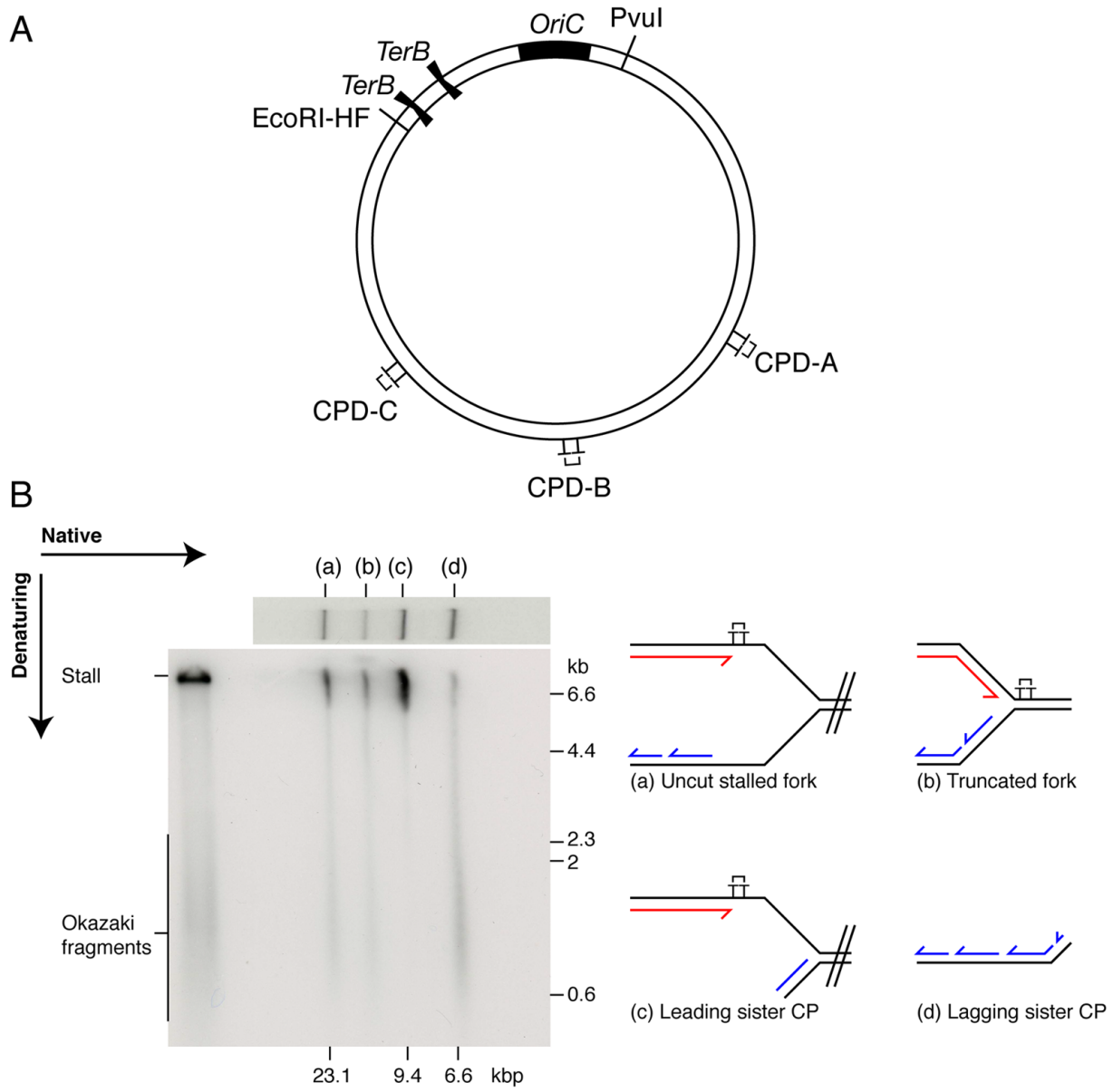

Figure S1. Related to Figure 1. Identification of stalled fork restriction enzyme mapping products.

(A) Diagram of the plasmid template used for DNA replication.

(B) Two-dimensional gel analysis of stalled replication forks cleaved by enzyme A (Kpn I). The CPD – C template was replicated under standard replication conditions. 30-seconds post-EcoR I addition a 25-fold excess of unlabelled dGTP chase was added and the reaction was arrested 60-seconds post-EcoR I addition. Products were immediately digested with enzyme A prior to two-dimensional gel analysis. Products (a) and (b) contain both leading and lagging strands suggesting that they correspond to uncut stalled forks and truncated stalled forks respectively. Product (c) is comprised solely of the leading-strand stall indicating that it is the leading-strand sister cleavage product (leading sister CP). (d) is predominantly composed of Okazaki fragments demonstrating that it is the lagging-strand sister cleavage product (lagging sister CP). There is a small amount of leading-strand stall in product (d), which we attribute to the dissociation of a proportion of replication forks post cleavage with enzyme A.

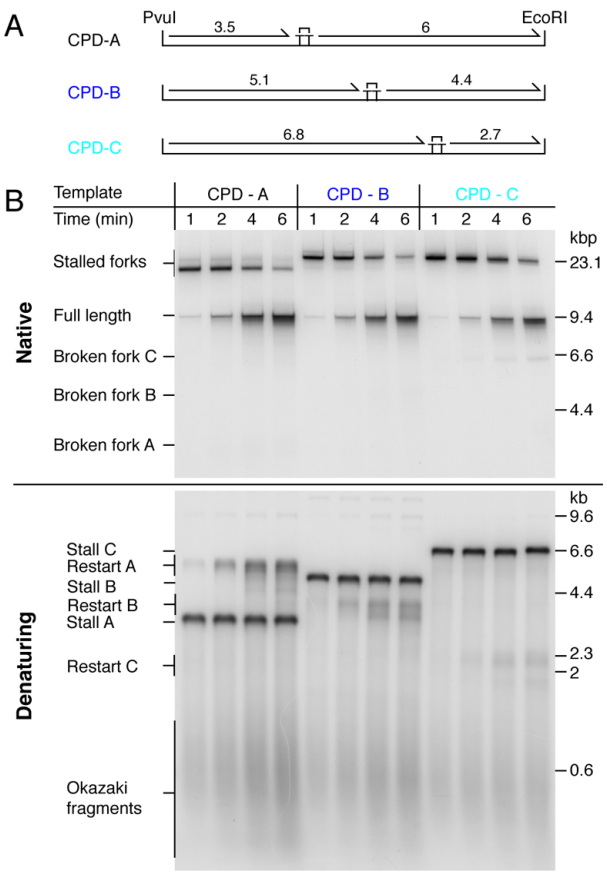

Figure S2. Related to Figure 2. Efficient leading-strand reinitiation occurs downstream of multiple different CPD locations

(A) Diagram of single-lesion replication templates illustrating the different locations of the CPDs, and the maximum lengths (kbp) of the putative leading-strand stall and restart products.

(B) Time courses conducted under standard replication conditions on the three single-damage templates.

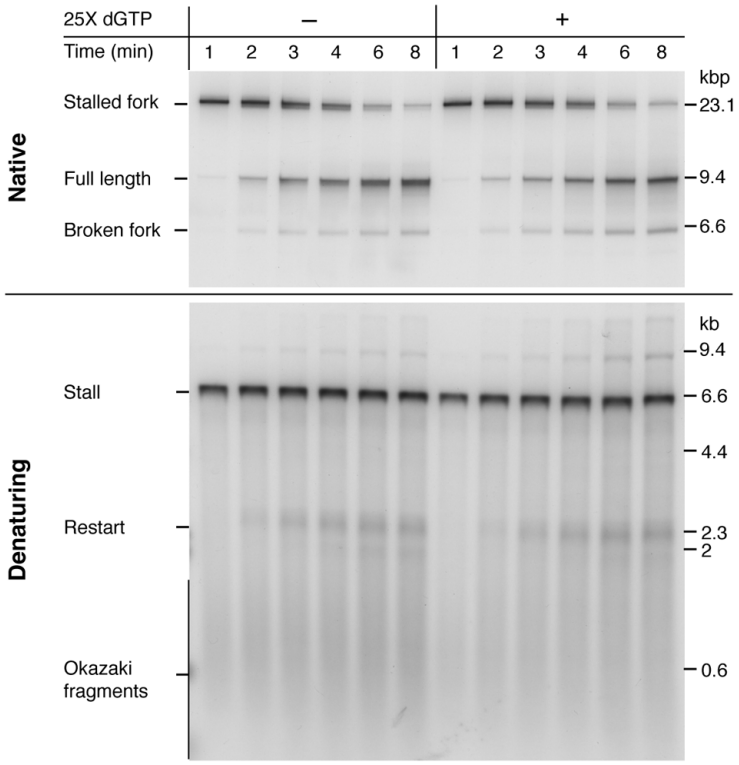

Figure S3. Related to Figure 2. Elevated dGTP concentration does not appreciably affect the kinetics or distribution of restart products. Standard replication reactions were conducted using CPD-C and [α-^{32}P]dATP. 45-seconds post EcoR I addition, a 25-fold excess (1 mM) of unlabelled dGTP was added to the reaction and aliquots were withdrawn at the indicated times post EcoR I addition.

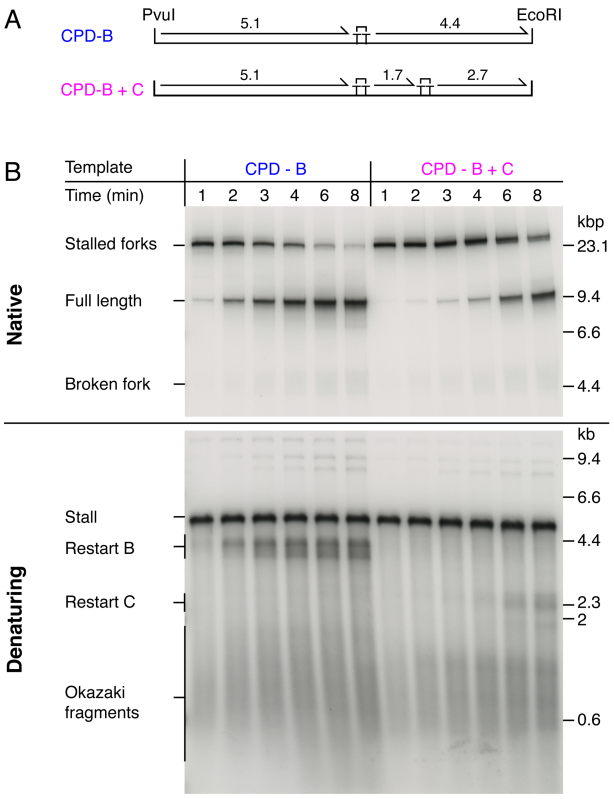

Figure S4. Related to Figure 3. Comparison of replication on the single-damage template CPD – B and the double damage template CPD – B + C.
(A) Illustration of the single- and double-damaged templates. Distances are given in kbp.
(B) Replication reactions were conducted under standard replication conditions for the indicated times post EcoR I addition.

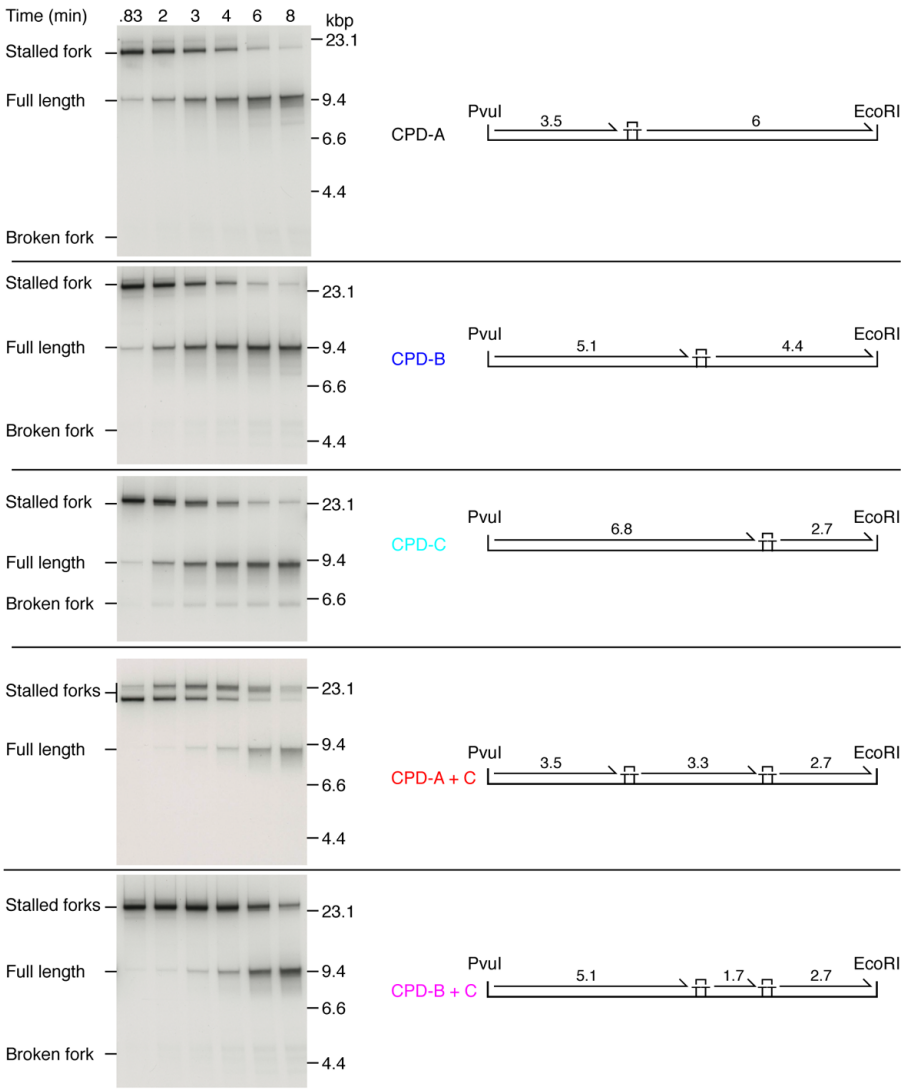

Figure S5. Related to Figures 2 and 3. Analysis of the kinetics of full-length product formation. Replication reactions were conducted under standard replication conditions with [α-^{32}P]dGTP and the indicated templates. 40 seconds post-EcoR I addition a 25-fold excess (1 mM) of unlabelled dGTP was added to prevent further incorporation of [α-^{32}P]dGTP into replication products. Aliquots were withdrawn at the indicated times post-EcoR I addition and were processed as described in materials and methods. The distances upstream and downstream of the CPDs are given in kbp. The data for the single-damage templates is as shown in Figure 2.

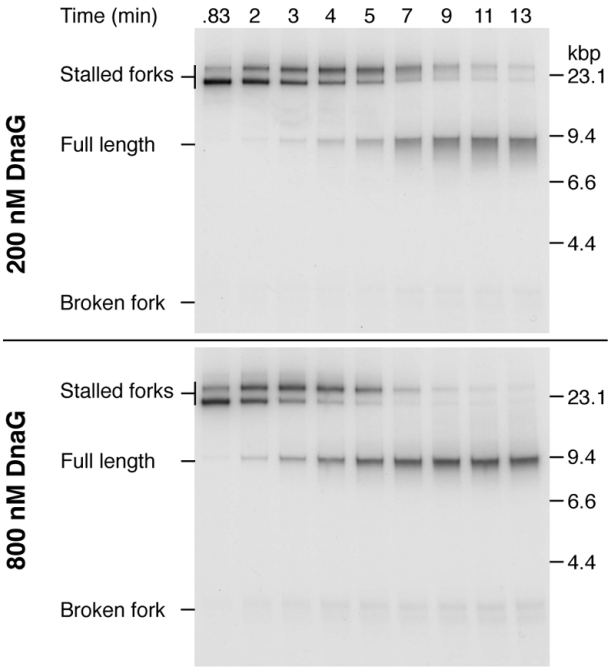

Figure S6. Related to Figure 4. DnaG concentration influences the kinetics of restart. Pulse chase experiments were conducted on the CPD A + C template using 200 nM and 800 nM DnaG as indicated. 40 seconds post EcoR I addition a 25-fold excess of unlabelled dGTP was added to prevent further incorporation of radiolabeled dGTP. Aliquots were withdrawn at the indicated times post EcoR I addition and were processed as described in materials and methods. Increasing concentrations of DnaG also resulted in an increase in fork breakage for all templates tested (Figures 4B, S6, and data not shown), with the effect being most visible on the CPD-C template. This effect is unlikely because of nuclease contamination, as it was observed with multiple independent DnaG preparations (data not shown).

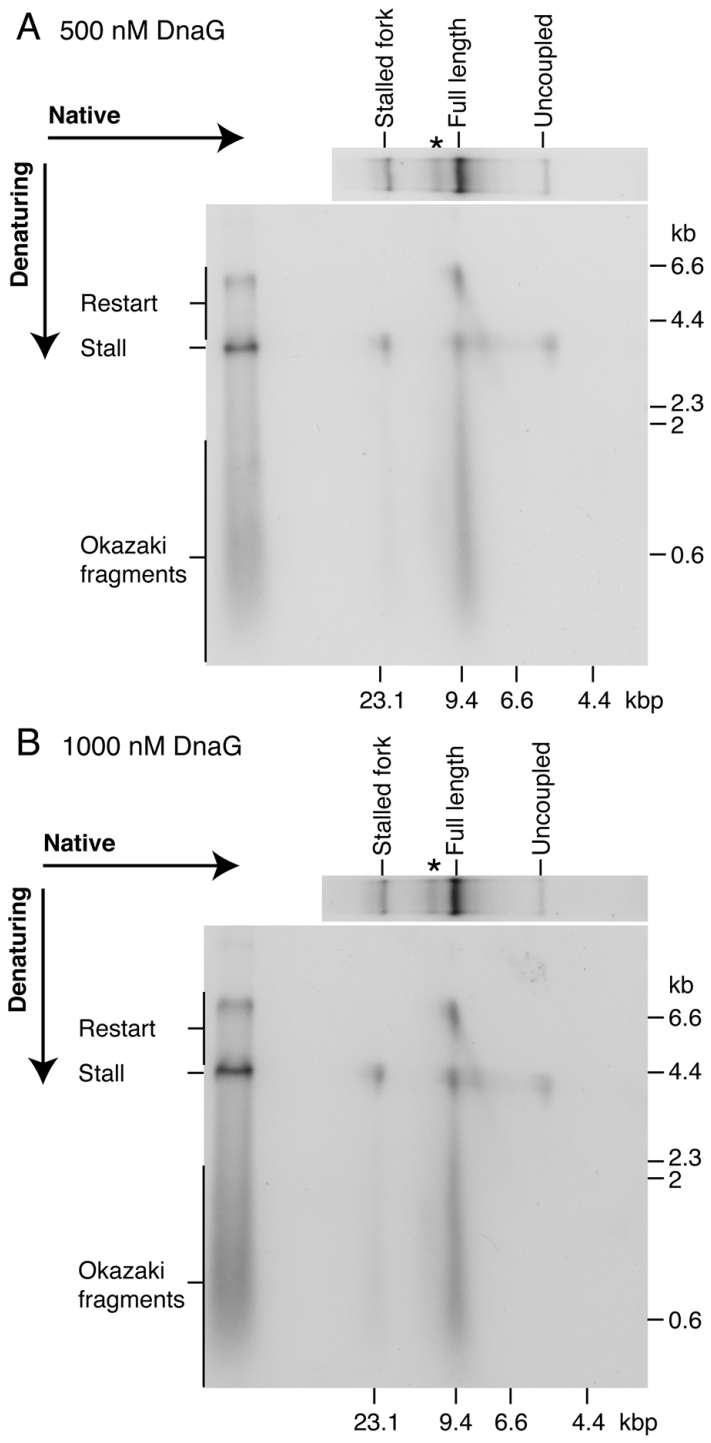

Figure S7. Related to Figure 5. Two-dimensional gel analysis of column-isolated replication products. CPD-A was replicated under standard column-isolated conditions for 6 minutes with either 500 nM (A) or 1 μM DnaG (B) post column as indicated. The products were resolved first through a native agarose gel followed by denaturing gel analsysis. * Labelled unresolved ERIs.

Supplementary Experimental Procedures

M13-JY13 Phage Construction

The 10,386 bp phage, M13-JY13, was generated by deleting a 58 bp region from the phage M13OriCT (Yeeles and Marians, 2011). 5′- phosphorylated primers, 7000delete_F 5′-GCCATCTCCTTGCATGCACC – 3′ and 7000delete_R 5′ – GTGCGCTCTCCTGTTCCGAC – 3′ were used to generate a PCR product that was subsequently ligated generating a 58 bp deletion. The sequences of the deleted region, *oriC* and the *terB* sites were confirmed by sequencing (MSKCC sequencing facility).

DNA Template Synthesis

CPD-A was synthesized using the oligonucleotide WF119 (3,472 bp from PvuI) 5′-CATTAAAGGTGAA(TT)ATCACCGTCACCG - 3′. CPD-B was synthesized with WF118 (5,138 bp from PvuI) 5′ - CAGAGGCGAATTA(TT)CATTTCAATTACC - 3′ (TriLink BioTechnologies). 5903CPD (6,744 bp from PvuI) 5′ - GGTCCACGCTGGT(TT)GCCCCAGCAGGCG - 3′ (Sigma) was used to generate CPD-C.

Standard Replication Reactions

Standard replication reactions (10 – 40 μl) were conducted in a buffer containing: 50 mM Hepes-KOH pH 8; 75 mM potassium glutamate; 10 mM $Mg(OAc)_2$; 10 mM DTT; 100 μg/ml BSA (NEB); 200 μM CTP, UTP, and GTP; 40 μM dGTP, dCTP, dATP, and dTTP; 1 mM ATP; 8 nM Tus; 1 μM SSB (monomer), and 2 nM DNA template. Unless otherwise indicated in the figure legends, reactions were initiated by addition of replication proteins to final concentrations of 140 nM DnaA, 200 nM DnaB (monomer), 180 nM DnaC, 250 nM DnaG, 12.5 nM HU_2, 30 nM β_2, and 20 nM Pol III*. Following 2-min incubations at 37 °C, topologically stalled replisomes in early replication intermediates were released by addition of EcoRI-HF (NEB) and [α-^{32}P]dATP (111 TBq/mmol) to final concentrations of 0.58 U/μl and 13.9 nM, respectively. Following incubation at 37 °C for the indicated times, reactions were quenched with 1.5X or 2X stop buffers containing: 50 mM Hepes-KOH pH 8; 75 mM potassium glutamate, 10 mM $Mg(OAc)_2$, 10 mM DTT, 100 μg/ml BSA, a 10-fold molar excess of 2′, 3′-dideoxyribonucleoside 5′-triphosphates (ddNTPs) over dNTPs, and a 10-fold molar excess of AMP-PNP over ATP. In all cases, reaction products were immediately digested for 15 min with PvuI, and additional enzymes (NEB) where indicated, prior to quenching with 30 mM EDTA. Prior to electrophoresis, samples from DnaG titrations were treated with 0.25% SDS and 0.2 mg/ml proteinase K for 30 minutes at 37 °C to remove SSB bound to ssDNA gaps. Prior to electrophoresis, radiolabeled nucleotide incorporation was measured by trichloroacetic acid precipitation. Loading volumes were then normalized to ensure that equal radioactive counts were loaded for each sample. For time course experiments, equal volumes of the reactions were loaded at each time point. Products were separated by electrophoresis through 0.8 % native agarose gels, or 0.6 % denaturing agarose gels as previously described (Yeeles and Marians, 2011).

Pulse-chase Replication Reactions

Pulse-chase reactions were conducted under the conditions described for standard replication reactions except that [α-^{32}P]dATP was substituted with [α-^{32}P]dGTP. To prevent further incorporation of labeled dGTP, 25-fold excesses (1 mM) of unlabeled dGTP were added from a

61 mM stock at the times stated in the figure legends. Samples were quenched and processed as described for standard replication reactions. Full-length replication products were quantified using ImageGuage software. The counts for full-length products at each time point were divided by the total lane counts at 50 seconds.

Column-isolated Replication Reactions

Replisome-associated early replication intermediates were generated in bulk reactions (30 μl) as described above. Following a 2-min incubation at 37 °C, KCl was added to a final concentration of 100 mM and the entire sample was applied to a 3 x 195 mm Sepharose-4B column (GE Healthcare) equilibrated in 50 mM Hepes-KOH (pH 8), 50 mM potassium glutamate, 7.5 mM $Mg(OAc)_2$, 0.015% IGEPAL CA-630, 10 mM DTT, 100 μg/ml BSA, 0.5 μM ATP, and 4 μM dATP, dTTP and dCTP. Fractionation was conducted at 21-23°C and fractions 23-27 (~110 μl), containing the ERIs and their associated replisomes, were pooled. Reactions were assembled at 21-23 °C by addition of NTPs, dNTPs, and $Mg(OAc)_2$ to the final concentrations used in bulk replication reactions from a 10-fold concentrated stock. [α-^{32}P]dATP (111 TBq/mmol), SSB and β_2 were added to final concentrations of 200 nM (monomer) and 30 nM respectively, and DnaG was added to the concentrations stated in the figure legends. Following a 20 sec equilibration at 37 °C, replisomes were released from the ERIs by addition of EcoRI-HF to a concentration of 0.19 U/μl. Reactions were quenched with 0.111 volumes of a 9X-concentrated stop buffer containing 50 mM Hepes-KOH pH 8, 75 mM potassium glutamate, 10 mM $Mg(OAc)_2$, 10 mM DTT, 100 μg/ml BSA, 80 mM AMP-PNP, and 3.2 mM ddNTPs. Products were immediately digested with PvuI (NEB) for 15 min at 37 °C prior to quenching with 30 mM EDTA. For native agarose gel analysis, samples (8 μl) were treated with 0.2 mg/ml proteinase K for 30 min at 37 °C. Samples for alkaline gels (11.5 μl) were treated with 5 units of Calf Intestinal Phosphatase (NEB) for 30 min at 37 °C. Electrophoresis was conducted as described for bulk replication reactions.