Near-continuously synthesized leading strands in Escherichia coli are broken by ribonucleotide excision

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In vitro, purified replisomes drive model replication forks to synthesize continuous leading strands, even without ligase, supporting the semidiscontinuous model of DNA replication. However, nascent replication intermediates isolated from ligase-deficient Escherichia coli comprise only short (on average 1.2-kb) Okazaki fragments. It was long suspected that cells replicate their chromosomal DNA by the semidiscontinuous mode observed in vitro but that, in vivo, the nascent leading strand was artifactually fragmented postsynthesis by excision repair. Here, using high-resolution separation of pulse-labeled replication intermediates coupled with strand-specific hybridization, we show that excision-proficient E. coli generates leading-strand intermediates >10-fold longer than lagging-strand Okazaki fragments. Inactivation of DNA-repair activities, including ribonucleotide excision, further increased nascent leading-strand size to ∼80 kb, while lagging-strand Okazaki fragments remained unaffected. We conclude that in vivo, repriming occurs ~70x less frequently on the leading versus lagging strands, and that DNA replication in E. coli is effectively semidiscontinuous.

Significance

The antiparallel orientation of strands in a DNA duplex and unidirectional polarity of DNA polymerases necessitate that replication forks operate semidiscontinuously. That is, the strand replicates in the leading direction, the leading-strand, extends continuously, while the opposite-oriented “lagging” strand is synthesized in short “Okazaki” fragments, later joined by ligase. Semidiscontinuous replication is observed in vitro, even in reactions lacking ligase. However, in vivo, in ligase (lig) mutants, nascent DNA from both strands is found only in small pieces. Here, we report the continuous synthesis of leading strands in lig mutant Escherichia coli lacking all DNA excision-repair activities and, using an improved sucrose-gradient system, identify misincorporated ribonucleotides as the major driver of leading-strand fragmentation.

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E. coli continued to synthesize exclusively LMW RIs under maturation-deficient conditions (30, 34, 43), indicating that uracil excision was not a source of nascent leading-strand fragmentation.

That the leading strand could be synthesized by a continuous mechanism in the complete absence of ligase was amply demonstrated in various in vitro replication systems using highly purified replisomes (44–46). The resulting semidiscontinuous DNA-replication model (Fig. 1, Bottom) became a fixture of textbooks (47), even though it contradicted the bulk of in vivo results, and persisted despite our lack of understanding of the supposed fragmenting activities (22). Several attempts to identify the in vivo DNA-repair activities responsible for the putative postsynthesis fragmentation in E. coli were unsuccessful (30, 33, 34). The most recent study went so far as to inactivate all eight individual DNA glycosylases (eliminating base-excision repair), in addition to blocking nucleotide-excision repair (uvrA), mismatch removal (mutS), and alternative excision repair (nfi), yet even this dodecuple mutant [including the lig425(Ts) defect] showed no increase in RI size (31). Still, those authors were aware of the possibility that the tails of intermediate molecular weight (IMW) species could represent the leading-strand RIs (31). Besides, other work showed that only ~50% of Okazaki fragments from wild-type E. coli harbored the 5′-RNA moiety indicative of priming, suggesting that the other half was generated by excision (48).

Fig. 1. Continuously synthesized leading strand may appear discontinuous due to excision repair. (A) A scheme of fully discontinuous and semidiscontinuous replication highlighting the possible effects of excision repair. (B) Analysis by high-resolution alkaline-sucrose gradient of ligase-deficient replication intermediates reveals a substantial shoulder population of higher molecular weight intermediates. Here and henceforth, all gradients are sedimented from right (Top) to left (Bottom), with HMW species on the left, and LMW species on the right. The main panel shows the distribution of the [3H]dT pulse-labeled replication intermediates (red; at 42 °C for 2 min) or chronically labeled total chromosomal DNA (blue; at 28 °C for 35 min). (B, Bottom) The position of 32P-labeled molecular weight markers of the individual gradients (typically MWM-9, which is a 9.3-kb PCR fragment), color matched to the main panel. Specifically, for this panel, the strain is lig (GR501). The values are means ± SEM of 31 to 44 independent repetitions. Due to the significant number of repetitions, most error bars are completely masked by the symbols. (C) Same as in B, but the strain is lig ZER (LA111, the excision-minus GR501). The values are means ± SEM of four to nine independent repetitions. (D) The rnhB mutants accumulate significant density of single rNs in their DNA, as measured by supercoiled plasmid relaxation with RNase HII in vitro. RD, relaxed dimer; RM, relaxed monomer; SCD, supercoiled dimer; SCM, supercoiled monomer. Strains: WT, AB1157; rnhB, L-415. (E) Accumulation of alkaline-sensitive material in the DNA of excision-minus, rnhB, or combined mutants. Unlike in D, plasmid DNA was linearized with MluI and then treated as follows: nt, no treatment; 0°, 0.2 M NaOH, 0 °C × 5 min (background #1); f, 100% formamide, 37 °C × 5 min (background #2); 45°, 0.3 M NaOH, 45 °C × 90 min. Plasmid species: dsl, double-strand linear; ssl, single-strand linear. Strains: lig, GR501; lig ZER, LA111; lig ΔB (GR501 ΔrnhB), eGC193; lig ZER ΔB, eGC197. (F) The density of alkaline-sensitive sites was determined from several gels such as in E and is presented as the number per genome equivalent (±SEM). (G) Neutral 0.8% agarose gel separation of the chromosomal DNA prepared in agarose plugs and denatured in 0.2 M NaOH at 0 °C for 30 min to reveal any ss breaks. An inverted ethidium bromide (EtBr)-stained image is shown. As a positive control for ss-break detection, we incubated the two control lanes, marked HL, in 0.3 M NaOH at 45 °C for 90 min to quantitatively hydrolyze single DNA-rNs; this nicks on average every 10 kb of the DNA strands of the lig ΔB mutant; the lig DNA remains intact. Strains are like in E.
Two recent developments prompted our reevaluation of this conundrum. First, development of a higher-resolution AS-gradient system, buttressed by MW markers from 1 to 170 kb, revealed a distinct IMW (5- to 25-kb) shoulder adjoining the main LMW (~1.2-kb) Okazaki fragment peak formed under ligase-deficient (lig) conditions (Fig. 1B). Furthermore, the excision mutant used in previous work, deficient in all DNA-only excision-repair systems (now called lig ZER; zero excision repair), that previously produced exactly the same RI distribution as excision-proficient cells in regular AS gradients (31), now developed a clear separation of the two RI peaks in the improved AS-gradient system (Fig. 1C). Thus, this high-resolution analysis confirmed the presence of two distinct RI populations of significantly different molecular weight. Second, a new DNA contaminant was identified: Monoribonucleotides (rNs) were found to misincorporate into the chromosomal DNA of eukaryotes at high frequency (49, 50), even though in B. subtilis the density of a single DNA-rN misincorporation initially appeared qualitatively low (51). Recently, the density of single DNA-rNs in the mnhB mutant of E. coli was found to be one in 14,000 nt (52) (Fig. 1D). As DNA-rNs are efficiently excised (52), one chain break per 14,000 nt in the leading strand could produce fragments similar in size to the observed IMW fragments (Fig. 1 B and C). Unexpectedly, this significant accumulation of DNA-rNs was without any effect in E. coli, as mnhB mutants showed essentially wild-type behavior (52).

To test DNA-rNs as a potential source of RI breakage, we added an mnhB defect in ribonucleotide excision repair to our lig ZER strain (31). The resulting lig ZER ΔB mutant, in addition to the ligA251 temperature-sensitive ligase deficiency, carries 12 additional mutations completely inactivating all known excision-repair pathways in E. coli, including ribonucleotide-excision repair. By preventing the excision of all known “incorrect” bases, we hoped to increase the stability of nascent DNA and preserve the true size of RIs for examination. However, AS gradients appeared chemically inadequate for the task of separating the ribonucleotide-containing RIs from mnhB mutants, as the strongly basic pH of AS gradients, required to denature RIs from their template strands, efficiently hydrolyzes mono-rNs in DNA (52) and also destabilizes abasic sites (53). Therefore, we developed a pH-neutral denaturing gradient system to separate RIs without inducing breakage at alkaline-sensitive sites. Employing this gradient system, in concert with traditional alkaline-sucrose methods and strand-specific hybridization of DNA from gradient fractions, we demonstrate that the lig ZER ΔB mutant of E. coli replicates its leading strand by an essentially continuous mechanism.

Results

The Density of Alkaline-Sensitive Sites in Nascent DNA. Our basic protocol for RI detection follows the classic outline (15) and includes shifting exponentially growing cultures of ligA251 (Ts) mutant strains from 28 to 42 °C for 4 min to inactivate DNA ligase, followed by addition of a [3H]thymidine label, and continuous incubation at 42 °C for 2 additional minutes to label RIs. Following RI labeling, cellular metabolism is stopped instantly, the cells are lysed to release their genomic DNA, and the DNA is then purified and sedimented on alkaline-sucrose gradients. We never exceeded 6 min as the total incubation time of the ligA251 (Ts) mutant at 42 °C, avoiding chromosome fragmentation that begins after 20 min at the nonpermissive temperatures in this mutant (54) (SI Appendix, Fig. S1). The basic pH of alkaline sucrose denatures labeled RIs, freeing them from the unlabeled template strands, so they can sediment as single-stranded DNAs to be separated according to their length (molecular weight) (15). Importantly, the same alkaline pH at 0°C treatment of the chromosomal DNA of all our experimental strains [all ligA (Ts) mutants] after incubation at 42 °C for up to 25 min reveals no accumulation of single-strand breaks in bulk genomic DNA (Fig. 1G and SI Appendix, Fig. S1).

In this work, resolution of AS gradients was optimized, particularly for the IMW size range of 5 to 50 kb, by two modifications to the previous methodology: (i) The significantly reduced total applied g force maintained the majority of chromosomal DNA within the gradient instead of sedimenting it into the cushion at the bottom of the tube; and (ii) the apparatus used for the pouring and fractionation of gradients were completely reworked (Material and Methods). A 9.3-kb 32P-labeled DNA was included in every gradient, as both an MW marker and positional control (Fig. 1B and C, Bottom). In some experiments, virion DNAs from phages lambda (48.5 kb) and T4D (~170 kb) were also used as, correspondingly, IMW and HMW marker DNAs, and to build an MW standard curve (SI Appendix, Fig. S5) (55). Additionally, the MW marker DNAs served as internal controls against nonspecific DNA hydrolysis.

As stated in the Introduction, it became apparent that our previous conclusions concerning LMW RIs in the ligA251 mutant at 42 °C (30, 31) should be revisited, as the IMW shoulder in the RI distribution of the excision+ lig strain clearly shifted to higher molecular weight in the lig ZER mutant (Fig. 1B versus Fig. 1C, compare fractions 16 to 21; for quantification, see SI Appendix, Fig. SS5). Meanwhile, in vitro plasmid relaxation by RNase HIII indicated a significant density of single ribonucleotides in the DNA of mnhB mutants (Fig. 1D) (52). Since for this project it was more relevant to assess the density of alkaline-sensitive sites in DNA, we treated linearized plasmid DNA with alkali (0.3 M NaOH, 90 min at 45 °C), analyzing the intactness of the strands in neutral agarose gels (Fig. 1E). We found alkaline-induced nick densities in the lig A and lig ZER ΔB mutants of one per 8,000 to 9,000 nt, translating into 500 to 600 alkaline-sensitive sites per genome equivalent (Fig. 1E and F). The increase of alkaline-sensitive sites in the DNA of the lig ZER mutant compared with the lig mutant (both strains RnhB+)= was insignificant (Fig. 1E and F). Therefore, the density of DNA-rNs in the mnhB mutants pointed to ribonucleotides as the major genome contaminant, suggesting that in AS gradients, alkaline hydrolysis of DNA-rNs masks the true size of RIs. This prompted us to develop a pH-neutral denaturing sucrose-gradient system for separation of rN-containing DNA.

Formamide-Urea Gradients Reveal HMW Replication Intermediates. Formamide and urea are known to destabilize duplex DNA without breaking strands at rNs or other modified bases (56–58). In fact, formamide-sucrose gradients were used before to separate RIs, including the true size of RIs for examination. However, AS gradients appeared chemically inadequate for the task of separating the ribonucleotide-containing RIs from mnhB mutants, as the strongly basic pH of AS gradients, required to denature RIs from their template strands, efficiently hydrolyzes mono-rNs in DNA (52) and also destabilizes abasic sites (53). Therefore, we developed a pH-neutral denaturing gradient system to separate RIs without inducing breakage at alkaline-sensitive sites. Employing this gradient system, in concert with traditional alkaline-sucrose methods and strand-specific hybridization of DNA from gradient fractions, we demonstrate that the lig ZER ΔB mutant of E. coli replicates its leading strand by an essentially continuous mechanism.

As expected, we found that inactivation of RNase HII in the original ligA mutant shifted IMW RIs even further toward HMWs (Fig. 2B, lig ΔB @ 42 °C), showing that “contamination” of nascent DNA by ribonucleotides exceeded contamination by
all other DNA modifications removed by all-DNA excision-repair systems inactivated in the lig ZER mutant. A crucial finding came from introduction of the mbB defect into the lig ZER strain, to yield the lig ZER ΔB mutant deficient in all known initiators of excision repair in _E. coli_. In the FUS gradients, RIs from the lig ZER ΔB strain form two well-separated peaks: the standard LMW peak of Okazaki fragments and a now fully separate HMW peak similar in size to the chronically labeled chromosome (Fig. 2C). Importantly, in all these mutants (Fig. 2; also, the four gradients are overlaid in Fig. 3B), while the IMW peak gradually becomes HMW, the LMW peak does not shift to higher molecular weights, confirming that it is insensitive to repair activities and therefore likely represents true Okazaki fragments.

The MW standards for FUS gradients show a uniform separation between Okazaki fragments (1.2 kb), the 9.3-kb PCR fragment, lambda chromosome (48.5 kb), and T4 chromosome (∼170 kb) (Fig. 2D). This allowed us to estimate the size of the HMW RI peak as stretching from ∼20 kb to 300 kb, with the major fraction found between 50 and 150 kb (Fig. 2 C and D; also see SI Appendix, Figs. S4 and S5). We conclude that when all known excision-repair activities are eliminated (in the lig ZER ΔB mutant), and alkaline hydrolysis of DNA-rNs is prevented (in FUS gradients), RI distribution becomes strictly bimodal, with the HMW fraction appearing to result from continuous synthesis.

**Return to Alkaline-Sucrose Gradients Facilitates Testing Strand Identity of RIs.** Continuous synthesis of half of the RIs is consistent with the semidiscontinuous model of the replication fork (Fig. 1A), which makes a strong prediction about strand identity: According to the semidiscontinuous model, LMW RIs represent the lagging strand, whereas HMW RIs come from the leading strand. In previous studies, LMW RIs from maturation-deficient (both excision-proficient and -deficient) mutants hybridized equally to both strands (17, 20, 27, 31, 33, 60). Unique replication origin and terminus of bacterial chromosomes (61), by ensuring a fixed direction of movement of replication forks through specific loci, facilitate unequivocal determination of the leading versus lagging source of RIs. Would hybridization of lig ZER ΔB mutant RIs reveal a mixture of leading and lagging strands within each peak or their partitioning between the two peaks?

To answer this question, we sought to isolate different populations of RIs directly from gradient fractions and to query their strand identity by strand-specific hybridization. In so doing, technical considerations forced us to return to the alkaline-gradient system, which is more rapid, taking 1 d of centrifugation instead of 2 for FUS gradients, and whose lower viscosity greatly increased the speed with which fractions could be centrifugally dialyzed. We managed to dramatically reduce the problem of DNA-rN hydrolysis in alkaline pH due to our previous serendipitous finding that rN-containing DNA oligos become stable at alkaline pH if kept on ice (ref. 52; Fig. 1E, lanes 0° for lig ZER ΔB and lig ΔB strains). As a proof of concept, rRNA stability in 0.2 M NaOH was greatly increased at 0 °C, compared with 16 °C and especially 37 °C (Fig. 3A, lines c to e), while the same material incubated in the FUS loading buffer was stable at all temperatures (Fig. 3A, lines k to m). Calculations of the rate of hydrolysis of rRNA at 0 °C (SI Appendix, section 10) show that a similarly slow hydrolysis of sparse DNA-rNs should have only a minor effect on the final size of the HMW distribution.

We estimated the degree of alkaline hydrolysis in AS(4 °C) gradients using the DNA species most sensitive to breakage, the HMW (chromosomal) DNA. Separation of chromosomal DNAs from the RnhB+ strain (lig) and its rnhB mutant sibling (lig ΔB) that accumulate DNA-rNs in AS(4 °C) gradients revealed a difference of two fractions between the peaks of HMW material (Fig. 3C), consistent with a minor instability of single DNA-rNs under these conditions. Subsequent experiments confirmed these

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**Fig. 2.** Formamide-urea-sucrose gradients reveal HMW replication intermediates in the lig ZER ΔB mutant. (A) The separation pattern of FUS gradients for the lig mutant (GR501). The temperature of [3H]dT labeling is indicated. The corresponding alkaline-sucrose gradients from Fig. 1A are shown in faded colors for comparison. (B) The effect of removal of DNA-rN excision (lig ΔB) versus all other excision-repair systems (lig ZER) in FUS gradients. Strains: lig ZER, LA111; lig ΔB, eGC193. (C) The effect of the removal of both DNA-rN excision and all other excision-repair systems in one strain (lig ZER ΔB, eGC197) in FUS gradients. (D) FUS-gradient separation of our standard molecular weight markers: the 9.3-kb PCR fragment, phage lambda (48.5 kb), and phage T4 (∼170 kb), relative to the Okazaki fragments peak (∼1.1 kb) from the lig mutant (GR501).
predictions in ligase-minus conditions. If DNA-rNs were significantly hydrolyzed during AS-gradient centrifugation at 4 °C, the distribution of RIs from the lig ΔB mutant would sediment similar to those from its lig (RnhB+) parent. This is clearly not the case. Comparison of the four RI profiles from the FUS gradients (Fig. 3B), with identical material sedimented through AS gradients at 4 °C (Fig. 3D), clearly demonstrates conservation of the relative sedimentation patterns between the two systems. In particular, a large gap between the HMW and LMW species of RIs from the lig ZER ΔB mutant was essentially unchanged in AS gradients (Fig. 3D), allowing for a clean isolation of the two molecular weight populations with minimal cross-contamination.

Strand-Specific Hybridization of Tritiated RIs. To quantify the strand identity of HMW versus LMW RIs, we pooled distinct populations of labeled DNAs from AS(4 °C) gradients (Fig. 4A) for hybridization. The pooled fractions were concentrated to a small volume by centrifugal dialysis, sonicated (SI Appendix, Fig. S6), and split in half for hybridization with one of the two complementary strand-specific ssRNAs. The sonication step was important to reduce solution viscosity and to minimize “sandwich hybridization” (62) (SI Appendix, Fig. S8).

The strand-specific ssRNAs used as hybridization targets were generated by in vitro T7 transcription of an 8.8-kb genomic fragment containing the gspD-M operon. The gsp operon, located at position 3456 to 3465 kbp on the E. coli chromosome, was chosen for its distance from oriC (≤500 kbp), transcriptional silence (63), and relative lack of predicted secondary structures, ssRNAs produced in these in vitro reactions were cross-linked to nylon membranes in quantities sufficient to prevent saturation of probe binding (SI Appendix, Fig. S7) and tested for strand specificity in model hybridization reactions using 32P-end-labeled ssDNA oligo probes (SI Appendix, Fig. S8A). Input control DNA containing equal amounts of tritiated Watson and Crick strands was obtained from pooled fractions 5 to 13 (60 to 150 kb) of DNA labeled for 35 min at 28 °C (LigaA) conditions. As expected, this chronically labeled genomic DNA hybridized equally well to either strand, yielding a lead/lag ratio close to unity (Fig. 4A, Left pie chart). To assay RIs formed under ligA-deficient conditions, we labeled the lig mutant for 2 min at 42 °C and, following sedimentation, separately pooled the HMW/IMW RIs from fractions 8 to 20 (20 to 100 kb), while the LMW RIs were pooled from fractions 23 to 27 (0.5 to 7 kb). In contrast to the hybridization ratio obtained from the chronically labeled chromosomal DNA, the lead/lag ratio for the HMW RIs was 1.95 (Middle pie chart in Fig. 4A), while the lead/lag ratio for LMW RIs was 0.31 (Right pie chart in Fig. 4A). In other words, even under excision-proficient conditions where separation between LMW and HMW RIs was minimal, there was a ninefold difference (2.81/0.31) in the strand preference between the two pools of RIs.

When we repeated the strand-specific hybridizations with the same two pools of fractions from the lig ZER ΔB strain, in which HMW RIs were clearly separated from the LMW RIs (Fig. 4B), we observed a dramatic increase in strand specificity. For this strain, the lead/lag ratio reached 7.33 for HMW RIs (Left pie chart in Fig. 4B), while decreasing to 0.095 for the LMW RI population (Right pie chart in Fig. 4B). This 77-fold difference (7.33/0.095) in strand preference between RI pools from the lig ZER ΔB mutant reflects a mostly leading-strand origin for HMW RIs, and a mostly lagging-strand origin for LMW RIs. The actual purity of the leading- and lagging-strand fractions should be even higher, as it is compromised by the alkaline hydrolysis of DNA-rNs. Vertical arrows indicating peak fractions are color matched to the corresponding gradients. Mean ± SEM for multiple experiments (ligGR501 n = 44; ligΔB (egC193) n = 10). (D) As in B, but AS gradients at 4 °C. Each curve represents the mean of between 7 and 45 separate experiments; error bars are omitted for clarity. The visible shift to the right of the HMW peaks compared with the FUS gradients in B is ostensibly due to DNA-rN hydrolysis.
replication neither influenced the 1:1 hybridization ratio for the control ligase-proficient conditions (Fig. 5B, Left pie chart), nor did it alter the bimodal nature of the ligase-deficient RI distribution (Fig. 5B, red $^3$H profile). However, 42 °C RI profiles from the oriF strain did display an altered ratio of areas under the HMW and LMW RI curves, with ~70% of total RIs now found under the HMW peak (Fig. 5B). The reason for this altered proportion of label in the HMW peak is unknown, but we suspect residual activity of the LigA251(Ts) enzyme at 42 °C in this background, possibly reflecting strain-specific variations in the protein-degradation levels (65, 66).

Nevertheless, the oriF strains produced broadly similar hybridization results to those obtained for the oriC strains. The lead/lag ratio was 3.1 for the HMW RIs (Middle pie chart in Fig. 5B), while it was 0.1 for LMW RIs (Right pie chart in Fig. 5B), yielding a more than 30-fold difference in the relative strand preference between the two pools of RIs. When hybridization results from the oriC and oriF strains were plotted side by side, the chronically labeled DNA showed an even distribution of label between the two strands, regardless of the replication origin tested (Fig. 5C, gray bars). Importantly, shifting the origin of replication relative to the hybridization region reversed the ratios for pulse-labeled fractions: If, in the oriC strain, the Watson strand signal was low in a particular MW pool, in the oriF strain that pool showed increased signal, and vice versa (Fig. 5C), confirming the theoretical considerations (Fig. 5A). We conclude that, for at least the gsp locus of the chromosome, the leading strands are synthesized as HMW species, while the lagging strands are synthesized as LMW species (Okazaki fragments), independent of the direction of replication.

**Periodic Repriming of the Leading Strand.** Are the nascent leading strands truly continuous with the rest of the chromosome, or are they periodically reprimed? To detect LMW RIs, the Okazaki group pulse-labeled growing cells with $[3^H]dT$ for several seconds (11, 19). They later found that, under ligase-deficient conditions, even with increased pulse length, LMW RIs continued accumulating linearly over at least 1 min without significant increase in length (18). In our hands, with the ligA251(Ts) mutant, incubation at 42 °C up to 3 min did not change the overall RI distribution (31). To maximize the yield of material for hybridization, we typically labeled our lig mutants at 42 °C for 2 min. Under this long period of labeling, HMW RIs from the ZER mutant appeared similar in length to the chromosomal DNA (Fig. 3B and D), as would be expected if the leading strands were synthesized continuously, without periodic repriming, and therefore were contiguous with the rest of the chromosome (Fig. 6A, Left) (within the ~100-kb size limit of intact DNA strands due to our cell-lysis procedure; SI Appendix, Fig. S2). At the same time, incubation at 42 °C for 2 min allowed for polymerization of 120 to 150 kb of nascent DNA (54), making it possible that this long pulse time could obscure periodic repriming events (Fig. 6A, Right).

To detect possible periodic repriming on the leading strand, we labeled the lig ZER ΔB mutant under ligase-deficient conditions for times between 10 and 270 s. Since this experiment is exquisitely sensitive to any traces of remaining ligase activity, the lig ZER ΔB mutant was labeled at an elevated temperature of 45 °C, 3 °C above normal (but similar results were also obtained at 42 °C). There is little to no active ligase present in these cells, as can be seen from the labeling-time insensitivity of the position and height of the Okazaki fragment peak (Fig. 6B). While the Okazaki (LMW) peak remained unchanged, increased labeling time caused the position of the (very broad) leading-strand distribution to shift toward higher molecular weights, while the trough between the two distributions deepened (Fig. 6B). Specifically, the leading-strand distribution was centered at ~20 kb following a 10-s pulse of $[3^H]dT$, but shifted to ~50 kb following 270 s of labeling. This result is consistent with the periodic repriming of the leading strand, at least every ~50 kb, although this value is likely an underestimate due to the limited hydrolysis of RIs in alkaline sucrose (Fig. 3 C and D).
Discussion

The results presented here largely resolve one of the oldest puzzles in molecular biology—that of the divergent observations of fully discontinuous leading-strand replication in vivo versus continuous replication in vitro (22, 67). Employing the classical techniques of alkaline sucrose-gradient sedimentation and Southern hybridization, in tandem with a unique pH-neutral denaturing gradient system, we revealed the newly continuous replication of the leading strand in E. coli. This highly continuous in vivo replication was observed only in excision repair-deficient mutants, and ribonucleotide excision repair was identified as the major driver of leading-strand scission. Pulsed-labeled RIs from the ligZERΔB strain, which lacks all known pathways for excision repair, appeared as two distinct populations of either LMW (0.5- to 5-kb) Okazaki fragments or HMW (20- to 300-kb) pieces, the latter being similar in size to the chronically labeled chromosomal DNA (Fig. 2C). Strand-specific hybridization of these nascent DNA strands revealed a leading-strand origin for HMW RIs, while the LMW RIs were mostly derived from the lagging strand. Comparisons of LMW and HMW specificity ratios (Fig. 4B) indicate a relative lead/lag ratio enrichment factor of nearly 80-fold between the fractions. Control hybridization experiments suggest that the observed enrichment factor approaches the maximum possible for this assay in its current form (SI Appendix, Figs. S7 and S8). Therefore, it appears that, save for the occasional repriming, the leading strand is synthesized by a nearly continuous mechanism, only to become fragmented by excision repair soon after replication-fork passage. In other words, newly synthesized DNA is quite “dirty,” being initially synthesized with a significant number of chemically incorrect bases, mostly ribonucleotides, and undergoes significant excision repair before its maturation into chromosomal DNA. Since the size of LMW RIs is unaltered by the inactivation of excision-repair systems, the LMW population likely constitutes bona fide RNA-primed Okazaki fragments.

As excision repair must be inhibited to produce a more continuous leading strand, inactivation of only a subset of repair systems allows for determination of the contribution of individual repair pathways to the overall RI fragmentation. While our study lacked granularity regarding DNA-only excision repair, we did examine the contribution of ribonucleotide excision repair to leading-strand fragmentation. Tests for the individual contributions of mono-rN and DNA excision activities revealed that RNase HIII cleaves the leading strand at a frequency similar to the combined effects of all other excision-repair systems (Fig. 3 B and D). Supporting this result, our in vitro data indicate a mono-rN density in the DNA of mibB mutants of one in 8,000 to 9,000 nt (Fig. 1 D–F). Therefore, excision-proficient cells produce LMW RIs that are a mixture of lagging-strand Okazaki fragments and excision-fragmented leading strands. However, these leading- and lagging-strand fragment populations are not entirely overlapping; hybridization of the “shoulder” population from an excision-proficient strain displayed a clear leading-strand bias, whereas the classic Okazaki peak was mostly lagging-strand in origin (Fig. 4A). As such, our results for the repair-proficient lig4(ΔTs) strain closely mirror those obtained by Louarn and Bird during their isolation and hybridization of RIs from pol4(ΔTs) mutant cells (27).

Is leading-strand replication completely continuous in vivo, as depicted in textbooks? Our data suggest that it is not. When we pulsed ligase-deficient cells for shorter periods than normal (Fig. 6B), the leading-strand fragments steadily decreased in size with shorter pulses. If the leading-strand synthesis were primed only once at the origin, as implied in some replication models (68), then no more than 1% of asynchronously dividing E. coli cells would possess leading strands shorter than 50 kb, irrespective of pulse length. Since pulse-length invariance is not observed, leading-strand repriming events occur with a minimum periodicity of 50 to
70 kb. It is of course possible that leading-strand synthesis is more continuous than seen here, and that as-yet unrecognized excision-repair activities impart a low-level residual fragmentation.

That leading-strand repriming catalyzes the bypass of fork-blocking lesions in template DNA was demonstrated 50 y ago for UV-induced pyrimidine dimers (69). While the mechanisms underlying leading-strand repriming in vivo remain unexplored, direct bypass of pyrimidine dimers by a primase-dependent pathway has been demonstrated in vitro (70, 71), where blocking extension by the leading-strand polymerase slowed helicase progression until repriming could occur on the accumulating ssDNA of the leading-strand template. This scenario is especially relevant for our experimental system, as some fork-blocking lesions may accumulate in the lig ZER Δβ mutant (eGc197), either labeled chronically (28 °C × 35 min) for full-length chromosomal DNA or pulse labeled at 45 °C for 10, 30, 90, or 270 s to label RIs.

In general, leading-strand repriming assumes specific inhibition of the leading-strand polymerase relative to the replicative helicase, to generate ssDNA on the leading-strand template. Besides blocking DNA lesions, other potential replication irregularities may cause leading-strand repriming, such as incorporation of chain-terminating base analogs such as azidothymidine (72), or malfunctioning replisomes [for example, in holC and holD mutants (73)] or depletion of a specific DNA precursor, as during thymine starvation (74). Finally, although not observed in vitro, a regular, damage-independent leading-strand repriming, as first hypothesized by Okazaki et al. (11), could function similar to the “clock”-type mechanism of helicase-primase binding for lagging-strand priming (75), perhaps at a greatly reduced cadence. Another primase-independent pathway has also been demonstrated in vitro (76), according to which the replisome recruits nascent mRNA to directly reprime the leading strand after collision with, and displacement of, a codirectional transcription-elongation complex.

In summary, this work unifies 50 y of incongruent in vivo and in vitro results concerning the continuity of leading-strand replication, by showing that in vivo demonstrations of Okazaki fragment formation on the leading strand were the result of fragmentation of nascent strands by excision DNA repair. Although this fragmentation was long-suspected, a definitive demonstration of continuous leading-strand replication in the absence of repair activities had remained elusive (22). It now appears that in vivo nascent DNA contains at a minimum 1 incorrect base per ∼10,000 nt synthesized, with most of these “wrong” bases consisting of ribonucleotides. That in vitro replication assays have generally failed to observe leading-strand discontinuities is likely due to the omission from replication reactions of nucleotide contaminants and their cognate excision enzymes. Indeed, it now appears highly unlikely that E. coli leading strands exhibit the level of continuity seen in vitro, where leading-strand length can exceed one-quarter of a megabase (77). However, in vitro studies addressing the mechanisms of base misincorporation, lesion bypass, and replication restart are coming to the fore (78).

The in vivo methods presented here produce a clean separation of bona fide lagging-strand Okazaki fragments from the mostly continuous leading strands, and so offer a new avenue for the study of strand-specific effects of mutations or conditions perturbing replication. The nature and frequency of periodic leading-strand repriming are the next questions to answer.

Materials and Methods

Bacterial strains and plasmids are described in SI Appendix and in SI Appendix, Table S1.

Growth Conditions, Radiolabeling, Stop Solution, and DNA Isolation. Strains were grown at 28 °C, either in LB (10 g tryptone, 5 g yeast extract, 5 g NaCl, pH 7.2 with NaOH) or on LB plates (15 g agar/L of LB broth). Incubation of liquid cultures was performed in appropriately sized vessels and with sufficient shaking to effect robust aerobic growth. For labeling experiments, a single colony was grown overnight at 28 °C to saturation in LB and diluted 30 to 100× into 5 mL of LB in the morning. The starting OD600 of subcultures was lig and ligΔβ OD 0.02, lig ZER OD 0.05, and lig ZER Δβ OD 0.06. Subcultures were grown at 28 °C for ~3.5 h to OD ~0.5 to 0.6 and labeled by addition of 5 to 10 μCi/mL of 60 to 90 Ci/mmol [3H]thymidine (PerkinElmer). We found that growing strains past an OD of 0.4 in LB allowed for much increased thymidine incorporation (SI Appendix, Fig. S3), likely due to exhaustion of dt in LB. Bulk chromosomal DNA was labeled at 28 °C for 35 min. To pulse label replication intermediates, we shifted 5 mL of culture to 42 °C for 4 min before adding label and incubating for an additional 2 min. Labeling was stopped by mixing the culture with the stopping solution [five volumes of 10 mM KCN, 10% pyridine (79), kept on ice throughout the experiment]. Direct comparison of this ice-cold KCN/pyridine mixture with the phenol-ethanol-acetate stopping solution (80), used by Okazaki (15) and by us before (30, 31), showed no difference in RI distribution, but we found cell pellets stopped with KCN/pyridine were easier to resuspend before lysis. Once mixed with the stopping solution, cells were pelleted, and the pellets were resuspended in 1 mL of 30% sucrose, 50 mM Tris (pH 8.0), moved to Eppendorf tubes, and again pelleted at 13,000 × g for 2 min. Pellets were then resuspended in 50 μL of the same buffered 30% sucrose and lysed by addition of 350 μL of 2% SDS, 40 mM EDTA, 50 mM Tris (pH 8.0) and incubation at 65 °C for 5 min. Lysates were sequentially extracted with equal volumes of phenol (pH 8), 50:50 phenol/chloroform, and chloroform (SI Appendix, Fig. S2). The aqueous phase was moved to DNA LoBind microcentrifuge tubes (Eppendorf) and precipitated twice with ethanol in the
presence of 150 mM KCl. The final DNA pellet was resuspended in 100 μL of TE buffer (10 mM Tris HCl pH 8.0, 1 mM EDTA).

Plasmid Assay for RNA Density and Alkaline-Labile Sites. Assays of RNAse HII sensitivity and alkali sensitivity of plasmid DNA were performed as described previously (52), but the plasmid was pcYG566 (81). For linear plasmid species treated with formamide or NaOH, the average number of strand breaks was derived from the zero class of the Poisson distribution, taking into account the background level of nicks in parental molecules (82), by the equation –ln(F(t)) = F(full-length) – F(fractional), where F(full-length) is the fraction of the full-length ssDNA in 0.3 M NaOH, 45 °C × 90-min treatment, and F(fractional) is the fraction of the full-length ssDNA in 0.2 M NaOH, 0 °C × 5-min treatment or dissolution in 100% formamide. The average density of the nicks was determined by dividing the total single-stranded plasmid length in nucleotides by the average number of nicks per molecule.

Alkaline-Sucrose Gradients. Alkaline-sucrose gradients were made essentially as described (15). Briefly, 15.6-mL linear gradients of 5 to 20% sucrose in 0.1 M NaOH, 0.9 M NaCl, 1 mM EDTA were formed in 16 x 102-mm polypropylene ultracentrifuge tubes (Beckman; 337986) using an SG15 gradient maker (Hoefer). Gradients were poured from the bottom up by pumping the solution at ~1.5 mL/min through a length of tubing capped with a glass capillary tube. During pouring, just as the final 20% of solution exited the mixing chamber, flow was stopped, and a 1.2-mL shaft of 80% sucrose, in the same buffer, was added. The bottom of the tube was then completely advanced and chilled at 4 °C for at least 1 h before loading. Our DNA-treatment protocol includes, for historical reasons, a mock alkali treatment step: to each 100-μL DNA solution was added 100 μL of 0.6 M NaCl, and the mixture was incubated at 45 °C for 1 h. Following this mock treatment, a few microdrops of [32P]Pharker DNA was added, and the mixture was chilled in an ice/water bath for 20 min. Denatured DNA was from pGEM-4::gspD-M plasmids described above, which contain the gap operon in either of the two orientations, were linearized with BamHI for use as templates for production of 8.8-kb stand-specific ssRNAs using an AmpliScribe T7-Flash Kit (Epicerin) according to the manufacturer's instructions. For hybridization, ssRNAs were denatured in formamide/formaldehyde as described (84), and applied by Hybond-XL nylon membrane (GE Healthcare) in 3-mm-diameter circles using a dot-blot vacuum manifold (Bio-Rad). Twenty micrograms of ssRNA (SI Appendix, Fig. 5) was applied per spot and immobilized by UV cross-linking (UVP Scientific). Membranes were cut into 8 x 16-mm pieces containing individual spots of ssRNA and placed on a shelf in 1.5-mL microcentrifuge tubes (Sigma). One milliliter of hybridization buffer (8% SDS, 0.5 M NaPO4, 10 mM EDTA, pH 7.2) was added and the tubes were rotated end over end at 55 °C for at least 1 h. Gradient fractions for hybridization were pooled and concentrated to 200 μL in TE buffer by centrifugal dialysis (Amicon Ultra-4, 10K NMWL; Millipore). Dialysates were moved to screwcap Eppendorf tubes and sonicated (SI Appendix, Fig. 56) at 75% power, 100% duty cycle, in the "cup horn" attachment of a Misonix S-4000 sonicator chilled to 4 °C, and then brought to a total volume of 1.5 mL in 1X hybridization buffer. Hybridization mixtures were boiled for 5 min and kept at 65 °C before each was split into two tubes containing the membrane-attached Watson or Crick gap ssRNA targets. Hybridization proceeded overnight at 55 °C, after which membranes were removed to trays and washed successively for 30-min intervals with 5 mL each of 2× SSC, 0.5× SSC, and 0.1× SSC, each containing 0.5% SDS. To improve the efficiency of tritium counting, membranes were cooled down and stripped by boiling in 250 μL of 0.2 M NaOH. Stripping solution was then neutralized by addition of 250 μL of 0.2 M acetic acid, 100 mM Tris (pH 7.5), added to 4 mL of scintillation fluid (Bio-Safe II; RPI), and counted to 2% error in a liquid scintillation counter.

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