SUPPLEMENTAL MATERIAL

In vitro reconstitution of DNA replication initiated by genetic recombination: A T4 bacteriophage model for a type of DNA synthesis important for all cells

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Supplemental Text

As implied in the main text, we lack a definitive explanation for the radioactive ‘19kb’ DNA single-strands that are observed as products of RDR when a linear double-stranded DNA molecule is the template in our system (see for example Figure 3, reaction 4). The Figure 3 data reveal that the appearance of the ‘19kb’ band requires both that the gp41 DNA helicase be loaded onto the DNA and that lagging strand DNA synthesis be blocked by the absence of gp61. This ‘19kb’ DNA could be produced if the initial 14kb hairpin product has its 3’OH end repositioned by gp41 in such a way that this growing end either turns around to allow the DNA polymerase to copy its own strand (producing a second hairpin) or pairs with a region of partial homology on a second double-stranded molecule, allowing this end to be further extended using that template. The hydrolysis of bound nucleoside triphosphate by gp41 propels it along a DNA single-strand in the 5’ to 3’ direction, and if gp41 can affect how the 3’OH end finds a region of homology, this could explain genetic evidence for a gp41 involvement in genetic recombination events (Kuroki and Yonesaki, 1999).

An alternative explanation, derived from a suggestion of Dr. Scott Morrical, is based on the fact that a small fraction of our linear, double-stranded template molecules contain a nick in one of their two DNA strands. In the presence of gp41, these nicks can prime strand-displacement DNA synthesis in the absence if any recombination event (see data revealing a low level of such synthesis in Supplemental Table S1). When RNA primer synthesis is blocked in the absence of gp61, the DNA strand downstream of each nick will be displaced as a long free single strand as DNA synthesis proceeds. Once the end of the double-stranded linear template is reached, this displaced strand’s 3’OH is freed to fold back on itself to prime “snap-back” DNA synthesis. This produces a double helix that has a covalent hairpin “crosslink” joining its opposite strands at one end. This is the same reaction, described in the main text, that generates the 14kb single strands observed after the release of template-length, single-stranded products in the Figure 3 experiment (as diagrammed in Supplemental Figure S5A). However, the crosslinked double helices generated by the above nick-primed DNA synthesis will be shorter than the linear template and, assuming that the nicks are randomly located, half of them will be crosslinked at the end that is opposite to the end that is crosslinked in the 14kb single strands.

Once homologous double-stranded DNA molecules form that are cross-linked at opposite ends, subsequent recombination-initiated DNA synthesis would be expected to produce DNA strands longer than 14kb -- strands that migrate in alkali similarly to the mixture of species that we have designated as ‘19kb’. For clarity, one specific example is diagrammed in Supplemental Figure S5B. Here the nick-primed DNA synthesis begins 5kb downstream from the ‘left end’ of the linear DNA double helix, and it
uses as its template the strand opposite to that used for recombination-initiated DNA synthesis. The product of the reaction in Figure S5B then reacts with the product of the reaction in Figure S5A to form the observed strands longer than 14kb, as diagrammed in Supplemental Figure S5C.

Note that a 5’ end-labeled experiment (Supplemental Figure S1) suggests that the “19kb” band is a much more minor product than do experiments using [α-32P]dTTP to label the DNA (as in Figure 3). This result would be expected if the model proposed for that band’s generation in Supplemental Figure S5 is correct.

Supplemental Figures

**Figure S1.** Only the expected leading strand products of recombination-initiated DNA synthesis are detected by following the fate of a 5’ 32P end-labeled, homologous DNA single strand that primes DNA synthesis on a linear double-stranded DNA template. Standard reactions for recombination-initiated DNA synthesis were carried out as in Figure 3 in the main text, with and without the 61 protein (gp61) present. But here the only label present was a radioactive phosphate at the 5’ end of the single-stranded 1623 nucleotide primer. The radioactively labeled DNA products at (a) 8 min and (b) 16 min have been analyzed by electrophoresis through a 0.6 % alkaline agarose gel followed by autoradiography.
Figure S2. Topoisomerase-dependent recombination-initiated DNA synthesis on a supercoiled template requires a homologous single-stranded primer and the UvsX and UvsY proteins. Standard reactions for recombination-initiated DNA synthesis in the presence of gp61 and T4 DNA topoisomerase -- with the UvsX and UvsY proteins and the single-stranded 1623 nucleotide primer present where indicated -- were prepared and carried out as described in Figure 6. After (a) 7 min and (b) 14 min of synthesis, DNA products were analyzed by both neutral and alkaline gel electrophoresis as for Figure 6. Autoradiographs of the gels are shown.
Figure S3. Characterization by alkaline agarose gel electrophoresis of the leading strand products of recombination-initiated DNA synthesis on a supercoiled template. Only the leading strand products are detected here, because the only radioactivity present is from the 5' $^{32}$P end-labeled, 1623-nucleotide primer. The results are those expected from the reactions diagrammed in Figure 5. The reactions for recombination-initiated DNA synthesis were carried out exactly as described for reactions 1-3 in Figure 6, with gp61 added where indicated to complete the primosome. The DNA was analyzed on a 0.6% alkaline agarose gel at (a) 8 min and (b) 16 min following substrate and primer addition. An autoradiograph of the gel is shown (see Materials and Methods).
Figure S4. Electron microscopic examination of stalled replication bubbles with supertwists removed. The molecules produced after 7 min of DNA synthesis in reaction 2 of Figure 6 (active primosome without topoisomerase) were examined following a brief DNase I treatment (see Materials and Methods). After such nicking, the supertwisted circles with a stalled replication bubble seen in Figure 7C are converted to relaxed circles with a stalled replication bubble. Three of the ten molecules analyzed to determine the parameters given in the text are shown in panels A, B, and C. Panel D defines the lengths measured for each of the ten molecules, and the results thereby obtained are listed in panel E. The mean stalled replication bubble size, \( \frac{(w+x)}{2} \), is 1.36 ± 0.19kb. The mean value for \( Y \) is 0.52 ± 0.22kb. (For those molecules with extensions only from one end, this was assumed to be the end \( Y \) that contains the initial primer DNA (see Figure 8, molecule 2B).

As explained in the text, branch migration has clearly occurred to enlarge the bubbles observed in these preparations following the DNAse nicking. In a rolling circle from a small moving bubble, such as the schematic molecule 2B in Figure 8 and the molecule shown in an electron micrograph in Figure 7B, the replication bubble is much smaller, ranging in size from 314-445 nucleotides (four molecules measured). This agrees with our estimate from the measurements above that the 1623 nucleotide primer has been extended by only about 330 nucleotides (see text). For an explanation of these forms, see Figure 8.
Figure S5. Diagrams of “snap-back” DNA synthesis and its possible consequences in our in vitro reactions. It has long been known that the free 3’OH end of a single-stranded DNA molecule can readily form a short hairpin helix that serves as the primer required by DNA polymerase to start DNA synthesis (Goulian et al, 1968; Englund, 1971). This produces a DNA double-helix whose two strands are covalently joined, or “cross-linked”, at one end. Any such molecule is said to be “reversibly denaturable”, since the helix will immediately reform when a treatment that disrupts its base-pair hydrogen bonds --such as alkali or high temperature-- is removed (Alberts and Doty, 1968). (A) The reactions that form the 14Kb single strands that are observed in Figure 3 in the absence of a functional primosome. (B) In the absence of a functional primosome, the type of result that is expected from a small amount of nick-primed DNA synthesis that occurs as a side-reaction in our system. Here we have chosen to illustrate only one of the many actual nick positions, as explained in the Supplemental Text. (C) The result expected from recombination-initiated DNA synthesis after the two types of cross-linked double-stranded molecules in A and B have formed, presented to account for the “19Kb” single-stranded observed.
### Supplemental Table

| Reaction | Percent Relative DNA Synthesis | Sizes of Major DNA Products in kb | Remarks |
|----------|---------------------------------|-----------------------------------|---------|
| Complete | 100                             | 7.16 0.5-7                       | The leading strand (primer extended) product is 7162 nucleotides. Okazaki fragments are 0.5 to 7 kb. (See reaction 6, Figure 3). |
| - dda    | 58                              | 7.16 0.5-7                       | The products are identical to those in the complete reaction. |
| - 61*    | 44                              | 7.16 "19" 14                     | No Okazaki fragments are synthesized. The 7162 nucleotide, single-stranded product of leading strand synthesis can be extended by snap-back synthesis, primed from its 3’OH end to give a double-length product at 14kb. Providing that both the 41 and 59 proteins are present, a “19” kb product also appears (e.g., compare reactions 1 and 2 in Figure 3). |
| - ds DNA | 7                               | 0.1-1.5                          | The 1623 nucleotide primer now serves as a single-strand template. The primosome randomly primes RNA-dependent DNA synthesis using it, producing small fragments. |
| - ds DNA - 61 | 1 | ~3 | The single-stranded 1623 nucleotide primer can be extended by snap-back synthesis, primed from its 3’OH end to give a double-length product. |
| - ss DNA | 24                              | 7.25 0.5-7                       | DNA synthesis initiates at a limited number of nicks. Some of the Okazaki fragments produced are displaced as single strands by “onion skin” synthesis and serve as primers for recombination-initiated synthesis. Consequently, the reaction rate accelerates. Reaction products <7.25 kb predominate. |
| - ss DNA - 61 or - 59 | 2 | 7.25 | Leading strand synthesis initiates at a limited number of nicks. The reaction ceases within 7 min. |
| - uvsX - uvsY | 21 | 7.25 0.5-7 0.1-1.5 | Half of the products are 0.1-1.5 kb, synthesized by the reaction described above for the minus dsDNA reaction. The remaining products are produced by DNA synthesis that is initiated at a limited number of nicks in the double-stranded template: this displaces a DNA single strand ahead of the polymerase, on which Okazaki fragments are made. |
| - uvsX, Y - ss DNA | 13 | 7.25 0.5-7 | DNA synthesis initiates at a limited number of nicks in the double-stranded template: this displaces a DNA single strand ahead of the polymerase, on which Okazaki fragments are made. |
| - uvsX, Y - 61 | 3 | 7.25 | Leading strand synthesis initiates at a limited number of nicks in the double-stranded template. The reaction stops within 7 min. |

*When dda is omitted from this reaction, the sizes of the indicated DNA products are unchanged.*
Table S1. Primer, template, and protein requirements for recombination-initiated DNA synthesis with Okazaki fragment production. The complete reaction here is identical to reaction 6 in Figure 3, with the 3’OH end of a linear, single-stranded DNA molecule of 1623 nucleotides priming DNA synthesis on a linear double-stranded DNA template. Each of the components omitted from the complete reaction is indicated with a minus sign. Aliquots of reaction mixture were taken after 7, 14, and 21 min of DNA synthesis and the amount of product determined as described in Materials and Methods, using [α-32P]dTTP (500 Ci/mol) as the labeled precursor. Percent relative DNA synthesis is based on the amount of synthesis in the complete reaction at 14 min, which is 10.4 nmol/ml, with the DNA products at this time being analyzed on 0.6% alkaline agarose gels (see Materials and Methods). Although the 7250 bp double-stranded template is present at 20 nmol (nucleotide)/ml, the maximum amount of DNA synthesis expected in the complete reaction is 15.7 nmol/ml, inasmuch as the site of priming by the 1623 nucleotide fragment (present at 1.23 pmol/ml) should allow only 76% of the leading strand template to be copied. However, DNA synthesis in the complete reaction exceeds 15.7 nmol/ml (it is 22 nmol/ml at 21 min), apparently because the displacement of some Okazaki fragments by the growing 3’OH end of adjacent Okazaki fragments (a process called “onion skin” synthesis; Barry and Alberts, 1994) liberates new homologous primers that can initiate additional rounds of recombination-dependent DNA synthesis. Consequently the amount of DNA product in the complete reaction at 14 and 21 min is, respectively, 2.3 and 3.1 times the amount found in the absence of lagging strand synthesis (the minus 61 protein reaction).