Amplification of Snap-back DNA Synthesis Reactions by the uvsX Recombinase of Bacteriophage T4*

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The uvsX protein of bacteriophage T4 is a recA-type recombinase. This protein has previously been shown to help initiate DNA replication on a double-stranded DNA template by catalyzing synapsis between the template and a homologous DNA single strand that serves as primer. Here, we demonstrate that this replication-initiating activity of the uvsX protein greatly amplifies the snap-back (hairpin-primed) DNA synthesis that is catalyzed by the T4 DNA polymerase holoenzyme on linear, single-stranded DNA templates. Amplification requires the presence of uvsX protein, the DNA polymerase holoenzyme, T4 gene 32 protein, and a T4 DNA helicase, in a reaction that is modulated by the T4 uvsY protein (an accessory protein to the uvsX recombinase). The reaction products consist primarily of large networks of double-stranded and single-stranded DNA. With alkali or heat treatment, these networks resolve into dimer-length single-stranded DNA chains that renature instantaneously to reform a monomer-length double helix. A simple model can explain this uvsX protein-dependent amplification of snap-back DNA synthesis; the mechanism proposed makes several predictions that are confirmed by our experiments.

The homologous genetic recombination machinery of bacteriophage T4 plays an important role in replication of the phase DNA. During the late stage of T4 infection of Escherichia coli, the modes of replication used earlier cease operating, and the recombination activity of the T4 uvsX protein (a recA protein-like DNA recombinase) is required to initiate DNA synthesis (1-4). The T4 uvsY protein, an accessory protein to the uvsX protein, is similarly required. The functional coupling between genetic recombination and DNA replication leads to the formation of the long concatemeric T4 chromosomes that are required for optimal DNA packaging; this coupling is therefore a central feature of the DNA metabolism of bacteriophage T4 (2).

In vitro studies of the purified uvsX and uvsY proteins have given us a preliminary understanding of how these recombination proteins function in T4 DNA replication. The 40-

kilodalton uvsX protein binds cooperatively to both ssDNA and dsDNA, and it catalyzes DNA pairing and branch migration reactions between homologous ssDNA and dsDNA species (5-9). The protein also hydrolyzes nucleoside triphosphates in a DNA-dependent manner and promotes reannealing of complementary ssDNA molecules. The 16-kilodalton uvsY protein binds to both ssDNA and dsDNA, but it has no detectable enzymatic activity of its own. Instead, the uvsY protein stimulates the enzymatic activities of the uvsX protein by helping it bind to ssDNA (10-12). As a result, the uvsY protein restores uvsX protein recombination activities under conditions that are suboptimal for uvsX-ssDNA interactions (e.g. low uvsX protein concentration, high salt, or high T4 gene 32 protein concentrations).

In a T4 in vitro DNA replication system, the uvsX protein was found to promote DNA synthesis on a double-stranded template by catalyzing synapsis between this template and a homologous linear ssDNA molecule (13). The product of the uvsX-catalyzed synthesis is a D-loop structure in which the 3'-end of the ssDNA molecule is annealed to the template, forming a primer-template junction. The T4 DNA polymerase holoenzyme assembles at this site, and leading strand DNA synthesis begins as nucleotides are added onto the 3'-end of the ssDNA molecule. The uvsY protein greatly lowers the concentration of the uvsX protein required to initiate this DNA synthesis (12). In addition to the uvsX and uvsY proteins, this reaction requires the following T4 DNA replication proteins: gene 43 protein (DNA polymerase), gene 45 and 44/62 proteins (DNA polymerase accessory proteins), gene 32 protein (helix destabilizing protein or SSB), and the dda protein (a DNA helicase). The T4 gene 41 protein, a DNA helicase and primosome component, will replace the dda protein in this form of recombination-dependent DNA synthesis, providing that the T4 gene 59 protein is also added. In addition to its role in initiating DNA replication, the uvsX protein can also participate in the chain elongation phase of DNA synthesis. Under some conditions, uvsX protein actively displaces the newly synthesized daughter strand from the template through a branch migration reaction, causing replication to occur by a conservative mechanism (13). This reaction appears to involve a direct mechanistic coupling between uvsX-catalyzed DNA branch migration and the movement of the T4 replication fork, and it may involve specific protein-protein interactions between uvsX protein and DNA replication proteins (18).

In the course of our studies of recombination-initiated DNA replication, we observed what appeared to be high levels of snap-back or hairpin-primed DNA synthesis on the linear

1 The abbreviations used are: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; RFI, supercoiled plasmid DNA; RFI, nicked circular DNA; kb, kilobase(s).
2 J. E. Barry and B. M. Alberts, manuscript in preparation.
ssDNA molecules present. Snap-back DNA synthesis occurs when the 3'-end of a linear ssDNA molecule undergoes intramolecular reannealing with an internal complementary sequence, forming a terminal hairpin that primes DNA synthesis (16-18). This snap-back reaction can be catalyzed by DNA polymerase alone; however, our data indicated that the reaction is somehow stimulated by the recombination activity of the uvsX protein. The current study explores the mechanism of this stimulation. Our results reveal that a novel, recombination-dependent mechanism amplifies the snap-back DNA synthesis reactions that are catalyzed by the T4 DNA polymerase holoenzyme.

**MATERIALS AND METHODS**

**Reagents**—Radioisotopes including methyl-[H]dTTP and γ[32P]ATP were purchased from Amersham. All nonradioactive nucleotides were from Pharmacia LKB Biotechnology Inc., and other chemicals were from Sigma, unless specifically noted. Restriction nucleases and T4 polynucleotide kinase were from New England Biolabs, creatine phosphokinase from Sigma, and S1 nuclease from Bethesda Research Laboratories.

**Proteins and Nucleic Acids**—Purification and storage conditions for T4 DNA replication and recombination proteins, including the genes 32, 41, 45, 44/62, 45, 59, dda, uvsX, and uvsY proteins, are described elsewhere (5, 8, 19-22). The concentration of uvsX protein in stock solutions was determined by the absorbance at 280 nm, using an extinction coefficient of 1.54 mg/ml/λ260 (8). All other protein concentrations were determined by Bradford assay, using bovine serum albumin standards. Circular single-stranded DNAs from bacteriophages M13 and M13mp19 were isolated by extraction from agarose gels as described (13). Linear single-stranded DNA molecules were labeled with [32P] at their 5'-ends using the exchange reaction of T4 polynucleotide kinase (25) and are expressed as micromoles of nucleotide residues per liter.

**Electron Microscopy**—DNA replication reactions were performed as described above, using the 1623-nucleotide fragment of M13 ssDNA as primer/template. uvsX, uvsY, and dda protein concentrations were 20, 5, and 16 µg/ml, respectively. After starting the reactions, samples were removed at various reaction times and brought to 30 mM Na3EDTA (final concentration). These samples were spread directly for electron microscopy in most cases. Alternatively, DNA samples were phenol-extracted and precipitated with ethanol prior to processing for electron microscopy. Sample spreading in formamide was accomplished by standard procedures (28). After platinum shadowing, samples were examined in a Phillips EM400 microscope.

**RESULTS**

**Replication of Linear ssDNA**—Time courses for the replication of three M13 ssDNA fragments, whose 3'-ends map to different HaeIII sites, are shown in Fig. 1. Since no primer has been added, only snap-back DNA synthesis is expected, resulting from self-primering by the folded back 3'-end of each linear DNA single strand (16-18). In the absence of uvsX protein, very small amounts of DNA synthesis occur on all three templates. Preincubation of the ssDNA molecules with T4 replication proteins prior to the addition of the uvsX protein failed to eliminate these delays (data not shown).
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The concentration of uvsX protein used in this experiment (70 µg/ml) represents the optimum concentration for these reactions (see below). At this concentration, uvsX protein is in excess over the ssDNA originally present in the reaction mixture, assuming a binding site size of 4 nucleotide residues per uvsX monomer (29). Similar replication time courses are also obtained at lower, subsaturating uvsX protein concentrations, provided that the uvsY protein is present (data not shown).

Rapid Renaturability of Products—The products of normal snap-back DNA synthesis reactions on linear ssDNA templates are long duplex hairpins, which undergo instantaneous (Ctel = 0) intramolecular renaturation following denaturation, since their complementary template and daughter strands are covalently linked (16-18). We studied the renaturability of the replication products in our reactions by measuring the resistance of heat-denatured samples to digestion by the single strand-specific endonuclease, S1. The data for these experiments are presented in Table I. The products of uvsX-dependent replication of the 849-, 1623-, and 2527-nucleotide M13 ssDNA fragments are instantaneously renaturable by this criterion. After heat denaturation followed by rapid cooling, greater than 90% of the ³H-labeled DNA remains resistant to S1 nuclease (Table I). In contrast, after heat denaturation of a ³H-labeled RFI molecule synthesized in vitro (in which the daughter strand is not covalently connected to the template), only 4% of the label remains S1-resistant (Table I). Thus, the products of uvsX-dependent ssDNA replication have renaturation properties consistent with those expected for the products of snap-back DNA synthesis.

uvsX Protein Concentration Effects—The rate of replication of linear ssDNA molecules is extremely dependent on the concentration of uvsX protein (Figs. 2 and 3). In the absence of uvsY protein (Fig. 2), the highest rate of replication occurs in the uvsX protein concentration range of 60-80 µg/ml. Very low levels of synthesis are observed at uvsX protein concentrations less than 40 µg/ml, suggesting that there is a critical concentration of uvsX protein for replication activity. Above 80 µg/ml uvsX protein, the rate of synthesis decreases markedly, indicating that higher concentrations of the recombinase inhibit DNA synthesis. These effects of uvsX protein concentration on ssDNA replication are similar to those observed for the uvsX-initiated replication of dsDNA templates that is primed by a homologous single strand (12, 13).

The uvsX protein concentration dependence of ssDNA replication is dramatically altered in the presence of 5 µg/ml T4 uvsY protein (Fig. 3). In this experiment, the highest rate of DNA synthesis occurs at 20 µg/ml uvsX protein (enough recombinase to bind 50% of the original ssDNA), and synthesis rates decrease steadily with increasing recombinase above this concentration. A very low level of synthesis is seen at 0 µg/ml uvsX protein (Fig. 3); therefore, uvsY protein itself does not stimulate ssDNA replication. Instead, uvsY protein appears to stimulate replication indirectly, by lowering the
critical concentration of uvsX protein for the reaction. The rate of ssDNA replication also strongly depends on the concentration of uvsY protein present (data not shown). The modulation of uvsX protein concentration effects by the uvsY protein in this replication system closely parallels that seen in uvsX-initiated dsDNA replication reactions (12).

The products of uvsX-dependent synthesis on ssDNA templates form a single major band on denaturing agarose gels (Figs. 2 and 3). For the 1623-nucleotide ssDNA template, the band corresponds to a product length of approximately 3.2 kb. This is the same size as the products that form (at a much lower level) in the absence of uvsX protein (Fig. 2, lane 1, and Fig. 3, lane 1). A dimer-length product is consistent with a snap-back mechanism of DNA replication, where the product is constrained to be less than or equal to twice the length of the ssDNA primer/template (17). The data suggest that the T4 DNA polymerase helazymase (43, 44/62, and 45 proteins) catalyzes a low level of snap-back synthesis on this template, and that the uvsX protein somehow amplifies the reaction.

The data in Figs. 2 and 3 also indicate that the synthesis observed is all-or-none, since nearly all of the labeled DNA molecules appear as either nonelongated starting material (lower band) or full length product (upper band) on the denaturing agarose gels. Since few intermediate length molecules are visible at any of the uvsX protein concentrations employed in these experiments, the initiation of DNA synthesis must be the rate-limiting step in this system. Therefore, we conclude that the uvsX protein primarily stimulates an initiation step.

Effect of DNA Helicases—The T4 dda protein is a nonprocessive 5' to 3' DNA helicase (21). This protein has been present in all of the reactions described thus far. Its effect on the uvsX-dependent DNA synthesis is shown in Fig. 4. In the absence of the uvsX and uvsY proteins (Fig. 4, lanes 1–5), only low levels of DNA synthesis are observed at dda protein concentrations ranging from 0–16 μg/ml. Therefore, the DNA helicase alone does not stimulate synthesis on a linear ssDNA template. However, in the presence of the uvsX and uvsY proteins (Fig. 4, lanes 6–10) or of the uvsX protein alone (lanes 11–15), the addition of dda protein greatly stimulates DNA synthesis. The rate of DNA synthesis in the uvsX-dependent reaction depends strongly on the amount of dda protein added. Increasing the concentration of dda protein increases the rate of DNA synthesis, with the highest rates seen at 16 μg/ml dda protein (the highest concentration tested in these experiments). The dda protein concentration effects observed here are similar to those seen in uvsX-initiated DNA strand exchange and dsDNA replication reactions, both of which are stimulated by dda protein (13, 30). Very low levels of DNA synthesis are observed at 0 μg/ml dda protein in reactions containing either 70 μg/ml uvsX protein alone or containing 20 μg/ml uvsX protein plus 5 μg/ml uvsY protein (Fig. 4). Therefore, the synthesis we observe appears to require the joint action of the uvsX recombinase and the dda helicase.

At all concentrations of dda protein examined (Fig. 4), replication proceeds in an all-or-none fashion, as observed above (Figs. 2 and 3). Therefore, initiation remains the rate-limiting step of DNA synthesis, independent of the concentration of DNA helicase. These data indicate that the uvsX and dda proteins synergistically stimulate an initiation step.
tein, we found that present (compare the reactions analyzed in Fig. 5, lanes 7-12, the gene 41 protein, in uvsX-dependent synthesis on ssDNA T4 gene 59 protein, which is required to load 41 protein onto 3' DNA helicase (31, 32). Its activity is stimulated by the template (Fig. 5). Gene 41 protein is a highly processive, 5' to 3' DNA helicase (31, 32). Thus, both the dda and the gene 41 T4 DNA helicases have the ability to stimulate the uvsX protein-dependent initiation of DNA synthesis regardless of the presence of gene 59 protein (data not shown). The products reflect the same all-or-none replication pattern seen with the dda helicase (Figs. 4 and 5). However, the addition of 41 protein to 59 protein-containing reactions (Fig. 5, lanes 13-18) restores a high level of DNA synthesis. The replication-stimulating activity of 41 protein is independent of the dda protein, but totally dependent on the presence of 59 protein (data not shown). The products reflect the same all-or-none replication pattern seen with the dda helicase (Figs. 4 and 5). Thus, both the dda and the gene 41 T4 DNA helicases have the ability to stimulate the uvsX protein-dependent initiation of DNA synthesis on linear ssDNA molecules.

Electron Microscopy of Replication Products—As shown above, the products of uvsX-amplified snap-back synthesis reactions run as dimer-length ssDNA chains on denaturing gels. However, when products from the same reactions were subjected to electrophoresis on nondenaturing gels, we observed that most of the DNA moved so slowly that it remained in the wells (data not shown).

The data in Table II represent a tabulation of the structures observed by electron microscopy at 15 min and 30 min, respectively, after the initiation of DNA synthesis, using the 1623-nucleotide M13 ssDNA fragment as primer/template. By the 30-min reaction time, the majority of the DNA in the reaction mixture is contained within large branched networks of ssDNA and dsDNA. These, evidently, are the slow-moving species observed on nondenaturing agarose gels. An example of this type of structure is shown in Fig. 6A. Although outnumbered by much smaller molecules (see Table II), branched structures containing at least 100 recognizable ssDNA and dsDNA segments account for at least 70% of the total mass of DNA in the solution by 30 min. Phenol extraction followed by ethanol precipitation of reaction products had no effect on either the amount or the relative size of the DNA networks (data not shown); therefore, these networks are held together primarily by DNA base pairing.3 DNA networks were also observed in the 15-min samples (Table II). These early-forming networks all contained less than 50 ssDNA and dsDNA segments per network, indicating that formation of the very large networks (more than 100 segments) occurs only later in the reaction.

In addition to large networks, samples examined at both reaction times contained several types of smaller structures

![Fig. 6. Electron micrographs showing typical structures formed during uvsX-amplified snap-back DNA synthesis reactions. Samples were prepared as described under “Materials and Methods.” The scale bar represents 1.6 kb of dsDNA, as determined from markers. A, branched network of ssDNA and dsDNA. Arrows indicate 2X-length (3.2 kb) dsDNA molecules linked to the network by ssDNA branches or tails. B, linear intermediates and starting material. 1X ss denotes 1.6 kb linear ssDNA fragment (starting material). 1X ds denotes 1.6-kb linear dsDNA (primary product of snap-back synthesis). 2X ds denotes 3.2-kb linear dsDNA (amplification intermediate). M ds denotes a marker dsDNA molecule (eX174RF1, 5.4 kb), which was added to the sample prior to spreading. C, D-loop intermediate.](image)
The most common small molecule was a 1.6-kb linear ssDNA (the starting DNA template). This was expected, since the replication reaction does not go to completion in 30 min under these conditions (see Fig. 3). Linear dsDNA species of approximately 1.6-kb (1x) and 3.2-kb (2x) length are also observed. (The ssDNA starting material was free of dsDNA molecules of either length; data not shown.) These dsDNA species are compared with the linear ssDNA template in Fig. 6B. The 1x-length dsDNA corresponds to the structure expected for the product of simple snap-back replication of this fragment, which should be an approximately 1.6-kb duplex hairpin. The uniform size of the product strands on alkaline agarose gels (Figs. 2 and 3) suggests that the 2x-dsDNA species is formed by the base-pairing of two of the double-length strands derived from a 1x-length snap-back dsDNA molecule. If so, the 2x-dsDNA molecules should have a head-to-head dimer structure. We have demonstrated the presence of head-to-head dimers by treating reaction products with a restriction endonuclease (Fig. 7). The 1623-base dsDNA molecule. If so, the 2x-dsDNA molecules should have a head-to-head dimer structure. We have demonstrated the presence of head-to-head dimers by treating reaction products with a restriction endonuclease (Fig. 7). The 1623-nucleotide ssDNA starting material contains a single XmnI site, which is located 584 nucleotides from the 5'-end of the fragment. As shown in Fig. 7, treatment of the 2x- and 1x-length dsDNA species with XmnI leaves fragments of approximately 2.1 and 1.0 kb, respectively. The 1.0-kb fragment is the product of XmnI digestion of the 1x-dsDNA snap-back product. The 2.1-kb fragment is expected from the loss of a 584-nucleotide fragment from both ends of a 3.2-kb, head-to-head dimer molecule.

The 2x-length dsDNA species also appears in segments of the DNA network shown in Fig. 6A, where arrows point to such molecules connected to the main network by a single-stranded tail. As will be described below, we suspect that these 2x-length, head-to-head dimers are important intermediates in the snap-back amplification process (see "Discussion").

The final class of structures that we observe by electron microscopy are D-loop molecules (Table II). One such structure is shown in Fig. 6C. It consists of a large dsDNA loop with a protruding tail. We postulate that this type of structure forms when part of a 1x ssDNA molecule invades a homologous region of a 1x duplex hairpin molecule.

The presence of branched ssDNA/dsDNA structures in the snap-back replication reaction mixtures indicates that the uvsX protein catalyzes high levels of recombination between the ssDNA starting material and the dsDNA products of these reactions. It is likely that some or all of these recombination events lead to the initiation of new DNA synthesis, given the ability of the uvsX protein to prime DNA replication through this type of recombinational process. In fact, we believe that these branched recombination intermediates represent the "template" on which the snap-back synthesis reaction is amplified (see Fig. 8, below).

**DISCUSSION**

Our results indicate that the T4 uvsX protein amplifies the replication of linear ssDNA molecules by the T4 DNA polymerase holoenzyme. The uvsX protein strongly stimulates DNA replication on three different ssDNA fragments with different sequences and 3'-ends. Although modulated by uvsY protein and co-dependent on DNA helicases, the reactions are uvsX-dependent since neither uvsY, dda, nor 41/59 proteins stimulate DNA synthesis in the absence of uvsX protein. Some aspect of the homologous recombination activity of uvsX protein would therefore appear to be important for the amplification of snap-back replication of linear ssDNA.

The initial DNA synthesis reaction in the replication of linear ssDNA is the snap-back synthesis that can be catalyzed by the T4 DNA polymerase alone. The only input DNA in our reactions is a purified linear ssDNA fragment, homogeneous in length and sequence. This fragment must serve as both template and primer for DNA synthesis. Rapid intramolecular pairing of the 3'-end with a complementary internal sequence creates a hairpin structure that acts as a primer-template junction (16–18). The T4 gene 43 protein (DNA polymerase) is known to initiate synthesis from this type of structure, both in the absence of other DNA replication proteins (16–18) and in the presence of polymerase accessory proteins and ssDNA binding proteins such as uvsX and gene 32 proteins (13). In our reactions, we observe low levels of replication of linear ssDNA in the absence of uvsX protein, uvsY protein, and DNA helicases. The products of these reactions are those...
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Fig. 8. A model for the amplification of snap-back DNA synthesis by the T4 uvsX protein, based on the replication-initiating activity of this protein. uvsX-catalyzed recombination between the linear ssDNA primer/template and the dsDNA product of snap-back replication initiates DNA synthesis that produces a 2X-length duplex with the same sequence as the snap-back product. Recombination-initiated DNA synthesis on this template amplifies the production of snap-back products. See "Discussion" for details.

expected for snap-back DNA synthesis, since they are approximately twice the length (3.2 kb for a reaction with a 1.6-kb ssDNA fragment) of the starting linear ssDNA, as seen on denaturing agarose gels. The same size products are produced in much larger amounts in reactions containing uvsX protein and a T4 DNA helicase, and they are rapidly renaturable, as predicted for replication by a snap-back mechanism (Table 1). Our results therefore indicate that the T4 DNA polymerase holoenzyme replicates a small number of linear ssDNA molecules through snap-back synthesis, and that uvsX protein somehow amplifies the production of these snap-back products.

The amplification appears to require recombination-dependent DNA synthesis initiated by the uvsX protein. The properties of the amplified snap-back reaction are remarkably similar to those of the "bubble migration" dsDNA replication reaction described in previous papers (12, 13). Similarities between the two reactions include the following.

uvsX Concentration Dependence—Both the snap-back and bubble migration reactions exhibit similar concentration curves for uvsX protein. This includes similar critical concentrations for uvsX initiation activity, similar optimum concentrations for uvsX activity, and similar inhibition of DNA synthesis at high uvsX concentrations.

Effects of uvsY Protein—In both reactions, the uvsY protein dramatically lowers both the critical concentration and the optimum concentration of uvsX protein for replication initiation. Also, the addition of uvsY protein at high uvsX concentrations strongly inhibits both snap-back and bubble migration DNA synthesis reactions.

DNA Helicase Requirement—Both reactions are strongly dependent on the activity of a T4 DNA helicase. The bubble migration reaction is stimulated at least 6-fold by the dda helicase (13), and the snap-back reaction requires dda protein along with uvsX for amplification to occur. In both reactions, the gene 41 helicase can replace the dda helicase if (and only if) the T4 gene 59 protein is also present. The addition of 59 protein without 41 protein causes a marked inhibition of the dda-facilitated snap-back reaction, and it also inhibits the bubble migration synthesis reaction under these conditions.

Other Protein Requirements—The amplified snap-back reaction requires both gene 32 protein and the T4 DNA polymerase accessory proteins 45 and 44/62. These proteins are also required for uvsX-initiated dsDNA replication (13). In contrast, simple snap-back synthesis catalyzed by the T4 DNA polymerase is not dependent on the 32, 45, or 44/62 proteins (16-18).

DNA Networks—The undenatured products of both reactions are involved in high molecular weight networks that contain both ssDNA and dsDNA (13). Branched networks of DNA are observed both in uvsX recombination reactions (14) and in recombination-dependent DNA synthesis reactions initiated by the uvsX protein in vitro (13). Therefore, it appears that recombination-dependent DNA synthesis initiated by the uvsX protein plays a central role in the amplification of snap-back DNA replication reactions.

Based on our observations, we propose the model in Fig. 8 for the amplification of snap-back DNA synthesis.

Step 1—The T4 helicosome replicates a linear ssDNA template by a snap-back mechanism. The product of this reaction is a long duplex hairpin, which is homologous to the linear ssDNA starting material.

Step 2—uvsX protein catalyzes synthesis between a linear ssDNA molecule and the duplex hairpin product of Step 1. Due to the 5' to 3' polarity (with respect to the invading ssDNA) of DNA branch migration catalyzed by uvsX protein (6), the 3'-end of the ssDNA is incorporated into a D-loop structure, where it is poised to prime DNA replication.

Steps 3 and 4—DNA synthesis primed in this manner resolves the D-loop intermediate into a dimer-length linear duplex, which may now serve as a template for further recombination-initiated DNA synthesis (Step 5).

Steps 5 and 6—uvsX protein catalyzes synthesis between a linear ssDNA molecule (starting material) and the homologous portion of the duplex dimer intermediate, priming DNA synthesis. Replication on this template by a "bubble migration" mechanism (13) gives rise to dimer-length linear ssDNA molecules that are internally complementary and that will rapidly renature.

Step 7—Renaturation of this product forms a hairpin structure identical with that formed in Step 1. These products may then participate in new rounds of DNA synthesis, thereby amplifying the amount of product. This model is consistent

4 S. Morrical, unpublished results.
5 Bacteriophage T4 DNA also exists in a rapidly sedimenting, branched network in vivo (33, 34). This form is observed primarily during late stages of T4 infection, when recombination-initiated synthesis is the major mode of DNA replication.
6 The duplex is homologous to the linear ssDNA in the looped-out region of the hairpin, which could make pairing of the 3'-end difficult. To create a stable primer/template junction, the 3' to 5' exonuclease activity of gene 43 protein (DNA polymerase) may be required to chew back past the unpaired region. Although the exonuclease is strongly inhibited by the combination of gene 32 protein and uvsX protein, limited degradation near the 3'-end of the ssDNA does occur (S. Morrical, unpublished observations).
with our observations that (a) the primary replication event is snap-back synthesis, (b) recombination-initiated synthesis amplifies the reaction, and (c) all other replication products are derived from copies of the original snap-back products. The key feature of this model is that recombination of primary replication products with starting material initiates DNA synthesis that ultimately produces more of the same replication products.

The model in Fig. 8 predicts the formation of specific D-loop and linear dsDNA intermediates in the amplification of snap-back synthesis. These types of structures occur in the snap-back reactions, as demonstrated by our electron microscopy studies (Fig. 6 and Table III). The key step in the amplification scheme would appear to be the formation of the 2x-length dsDNA intermediate (Fig. 6B), which according to our model must consist entirely of a large head-to-head dimer or inverted repeat structure (Fig. 8). We have confirmed the presence of the head-to-head dimer structure in snap-back amplification reactions by restriction enzyme analysis of reaction products (Fig. 7). We postulate that this intermediate is formed through recombination-initiated replication of the hairpin product of the primary snap-back reaction, with the linear ssDNA starting material as a primer (Fig. 8). The 2x-length dsDNA is equivalent to two denatured snap-back products annealed together, and it contains all of the information necessary to produce exact copies of the primary snap-back product upon replication. Recombination-initiated replication of this molecule sets up a true exponential amplification reaction, since the products of bubble migration synthesis on this template ultimately form more of the dimer dsDNA molecules (Fig. 8). Exponential amplification is consistent with the kinetics of the uvsX-stimulated reaction, where we see a rapid onset of DNA replication after a long time lag (see Fig. 1). Amplification is ultimately limited by the amount of ssDNA starting material present, since these molecules are needed as primers in all stages of the amplification mechanism.

The initial snap-back synthesis is not rate-limiting, since a preincubation of the ssDNA with the DNA polymerase holoenzyme and gene 32 protein does not eliminate the long time lag (data not shown). We suggest that the initial recombination between ssDNA and the hairpin product of snap-back synthesis is the slow step of the reaction. This recombination event is the first step in the formation of the 2x-length dsDNA intermediates which serve as the template for amplification. The rate-limiting nature of this step would explain the all-or-none replication of ssDNA at varying uvsX and helicase concentrations, since only those molecules which recombine in a uvsX- and helicase-dependent manner can go on to enter the amplification cascade. Because the hairpin molecules are present at very low concentrations early in the reaction, we see a large time lag in the onset of rapid DNA replication.

The biological consequences of snap-back amplification are unclear. In our in vitro system, lagging strand DNA synthesis initiated by the T4 primosome totally suppresses snap-back synthesis, by rapidly converting the ssDNA starting material into dsDNA.3 However there may be situations in vivo in which the 3'-end of a ssDNA molecule remains unreplicated long enough to undergo intramolecular snap-back replication. Snap-back replication would presumably be detrimental to T4, since information contained in the looped-out region of the hairpin would be lost. Under these conditions, recombination-initiated DNA synthesis could resolve the hairpin structure into a normal duplex containing all of the original information in a form usable by the phage. Therefore, the initial steps of the amplification scheme outlined in Fig. 8 could have important implications for the growth and packaging of the bacteriophage.

The model in Fig. 8 also represents an intriguing enzymatic mechanism for generating precise inverted repeats of DNA sequences. Conceivably, such a mechanism could play a role in gene amplification or in other large DNA rearrangements in higher organisms. Finally, the snap-back DNA synthesis reaction is an example of an isothermal DNA amplification process carried out with a high fidelity DNA polymerase in a relatively simple in vitro system. We are currently exploring the potential practical applications of this DNA amplification reaction.7

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REFERENCES

35. S. W. Morrical and B. M. Alberts, manuscript in preparation.