater than 50%, ranging from 53% in the shortest pulse (0.1 min at 20°C) to higher values with longer pulses (see legend to Fig. 4).

We have selected two fractions of the larger material for further study: fractions 12–14 and 20–22. The latter sediments at a rate corresponding to the sedimentation of the bacteriophage λ genome and represents DNA 15-20 microns long. In enzymatic studies this material may be compared directly to λ DNA, and such material will sediment again with the same velocity. The larger material is often fragmented during collection of fractions, but the DNA of these fractions is still considerably larger than λ DNA. This heavier material is included for qualitative comparison.

Fig. 1*C* presents the sedimentation profile of DNA from cells grown for 20 min at 37°C in a mixture of 5-bromouracil (BrUra) and [^{14}C]thymine and then placed at 20°C and pulsed for 0.5 min with [^{3}H]thymidine. 15T⁻ DNA con-

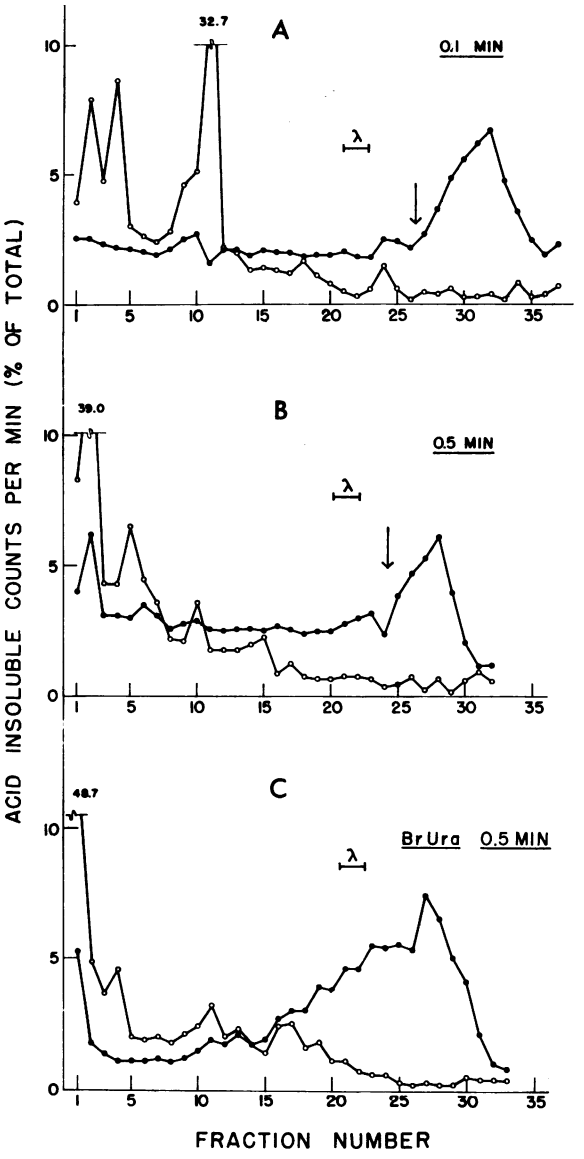

FIG. 1. Velocity sedimentation of pulse-labeled 15T⁻DNA through alkaline sucrose gradients (meniscus at right).

(*A*) 100 ml of a 15T⁻ culture was grown in glucose M9 at 37°C in [^{14}C]thymine (0.2 μCi/2 μg per ml) to a titer of 1.5 × 10^8 cells/ml. The culture was then placed at 20°C and 15 min later pulse-labeled with [^{3}H]thymidine (7 μCi/0.085 μg per ml) for 0.1 min. The DNA was extracted with alkali, layered on two sucrose gradients, and centrifuged through alkaline sucrose at 38,000 rpm for 2.5 hr in a Spinco L2-50 centrifuge, SW 41 rotor. Equal-volume fractions from both gradients were collected into one set of tubes and 0.1 ml of each fraction was assayed for radioactivity. The slight loss of resolution is the result of averaging the combined fractions from the two gradients. The percentage of total counts found in each fraction is shown: 100% = 13,650 cpm of ^{14}C; 29,900 cpm of ^{3}H. O, ^{14}C; ●, ^{3}H. The sedimentation position of λ DNA in this system is shown. Arrow indicates upper limit of sedimentation of low molecular weight fraction. (*B*) As in (*A*) except that [^{3}H]thymidine was incorporated for 0.5 min at 20°C. 100% radioactivity = 8,100 cpm ^{14}C; 64,000 cpm ^{3}H.

(*C*) 150 ml of a 15T⁻ culture was grown in glucose M9 medium at 37°C to a titer of 1.5 × 10^8 cells/ml. The culture was then transferred to 150 ml of medium containing a mixture of 5 μg/ml 5-bromouracil and 0.15 μCi/0.35 μg per ml [^{14}C]thymine in place of thymine. (Thymine was thus replaced by a 14:1 mixture of dBrUra–thymine.) After 20 min growth at 37°C the culture was placed at 20°C and, after 20 min, pulse-labeled for 0.5 min with [^{3}H]thymidine (6 μCi/0.073 μg/ml. Thereafter DNA was extracted and centrifuged as in *A* and *B* above. 100% radioactivity = 63,000 cpm ^{14}C; 360,000 cpm ^{3}H.

taining BrUra contains infrequent single strand breaks (C. Lark, unpublished data), and this leads to a greater fragmentation of the large molecular weight DNA than is observed with DNA from cultures grown in thymine. However, the results are similar to those in Fig. 1*A* and 1*B*.

We have examined the DNA from fractions 11–14 and 19–22 of Fig. 1*C* by sedimenting them to equilibrium in CsCl as follows. Each fraction was dialyzed to remove sucrose and divided into two portions. One was sonicated to break the DNA into small pieces the other was not. Both were centrifuged to equilibrium in CsCl. The results in Fig. 2 show that without sonication, the [^{3}H]-DNA sediments with a density approaching that of BrUra DNA. After sonication, however, the [^{3}H]DNA bands in a broad peak some 27 fractions lighter than the [^{14}C]BrUra DNA. We conclude that the [^{3}H]thymidine is incorporated into a homogeneous region (or a few such regions) attached to the BrUra-containing portions of the molecule. At least half of the [^{3}H]DNA present in the unsonicated preparation from fractions 19–22 bands at a density 7 fractions lighter than the BrUra DNA. Comparing this density difference with the minimum difference in density between the sonicated [^{14}C] and [^{3}H]DNA's we can conclude that a maximum of 7/27 (25%) of the unsonicated molecule is composed of regions of light [^{3}H]DNA. DNA the size of the λ genome will have a length between 15 and 20 microns and 25% of this length will correspond to between 4 and 5 microns of ^{3}H-labeled DNA. In a glucose culture, the entire chromosome (ca. 1200 microns) is replicated in 150 min at 20°C (J. Urban and K. G. Lark, unpublished data). We would therefore expect 1/300 of the chromosome (4

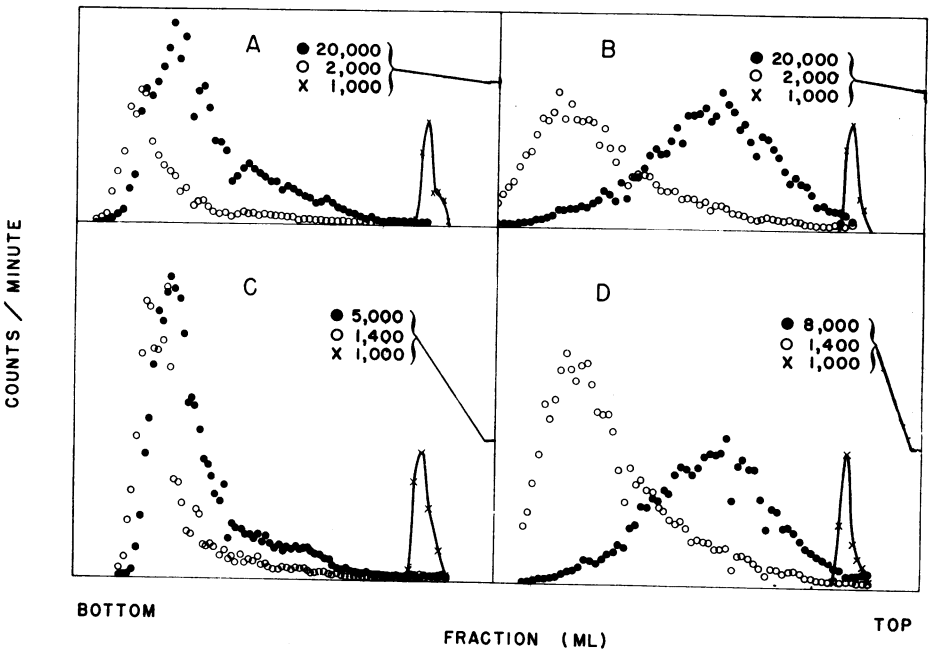

FIG. 2. Density distribution of ^{3}H- and ^{14}C-labeled DNA from the experiment in Fig. 1*C*. The fractions used were: (*A*) Fractions 19–22 (Fig. 1*C*), pooled to give a sample of DNA the size of the λ genome. (*B*) Half of the sample in (*A*), sonicated for 2 min at maximum power in a Raytheon Sonicator. (*C*) Fractions 11–14 (Fig. 1*C*), pooled to give a sample of DNA of higher molecular weight than the λ genome. (*D*) Half of the sample in *C*, sonicated as in (*B*).

Fractions from the velocity sedimentation in Fig. 1*C* were dialyzed overnight against 0.01 *M* Tris–0.001 M EDTA pH 8.5. After additon of [^{32}P]T_4-DNA as a marker, CsCl was added and the samples were centrifuged to equilibrium (40 hr at 40,000 rpm). ●, ^{3}H; ○, ^{14}C; ×, ^{32}P.

microns) of double-stranded DNA to be replicated in 0.5 min. Thus the density of the λ-size, ^{3}H-labeled, pieces indicate that they can contain the entire length of DNA that we expect to be synthesized during the 0.5-min pulse. The density shift of the unsonicated larger pieces (fractions 11–14) is much less, in agreement with their greater size.

We have examined the location within large pieces of the ^{3}H-labeled DNA. We digested pulse-labeled DNA with exonuclease I, which hydrolyzes single-stranded DNA specifically from the 3′ end.[14] (Activity at the 5′ end is less than 1/10,000 of that at the 3′ end (I. R. Lehman, personal communication). As a standard for comparison, we used [^{14}C]λDNA in which the 3′ ends were extended by a limited polymerase reaction incorporating [^{3}H]TTP opposite the cohesive 5′ ends (13). After denaturation this yielded a single strand of [^{14}C]DNA about 18 microns long, in which 0.1% of the molecule was labeled with ^{3}H at the 3′ end. Digestion of these molecules with exonuclease I is shown in Figs. 3 and 4. Fig. 3 shows that 80% of the ^{3}H-labeled λ DNA is rendered soluble before 20% of the total molecule ([^{14}C]DNA) is digested. This result serves as our standard for the expected digestion of a molecule in which all of the [^{3}H]DNA is located at the 3′ end. We have digested DNA pieces from

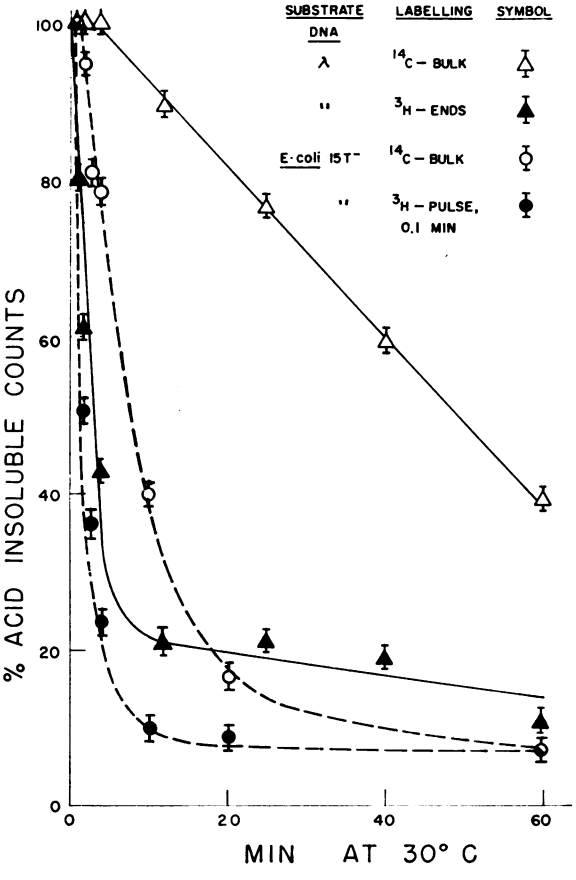

FIG. 3. Hydrolysis of λ DNA and ^{3}H-pulse-labeled DNA by exonuclease I. λ DNA uniformly labeled with ^{14}C and labeled with ^{3}H at the 3′ end (see *Materials and Methods*) was treated with exonuclease I. 0.1-ml samples were withdrawn at intervals and added to cold trichloroacetic acid. The initial counts per 0.1 ml aliquot were: ^{3}H, 2923 cpm; ^{14}C, 1578 cpm. Fractions 21–24 from the experiment in Fig. 1*A* were pooled and treated with exonuclease I. The initial counts per 0.1 ml aliquot were: ^{3}H, 184 ± 5.4 cpm; ^{14}C, 48 ± 3.3 cpm. Vertical bars denote ±SD.

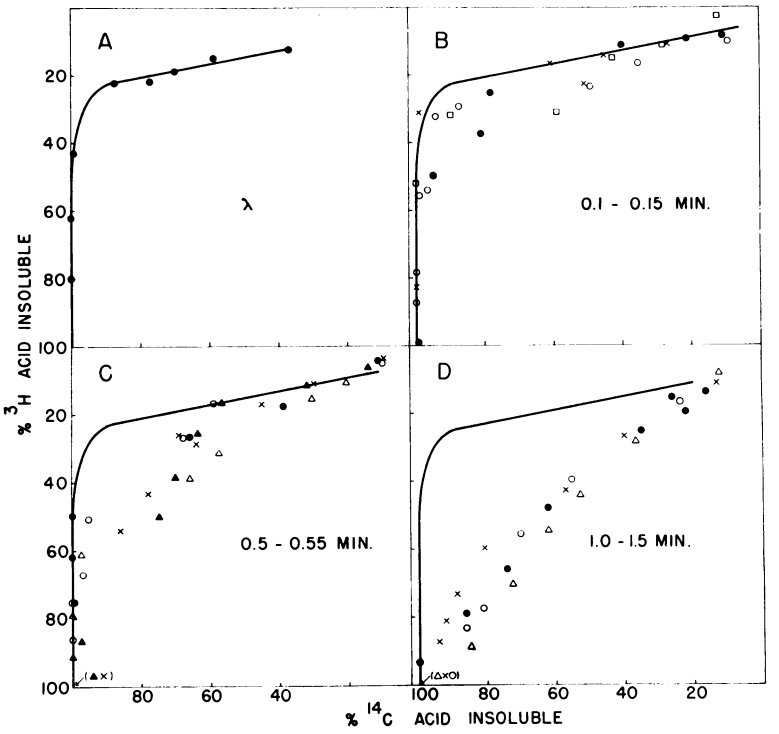

FIG. 4. Comparative hydrolysis by exonuclease I of ^{3}H-pulse-labeled and ^{14}C-prelabeled 15T$^-$ DNA.

The increasing hydrolysis of ^{3}H and ^{14}C DNA is measured as the decrease in acid-insoluble material. The hydrolysis of λ DNA in Fig. 3 is shown in (*A*). The solid lines in *B*, *C*, and *D* are reproductions of the λ-DNA curve. The DNA samples used were:

Sample	Pulse time (min) and medium	% High M.W. DNA	Approximate sed. rate relative to λ DNA	Initial cpm/0.1 ml aliquot ^{3}H	^{14}C
B ●	0.10, Glucose	53.1	1	184 ± 5.4	48 ± 3.3
B □	0.10, Glucose	53.1	1.8	261 ± 7.8	134 ± 4
B ○	0.15, Glucose	67.0	1	227 ± 12	300 ± 15
B ×	0.10, Aspartate	---	1	166 ± 8.3	199 ± 10
C ▲	0.5, Glucose	69.1	1	663 ± 13.2	38 ± 2.6
C ●	0.5, Glucose	69.1	1.8	623 ± 12.4	93 ± 4.6
C △	0.55, Glucose	63.4	1	2668 ± 53	941 ± 2.8
C ○	0.55, Glucose	63.4	1.8	642 ± 19.2	610 ± 18.3
C ×	0.5, Aspartate	---	1	856 ± 17	1650 ± 33
D ●	1.0, Glucose	73.4	1	2333 ± 23	47 ± 3.3
D ○	1.0, Glucose	73.4	1.8	1203 ± 36	446 ± 22
D ×	1.0, Aspartate	---	1	1590 ± 32	850 ± 25
D △	1.5, Glucose	74.1	1	14,742 ± 100	1157 ± 35

pulse-labeled cells with exonuclease I and compared the results to the hydrolysis of λ DNA. Cells growing at 37°C in glucose M9 or aspartate M9 media were labeled with [^{14}C]thymine and then pulse-labeled with [^{3}H]thymidine at 20°C for 0.1, 0.15, 0.5, 0.55, 1.0, or 1.5 min. Comparison of the relative ^{3}H and ^{14}C activities after pulses of different length indicated that our shortest pulse

(0.1 min) incorporated 1/10 of the activity incorporated by a 1-min pulse and that all of the pulses were within ±20% of the expected activity, assuming a linear rate of incorporation. (Thus, the 0.15-min pulse contained an amount of ^3H appropriate to an 0.18-min pulse and the 0.55-min pulse more closely resembled an 0.4-min pulse.)

DNA was extracted with alkali and centrifuged through alkaline sucrose as in the experiment in Fig. 1. Fractions corresponding to molecules the size of λ DNA or to larger molecules were dialyzed against exonuclease I assay buffer containing 0.001 M EDTA and then digested with the enzyme in the presence of added magnesium. The results are compared in Figs. 3 and 4 with the results of digesting λ DNA. Somewhat different rates of hydrolysis were observed in different preparations and there was a slight shoulder in the curve for the time course of hydrolysis (see Fig. 3). In material from cells pulse-labeled for 0.1 or 0.15 min (Fig. 4*B*) 70% of the ^3H label was hydrolyzed before 20% of the total piece had been digested, a result approaching that observed with the λ DNA in Fig. 4*A*. Digestion was somewhat slower for cells labeled for 0.5 or 0.55 min (Fig. 4*C*), although 70% of the ^3H label had been hydrolyzed when only 40% of the total DNA was digested. Longer pulses (Fig. 4*D*) led to an almost equal rate of hydrolysis of the ^3H and ^{14}C labels. In samples from cells labeled for 0.5 or 0.55 min, the larger pieces of DNA showed a faster solubilization of ^3H relative to ^{14}C than the smaller, λ-size pieces, a result consistent with the prediction that a smaller proportion of the molecule should be labeled with ^3H.

Discussion. Our data indicate that short pulses of [^3H]thymidine incorporated into the DNA of *E. coli* 15T$^-$ can be extracted by alkali in the form of both high and low molecular weight DNA, as previously shown with *E. coli* CR 34.[16] This high molecular weight material accounts for more than half of the extractable radioactivity. As observed,[11,16] such molecules are generally smaller than molecules of prelabeled [^{14}C]DNA. We suggest that this is the result of a higher probability of shear degradation when extracting and handling DNA in the vicinity of the replication fork.[17] Such large [^3H]DNA molecules are composed of newly synthesized DNA attached to previously synthesized material. After short pulses of [^3H]-thymidine equal to the synthesis of 1–5 microns of DNA, almost all the radioactivity in the larger molecular weight DNA is located at the 3′ end of the polydeoxynucleotide strand, as measured by its sensitivity to hydrolysis with exonuclease I, a sensitivity almost equal to that of the ^3H-labeled 3′ end of the λ-DNA standard, in which only 0.1% of the DNA is labeled with ^3H. We have obtained similar results with a mutant (pol A) lacking demonstrable polymerase.[18]

These results indicate a preferential location of newly synthesized DNA at the 3′ end of large deoxynucleotide strands, an asymmetry which mimics that of the *in vitro* synthesis of DNA by the Kornberg polymerase.

Radioactivity incorporated for longer periods of time does not appear to be preferentially digested by exonuclease I. This is due in part to the larger portion of the total molecule that is labeled with [^3H]thymidine. In addition, an appreciable number of ^3H-labeled small pieces must be joined to larger DNA in the period between 0.5 and 1.0 min at 20°C. This follows from the steady-state

number of small 1-micron pieces which remain unjoined at any time—five for glucose-grown cells and three for aspartate-grown cells[11]—and the amount of DNA replicated in one minute at 20°C (8 microns in glucose-grown cells, 5 microns in aspartate-grown cells). When short pieces become attached at the 5′ end of a growing deoxynucleotide chain (see below), the preferential hydrolysis of ^3H label by exonuclease I should be decreased.

However, two explanations still remain for the observed occurrence of short and long pieces: (*a*) synthesis occurs by the continuous extension of primer DNA from the 3′ end as well as by the synthesis of short pieces in a 5′ to 3′ direction[4,8] (models suggested by Okazaki and modified by Kornberg[4,6]); (*b*) synthesis occurs symmetrically by extension of deoxynucleotide strands from both the 3′ and 5′ ends, but asymmetric fragmentation also occurs such that only the 5′ strand contains single-strand breaks in the region of the replication fork. The latter possibility is rendered less likely by the finding[4,8] that small pieces of $15T^-$ and of T_4 DNA also are synthesized in a 5′ to 3′ direction.

The results described here are supported by recent studies on *B. subtilis* in which "Okazaki pieces" have been found to hybridize with only one strand of the chromosome (R. Kainuma and R. Okazaki, personal communication).

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Abbreviation: BrUra, 5-bromouracil.

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