Cell, Vol. 47, 793–806, December 5, 1986, Copyright © 1986 by Cell Press

DNA Synthesis Dependent on Genetic Recombination: Characterization of a Reaction Catalyzed by Purified Bacteriophage T4 Proteins

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Summary

To simulate a reaction that occurs in T4-infected cells, we have developed an in vitro DNA synthesis system that requires seven highly purified proteins encoded by this bacteriophage: the DNA polymerase "holoenzyme" (four proteins), gene 32 protein, dda DNA helicase, and uvsX protein—an enzyme that catalyzes homologous DNA pairing and is functionally homologous to the recA protein. In the reaction observed, the 3′-OH end of one single-stranded DNA molecule primes DNA synthesis using a double-stranded DNA molecule of homologous sequence as the template. The uvsX protein continuously removes the new DNA chain from its template, so that DNA is synthesized by a conservative mechanism. This type of reaction, which requires the cooperation of recombination and replication enzymes, seems likely to be a general feature of DNA metabolism.

Introduction

In vitro studies of DNA replication using a mixture of purified proteins produced by bacteriophage T4 have led to a working model for DNA replication fork movement (Alberts et al., 1983; Nossal and Alberts, 1983). However, little progress has been made toward reconstituting a biologically relevant mode of initiating DNA synthesis in this system. This is partly due to the complexity of the process, since several distinct modes of initiation appear to operate in T4-infected cells. The initial or primary mode acts early in T4 infections to start bidirectional DNA replication at one or more specific sites on the T4 genome; this reaction requires some function of the host Escherichia coli RNA polymerase (Mosig, 1983; Luder and Mosig, 1982). Later in the infection cycle the RNA polymerase is modified, and initiation by the primary mode ceases. Replication forks are then initiated by a secondary mode, which requires several T4 proteins involved in genetic recombination (Mosig, 1983; Luder and Mosig, 1982; Bernstein and Wallace, 1983; Kozinski, 1983; Epstein et al., 1964). Recent studies of two T4 DNA replication origins cloned in defective T4 phage suggest that a third mode of replication initiation also exists (Kreuzer and Alberts, 1985, 1986).

The secondary mode of initiating DNA synthesis is thought to act by converting genetic-recombination intermediates into replication forks (Mosig, 1983). Initiation by such a mechanism would complete the replication of the ends of the linear T4 genome, which would otherwise be expected to become shorter after each round of DNA synthesis (Watson, 1972; Broker, 1973). In addition to being of possible relevance to the mechanism of telomere replication in other organisms, a linkage between recombination and DNA synthesis seems likely to be involved in both genetic recombination (Meselson and Radding, 1975) and certain types of DNA repair (Friedberg, 1985).

In this report we describe the reconstitution of a recombination-dependent form of DNA synthesis in vitro with a mixture of purified T4 DNA replication proteins and the T4 uvsX protein. The uvsX protein is known to be required for secondary initiation in T4-infected cells (see Bernstein and Wallace, 1983), and it catalyzes homologous DNA pairing and strand exchange (Yonesaki et al., 1985; Yonesaki and Minagawa, 1985; Hinton and Nossal, 1986; Formosa and Alberts, 1986). In characterizing the in vitro DNA synthesis reaction, we have discovered that this protein also plays a central role in a novel, conservative form of DNA synthesis.

Results

A Model Reaction for Recombination-Dependent Initiation of DNA Synthesis

To test for recombination-dependent initiation of DNA synthesis in vitro, we required an assay in which the single-stranded 3′ end of one DNA molecule provides the only possible primer for DNA synthesis on a double-stranded DNA molecule (the template). Therefore, an intact double-stranded DNA circle was chosen as the template, and the 3′ end of a unique fragment of single-stranded DNA as the primer. This primer is a reasonable substitute for the proposed in vivo primer: a double-stranded DNA molecule with a single-stranded 3′ extension (Mosig, 1983).

The expected reaction is shown in Figure 1. When a fragment of single-stranded DNA is mixed with a homologous double-stranded DNA molecule, synapsis between the two DNAs should be catalyzed by the uvsX protein, resulting in the formation of a "D-loop." Like the E. coli recA protein (Kahn et al., 1981; Cox and Lehman, 1981; West et al., 1981), the T4 uvsX protein catalyzes branch migration in the direction required to tuck this 3′ end completely into the loop (Yonesaki and Minagawa, 1985). The resulting base-paired 3′ end is then expected to prime DNA synthesis, with the elongation of the single-stranded fragment being accompanied by a simultaneous displacement of a strand from the double helix that enlarges the D-loop. This reaction mimics major features of the proposed pathway for recombination-initiated DNA synthesis in T4-infected cells (Mosig, 1983; Dannenberg and Mosig, 1983).

Initiation of DNA Synthesis Requires Homology between Single-Stranded and Double-Stranded DNA Molecules

In our initial experiments, DNA synthesis was assayed by the incorporation of radioactively-labeled DNA precursors

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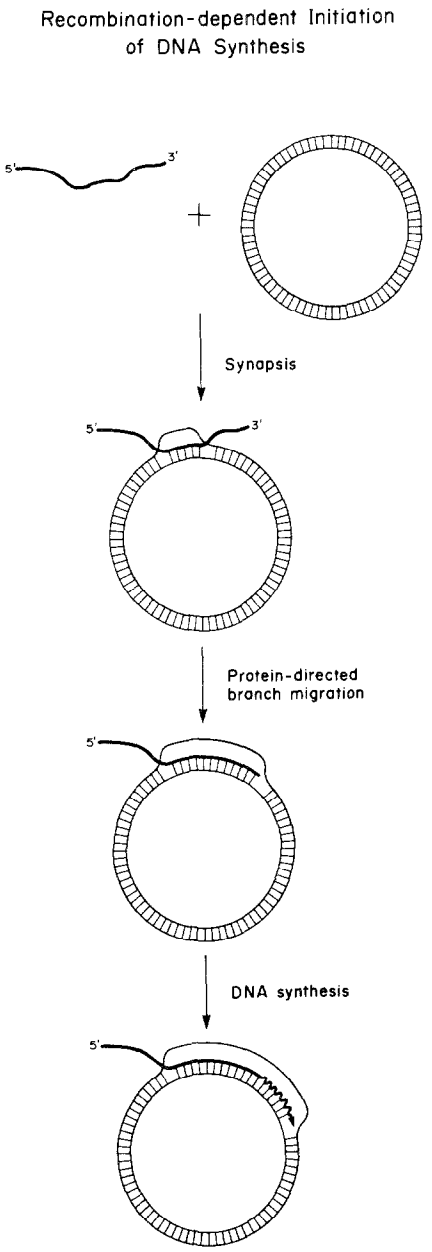

Figure 1. A Model Reaction Used to Test for Recombination-Dependent Initiation of DNA Synthesis In Vitro

A unique single-stranded fragment of DNA is mixed with an intact double-stranded DNA circle that contains a homologous sequence. Synapsis of the two DNA molecules, which is catalyzed by the uvsX protein, can in principle occur randomly with respect to the position along the single-stranded molecule (initially forming a "paranemic joint" [Flory et al., 1984]), but the uvsX protein then catalyzes a directed branch-migration reaction that inserts the 3′ end of the single-stranded molecule into the D-loop. In the last step shown, the T4 DNA polymerase "holoenzyme" (the DNA polymerase [43 protein] plus its accessory proteins [44/62 and 45 proteins]) uses this 3′ end to prime DNA synthesis, thus increasing the length of the single-stranded DNA molecule by using the double-stranded DNA molecule as a template.

into acid-precipitable material, using φX174 and M13 bacteriophage DNAs in various combinations as templates and primers. Efficient DNA synthesis was observed only when the single-stranded and double-stranded DNAs

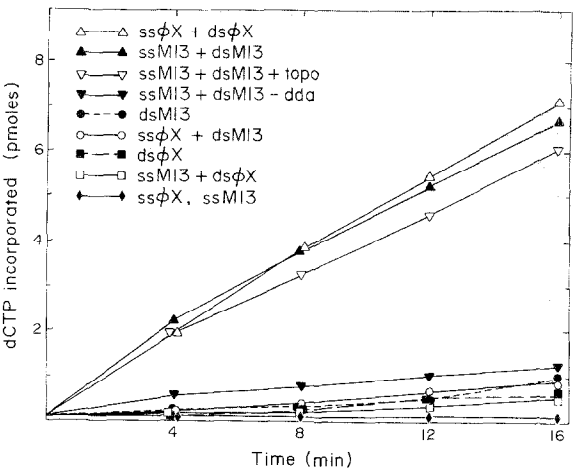

Figure 2. Recombination-Dependent DNA Synthesis Assayed by the Incorporation of Radioactive Nucleotide Precursors into DNA

DNA synthesis was assayed as described in Experimental Procedures. The single-stranded (ss) fragment was either the 766 nucleotide HaeIII fragment derived from M13 DNA or a HaeIII fragment of about 900 nucleotides derived from φX174 DNA; each was used at 2 μM (about 0.67 μg/ml), where indicated. The double-stranded (ds) template DNAs were the intact circular replicative forms of M13 or φX174 bacteriophages; each was used at 20 μM (about 6.7 μg/ml). Where indicated, the dda protein was omitted from the reaction, or 0.1 μg/ml of T4 DNA topoisomerase (topo) was added. Reactions are identified as shown in the key. Incorporation of nucleotides into trichloracetic acid–precipitable material was detected by removing samples at the times indicated and assaying for incorporation of [α-^{32}P]dCTP (1000 cpm/pmol) into acid-insoluble material.

present were homologous (Figure 2). The size of the DNA produced was examined by agarose gel electrophoresis under denaturing conditions, followed by autoradiography. As shown in Figure 3, when homologous primer and template DNAs are present, the DNA products become progressively longer as synthesis proceeds: the longest products seen, after 16 min of incubation, reveal that the circular DNA template has been copied more than four times around from a single growing 3′ end.

A small amount of DNA synthesis was detected in the presence of either the single- or the double-stranded DNAs alone. As explained in Experimental Procedures, such background synthesis is expected from two different side reactions that are well understood. We conclude that most of the DNA synthesis in our assay is initiated by a genetic recombination-linked mechanism that resembles the one outlined in Figure 1.

Seven T4 Bacteriophage Proteins Are Required for Efficient Recombination-Dependent DNA Synthesis

In addition to requiring homologous single- and double-stranded DNAs, the reaction characterized in Figures 2 and 3 absolutely requires ATP, the four deoxyribonucleoside triphosphates, and the following highly purified T4 proteins: the uvsX protein, gene 43 protein, gene 32 protein, gene 45 protein, and gene 44/62 protein (Table 1, Experiment A). In the absence of any one of these components, less than 10% of the normal amount of total DNA synthesis is obtained; moreover, the products of the reaction are of a different type than those observed with all

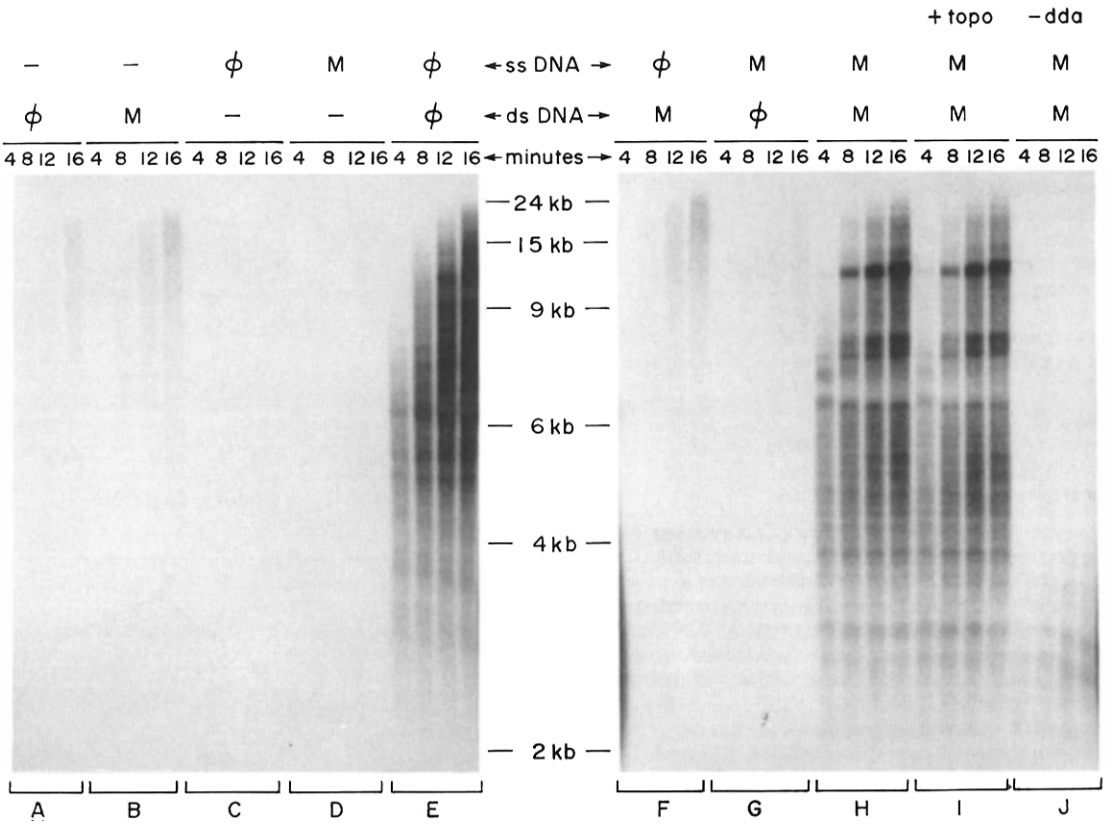

Figure 3. Analysis of the Products of Recombination-Dependent DNA Synthesis

Samples from the reactions shown in Figure 2 were removed at the times shown and were electrophoresed through 0.8% agarose gels under alkaline conditions as described in Experimental Procedures; the gels were then autoradiographed. The M13 (M) single-stranded DNA fragment used as a primer was 766 nucleotides long, and the φX174 (φ) single-stranded DNA fragment used was about 900 nucleotides long. In the lanes labeled "+ topo," 0.1 μg/ml of T4 DNA topoisomerase was added to the reaction. This amount of topoisomerase was sufficient to relax one-half of the original supercoils in the amount of double-stranded DNA present in approximately 1 min. In the lanes labeled "–dda," the dda DNA helicase was omitted from the reaction. Note that, in all of these reactions, discrete-length DNA products transiently accumulate that are different for reactions on the φX174 and M13 DNA templates; such bands reflect the DNA-sequence-specific pausing of the DNA polymerase that is normally detected on double-stranded templates (P. Bedinger, M. Munn, and B. M. A., unpublished results). The molecular weight standards used were bacteriophage λ DNA digested with HindIII and bacteriophage T4 DNA digested with XbaI (not shown).

components present, as judged by the size distribution of products after agarose gel electrophoresis followed by autoradiography (see Experimental Procedures; data not shown). The results in Table 1 (Experiments A and B) and Figures 2 and 3 also show that the reaction is strongly stimulated by the T4 dda protein (a DNA helicase; see Jongeneel et al., 1984b; Krell et al., 1979; Kuhn et al., 1979) and is unaffected by the addition of a DNA topoisomerase. These important aspects of the reaction will be described in detail later.

The observation that the protein products of T4 gene 43 (DNA polymerase) and of genes 44/62 and 45 (DNA polymerase accessory proteins) are all required to observe any recombination-dependent DNA synthesis indicates that the full T4 DNA polymerase "holoenzyme" is used in this reaction. When ATP is omitted from the reaction, recombination-dependent initiation of DNA synthesis is not observed, and instead some of the single-stranded DNA present is replicated in a self-priming reaction. The polymerase accessory proteins, the uvsX protein, and the dda DNA helicase all require ATP hydrolysis to perform their roles (Alberts et al., 1983; Jongeneel et al., 1984a, 1984b; Yonesaki et al., 1985; Hinton and Nossal, 1986; Formosa and Alberts, 1986).

When the rate of recombination-dependent DNA synthesis was measured as a function of the concentration of the uvsX protein added, the results plotted in Figure 4A were obtained. Below about 40 μg/ml of uvsX protein, no recombination-dependent DNA synthesis was observed. Maximum DNA synthesis was observed at about 100 μg/ml uvsX protein (2.5 μM), with higher levels being inhibitory as well as causing a decrease in the size of the DNA product molecules (data not shown).

The synapsis between two homologous DNA molecules that is catalyzed by the uvsX protein occurs at a maximum rate in the presence of 100 to 150 mM potassium acetate (Formosa and Alberts, 1986). However, the replication-fork movement catalyzed by the T4 DNA replication complex is progressively inhibited by increases in ionic strength above an optimal value of about 60 mM potassium acetate (Sinha et al., 1980). The reaction that we are studying displays an optimum at about 90 mM potassium acetate (Figure 4B). Thus, further increasing the efficiency of synapsis by increasing the salt concentration above 90 mM

Table 1. Requirements for Recombination-Dependent DNA Synthesis

Reaction Components	dATP Incorporated (pmol/ml)
Experiment A	
All components added	2670
– all proteins and ATP	3
– 43 protein	12
– 44/62 protein	201
– 45 protein	194
– 32 protein	306
– uvsX protein	119
– dda protein	624
– ATP	172
Experiment B	
All components added	890
– dda protein	150
– dda protein, +41 protein	150

For Experiment A, recombination-dependent DNA synthesis was assayed as described in Experimental Procedures except that dATP was radioactively labeled instead of dCTP. Reaction mixtures were warmed to 37°C for 2 min, and the reaction was then initiated by adding a mixture of the deoxyribonucleotides and the DNAs. After incubation at 37°C for 10 min, an aliquot of each reaction was spotted onto a glass-fiber filter and was washed with trichloroacetic acid as described in Experimental Procedures.

For Experiment B, reactions were carried out similarly except that the 32 protein was present at a lower concentration (50 μg/ml). The 41 protein was tested at 20, 40, or 80 μg/ml with identical results.

potassium acetate is probably counterproductive because of a loss of polymerizing capability.

The Rate of DNA Chain Elongation Is Increased by the dda DNA Helicase but Not by the 41 DNA Helicase

Our standard reaction mixture contains 2 μg/ml dda protein, a level that increases the total amount of DNA synthesis 4- to 6-fold (Table 1 and Figure 2). Further increasing the concentration of this DNA helicase increases the amount of DNA synthesis as judged by incorporation of radioactive nucleotides. However, a side reaction—the initiation of DNA synthesis primed from occasional random nicks in the double helix—is also greatly stimulated by such increases in dda protein concentration (Jongeneel et al., 1984a). This complication can be avoided by using a 5′-end-labeled DNA fragment as the single-stranded primer and by following its increase in length by alkaline agarose gel electrophoresis.

Figure 5 displays the results of such an assay. The addition of increasing levels of the dda protein is seen to cause a marked increase in the lengths of the DNA products produced in a 4 min incubation without having any appreciable effect on the number of radioactive single-stranded DNA fragments that serve as primers. We conclude that increasing the amount of this DNA helicase increases the rate of DNA polymerase movement on the double-helical DNA template in these reactions. The normal T4 DNA replication fork uses the gene 41 protein as its major DNA helicase (Liu et al., 1979; Venkatesan et al., 1982); this protein is not active in stimulating the recombination-dependent mode of DNA synthesis (Table 1, Experiment B).

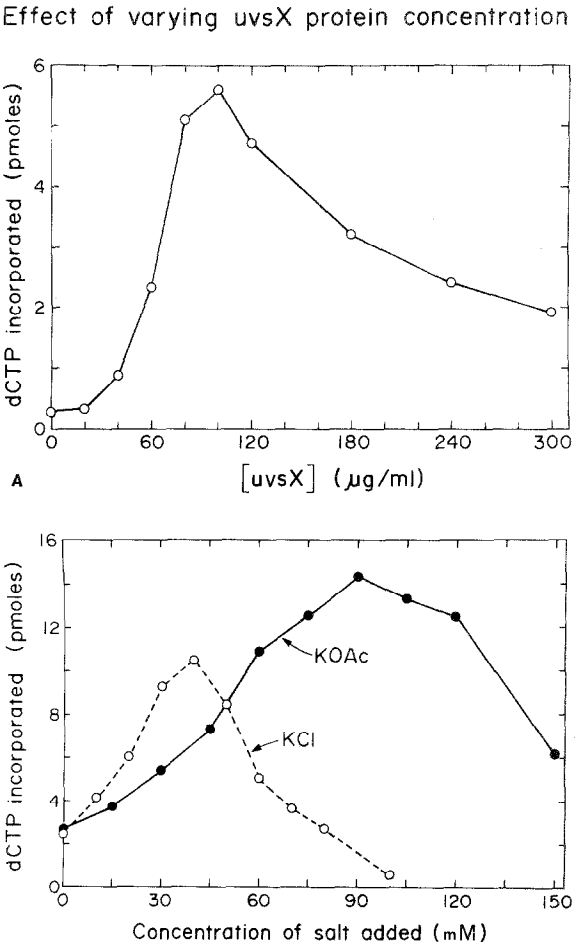

Figure 4. The Effect of Increasing Concentrations of uvsX Protein and Salts on the Recombination-Dependent DNA Synthesis

Recombination-dependent DNA synthesis was assayed as described in Experimental Procedures except that the concentrations of uvsX protein and salt were varied as indicated. (A) All reactions contained 60 mM potassium acetate and the indicated concentration of uvsX protein. After 5 min at 37°C, acid-precipitable label was measured as described in Experimental Procedures; the incorporation of dCTP per 8.3 μl of the reaction mixture is plotted. With regard to the single-stranded DNA present in the reaction, the stoichiometric ratio of one uvsX protein monomer per five nucleotides (Griffith and Formosa, 1985) is attained at 16 μg/ml uvsX protein. (B) All reactions contained 100 μg/ml uvsX protein, and the concentration of either potassium chloride or potassium acetate was varied as indicated. After 8 min at 37°C, the samples were tested for acid-insoluble label; the incorporation of dCTP per 10.7 μl of the reaction mixture is plotted.

Linear DNA Templates Can Also Be Used

The double-stranded template DNA that is used in the in vitro reaction described above does not need to be either supercoiled or circular. Figure 6 presents an autoradiograph that compares the products of reactions using circular (RFI) or linear (RFIII) double-stranded M13 DNA as templates. Similar DNA products are obtained with these two templates at early time points in the reaction. However, whereas the primer molecules can be extended indefinitely on the circular template, on the linear template most of the molecules replicate up to the end of the template and then stop, after being extended to just over full genome length (the primer DNA sequence used over-

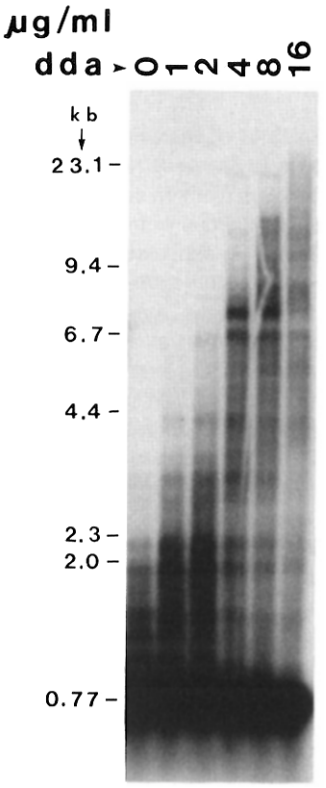

Figure 5. Increasing Concentrations of the T4 dda Protein Increase the Rate of DNA Chain Growth during Recombination-Dependent DNA Synthesis

DNA synthesis was assayed as described in Experimental Procedures except that ligase was omitted, the 766 nucleotide single-stranded HaeIII fragment of M13 ^{32}P-labeled at its 5′ end was used as the primer, and no radioactive nucleotides were present in the reaction. The single-stranded DNA fragment was present at a 1.6-fold molar excess over the complementary strand on the template DNA. The T4 dda protein was added to the concentrations indicated, and the reactions were carried out for 4 min at 37°C before being stopped by the addition of Na_3EDTA to 20 mM and NaOH to 30 mM. The samples were then electrophoresed through a 0.6% agarose gel under alkaline conditions, and the gel was processed for autoradiography as described in Experimental Procedures.

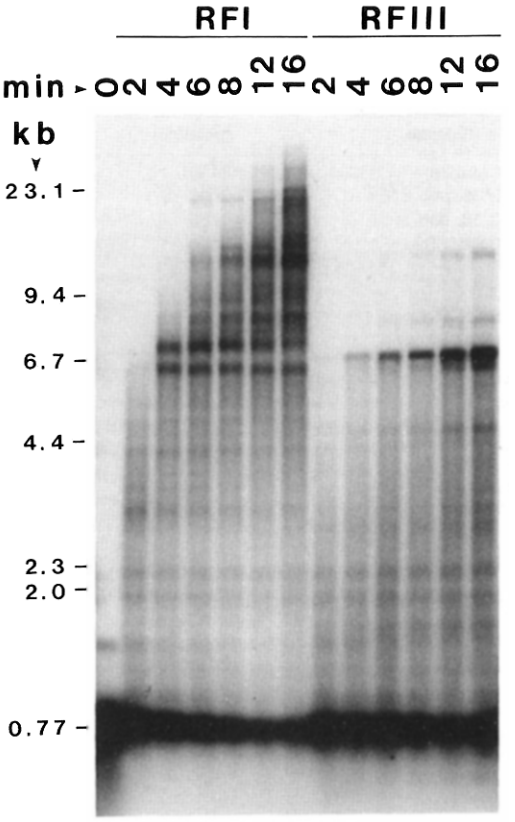

Figure 6. Recombination-Dependent DNA Synthesis Can Use Either a Circular or a Linear Double-Stranded DNA Molecule as the Template

Recombination-dependent DNA synthesis was carried out as described in Figure 5, with the single-stranded primer DNA labeled with ^{32}P at its 5′ end. RFI, reactions on a supercoiled circular double-stranded DNA template; RFIII, reactions on a linear double-stranded DNA template. The single-stranded DNA was present at about 1 μM, and the double-stranded M13 DNA was present at 20 μM (a 1.2-fold molar ratio of double- to single-stranded molecules). After incubation at 37°C, samples were withdrawn at the times indicated and were electrophoresed under alkaline conditions through a 0.6% agarose gel that was then processed for autoradiography as described in Experimental Procedures. DNA synthesis is reflected by the elongation of the original primer molecules, and the rate of elongation can be determined by comparing the average DNA length at each time with molecular weight markers run simultaneously (XbaI-digested T4 DNA and HindIII-digested λ DNA).

lapped the restriction site at which the double-stranded M13 DNA was linearized, and a final product that is 6800 nucleotides long is expected).

In Figure 6 only a minority of the single-strands have been elongated at early times of reaction. However, initiation events continue throughout the course of the reaction, so that the majority of the single strands have served as primers on the circular template by the 16 min time point. These delayed initiations account for the presence of small product molecules throughout the reaction. Somewhat less efficient initiation of synthesis is seen on the double-stranded linear DNA template, most likely because the superhelical tension in the circular template facilitates D-loop formation.

The average rate of DNA chain elongation in this replication system can be estimated at about 30 nucleotides per second from the data in Figure 6 (2 μg/ml of dda protein). The maximum rate observed with higher levels of dda protein (Figure 5) is about 4-fold faster. These rates are slower than those observed with the complete T4 DNA replication system, in which reactions typically start at a nick on a double-stranded DNA template and then create a normal replication fork (Alberts et al., 1983).

The E. coli recA Protein Fails to Substitute for the uvsX Protein

Addition of the E. coli recA protein, which is functionally analogous to the uvsX protein, does not interrupt the standard replication reaction catalyzed by the uvsX protein. However, as shown in Table 2, the recA protein fails to substitute for the uvsX protein, either with or without the E. coli SSB protein present to facilitate recA-catalyzed D-loop formation (Cox et al., 1983a, 1983b; West et al.,

Table 2. A Comparison of the Abilities of the T4 uvsX Protein and the E. coli recA Protein to Promote Recombination-Dependent DNA Replication In Vitro

Extra Proteins Added to the Reaction	dCTP Incorporated (pmol/ml)
uvsX and 32	2150
uvsX, 32, and SSB	2600
uvsX, 32, and recA	1760
uvsX and SSB	100
uvsX	280
32	230
32, SSB, and recA	270

The assay for recombination-dependent DNA synthesis was performed as described in Experimental Procedures, except that the uvsX and 32 proteins were omitted unless indicated otherwise. When present, the uvsX protein, 32 protein, recA protein, and SSB protein were added to final concentrations of 90 μg/ml, 100 μg/ml, 90 μg/ml, and 25 μg/ml, respectively. The SSB protein and the recA protein were gifts from Dr. J. Griffith; a second sample of the recA protein, generously provided by Dr. K. McEntee, gave identical results. Both preparations of the recA protein hydrolyzed ATP with about the reported specific activity, and catalyzed homologous pairing under our conditions. Reactions were incubated at 37°C for 18 min, and an aliquot was assayed for acid-insoluble radioactivity. Agarose gel electrophoresis of these samples indicated that all of the synthesis detected when less than 300 pmol/ml of dCTP was incorporated was caused by initiation from nicks in a small fraction of the circular double-stranded DNA molecules; all such products ran as unit-length or longer DNA strands even at early time points (not shown).

1982; Muniyappa et al., 1984). It therefore seems that the role of the uvsX protein in this form of DNA synthesis extends beyond the initial step of catalyzing formation of the D-loop.

Recombination-Dependent DNA Synthesis Appears to Occur without D-loop Enlargement

The mechanism in Figure 1 predicts that, as synthesis continues around a covalently closed circular template, a DNA topoisomerase will be required to relieve the winding strain introduced into the template DNA by formation of a longer and longer D-loop. However, addition of excess T4 DNA topoisomerase has a negligible effect on both the rate of incorporation of nucleotides (Figure 2) and the rate of DNA chain elongation (Figure 3). None of the other protein components added to the reaction has detectable topoisomerase activity (we observe no relaxation of DNA supercoils in a 30 min incubation under standard reaction conditions). It therefore appears that little, if any, winding strain accumulates ahead of the replication complex in this reaction, even though DNA products much longer than the circular template molecule are readily formed.

Direct examination of the products of the recombination-dependent DNA synthesis reaction by electron microscopy has proved difficult because large networks of DNA are formed under typical reaction conditions. (The same networks form with only uvsX protein and double-stranded DNA present.) Attempts to unravel this network are in progress; to date, those D-loops detected have all been about the same size (typically about 600 nucleotides long), irrespective of the extent of DNA synthesis (unpublished results of Mei Lie Wong, T. F., and Tom Kodadek). Both the lack of a requirement for a DNA topoisomerase and our failure to observe large D-loops suggest that the D-loop fails to enlarge as DNA synthesis proceeds.

The Major Product of Recombination-Dependent DNA Synthesis Is Single-Stranded DNA

Since the replication reaction can proceed to copy a circular template several times without interruption, the newly made DNA would be expected to be released from the template as a single strand after the first round of replication. However, when the template is a linear double-stranded DNA molecule, all of the newly made DNA should remain in a double-stranded form if it is formed by a semiconservative mechanism. When we treated the products of the recombination-dependent DNA synthesis reaction with the single-strand-specific nuclease S1 (Table 3), most of the DNA product was sensitive to S1 whether the template was circular or linear, suggesting that most of this DNA is single-stranded even when it is synthesized on a linear template.

These results, along with the failure of the D-loop to elongate, suggest the "bubble-migration" mode of DNA synthesis shown schematically in Figure 7. In this model, the D-loop propagates along the template helix without enlarging during DNA synthesis, while the new DNA chain is quickly displaced as a single-strand.

The uvsX protein resembles the recA protein in catalyzing unidirectional branch migration (Yonesaki and Minagawa, 1985), and one would expect this reaction to reanneal the back of the D-loop, removing the nascent DNA from its template as shown in Figure 7. Results obtained with the recA protein (Register and Griffith, 1985) suggest that this branch migration may be driven by the cooperative 5′-to-3′ assembly of a uvsX protein–DNA filament, which can be postulated to occur along the single strand that is ejected at a branch point. In this case, the rate at which the back side of the D-loop moves should increase as the concentration of the uvsX protein increases.

Rapid Dilution Reveals a Role for the uvsX Protein in the Elongation Phase of DNA Synthesis

Initiation of DNA synthesis in our system is not observed below a threshold concentration of the uvsX protein (Figure 4A). We have therefore tested for a role for the uvsX protein during the elongation phase of DNA synthesis by examining the effect of lowering the uvsX protein concentration by dlution after DNA synthesis begins. Typically, a 5-fold dilution was made into a solution of identical protein and salt composition, but lacking the uvsX protein. In some experiments the initial concentration of double-stranded DNA was maintained by its addition to the diluent; in other experiments this DNA concentration was reduced 5-fold along with the concentration of the uvsX protein. For comparison, the DNA concentration alone was reduced in some experiments.

Examination of the rate of incorporation of labeled nucleotides into acid-precipitable material (data not shown) permits the following conclusions: First, when the diluent contains both high concentrations of uvsX protein and double-stranded DNA, efficient DNA synthesis con-

Table 3. The Product of Recombination-Dependent DNA Synthesis Is Single-Stranded DNA

Reaction Number	DNA	% Resistant to S1 Nuclease Digestion		Estimated % Single-Stranded
		Native	Denatured	
1)	Double-stranded calf thymus DNA, labeled by chew-back and fill-in (control)	88.2	10.5	–
2)	Double-stranded circular M13 DNA synthesized in vitro, labeled in the minus strand (control)	119	25.4	–
3)	Product of recombination-dependent in vitro DNA synthesis on a double-stranded circular template	47.9	26.4	69
4)	Product of recombination-dependent in vitro DNA synthesis on a double-stranded linear template	42.3	29.4	77

DNA was synthesized under the conditions described in Experimental Procedures, using either linear double-stranded M13 DNA or intact circular M13 DNA as the template. The reactions were incubated for 15 min at 37°C and were stopped by adding sodium dodecylsulfate to 0.22%. As shown in Figure 6, the products of this reaction are longer than full-length M13 when the template is circular, and full-length (or less) when the template is linear. As controls, double-stranded DNA was synthesized either on a nicked calf thymus DNA template using the T4 proteins 32, 43, 44/62, and 45, or on an intact circular single-stranded M13 DNA template with the T4 proteins 43, 44/62, 45, and helicase-primase (41 and 61) under conditions that prohibit strand displacement.

Digestions with S1 nuclease were performed as described in Experimental Procedures. Note that, whereas the calf thymus DNA was about 90% digested by S1 nuclease after denaturation (reaction 1), all DNAs derived from M13 showed about 25% resistance to digestion. This may be due in part to the ease of renaturation of M13 DNA, caused by its lack of sequence complexity. In any case, our double-stranded M13 DNA control (reaction 2) was completely resistant to digestion, whereas the product of the recombination-dependent synthesis reaction was 50%–60% digested whether the template was linear or circular (reactions 3 and 4). Since the double-stranded M13 DNA control was completely resistant in its native conformation and about 25% resistant when denatured, we assume that 75% of the DNA was available to be degraded in reactions 3 and 4; therefore, the "estimated % single-stranded" values shown were obtained by dividing the percentage of degradation found for the native samples by 75. The major fraction of the DNA synthesized in the recombination-dependent reaction is thus judged to be single-stranded, even when no superhelical tension can be induced in the template (reaction 4).

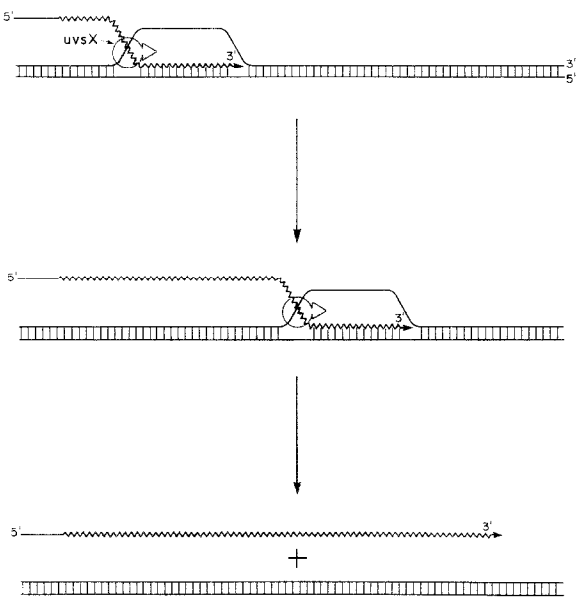

Figure 7. The Bubble-Migration Model for Recombination-Dependent DNA Replication

Recombination-dependent initiation of DNA synthesis is proposed to proceed as depicted in Figure 1 except that the D-loop is only briefly enlarged by DNA synthesis. The trailing edge of the bubble is continuously reannealed because of the branch migration catalyzed by the uvsX protein, as shown. The result of strand separation at the leading edge of the bubble, caused by DNA synthesis, and this reannealing at the trailing edge is a migrating "replication bubble" that produces a free, single-stranded DNA product. The replication bubble maintains a constant size as long as the strand-separation and reannealing reactions proceed at equivalent rates, as shown at top and center. However, if the reannealing reaction rate exceeds the rate of primer elongation, as expected at high uvsX protein concentrations (see text), the replication bubble shrinks, and DNA synthesis halts when the growing strand is displaced from the template (bottom). The termination of chain elongation is expected to occur predominantly at those sites on the DNA template where the DNA polymerase tends to pause.

tinues at a fairly constant rate (control reaction). Second, when the double-stranded DNA concentration but not the uvsX protein concentration is reduced by dilution, DNA synthesis is strongly impaired. Third, when the uvsX protein concentration but not the DNA concentration is reduced, DNA synthesis continues normally at early time points, but gradually diminishes in rate. Fourth, and most surprisingly, when both the double-stranded DNA and uvsX protein concentrations are reduced, the level of DNA synthesis is somewhat greater than that obtained when the double-stranded DNA concentration is reduced and the uvsX protein concentration is maintained. In other words, at a low concentration of the double-stranded DNA template, maintaining the normal uvsX protein concentration inhibits DNA synthesis, whereas one might have expected it to stimulate synthesis by allowing more initiation events.

Alkaline agarose gel electrophoresis of the products of these reactions reveals the basis for the above observations (Figure 8). When the uvsX protein concentration is maintained at a high level but the double-stranded DNA concentration is reduced (Figure 8, series A), the products are unusually short DNA fragments. Leaving the double-stranded DNA concentration high in these reactions leads to more DNA synthesis and a greater average product size: not only are more single-stranded primer molecules elongated, but each, on the average, is elongated further (Figure 8, series B). Control experiments in which double-

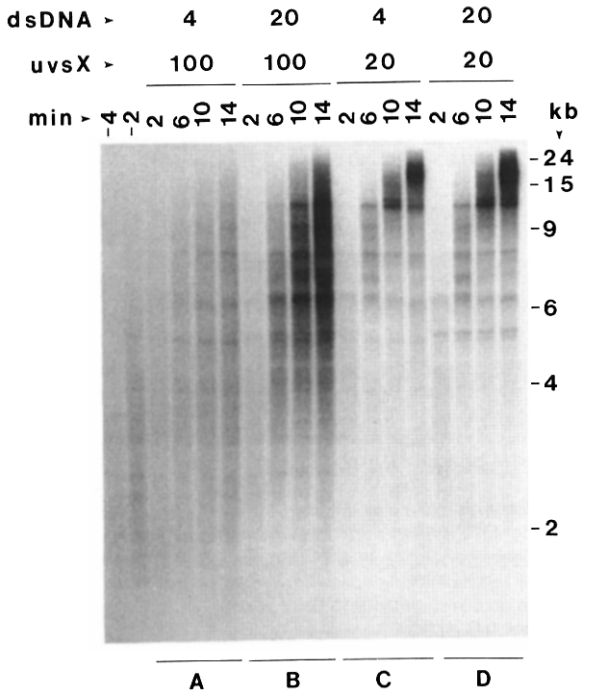

Figure 8. The Effect of Dilution on the Continuation of Recombination-Dependent DNA Synthesis

Recombination-dependent DNA synthesis was begun on a circular template as described in Experimental Procedures except that gene 32 protein was present at 100 μg/ml. After 6 min of synthesis at 37°C, 10 μl samples were withdrawn and diluted 5-fold into a reaction mixture identical to the original one except that no single-stranded DNA fragments were present. The final concentrations of uvsX protein and double-stranded DNA after dilution were as follows: (A) 100 μg/ml uvsX protein and 4 μM dsDNA; (B) 100 μg/ml uvsX protein and 20 μM dsDNA; (C) 20 μg/ml uvsX protein and 4 μM dsDNA; and (D) 20 μg/ml uvsX protein and 20 μM dsDNA. When uvsX protein was omitted from the diluent, its final concentration after dilution was 20 μg/ml, which is too low to allow the initiation of recombination-dependent DNA synthesis (Figure 4A). Samples were withdrawn at the times indicated, electrophoresed under alkaline conditions through a 0.8% agarose gel, and autoradiographed as described in Experimental Procedures. (The negative numbers in the left lanes indicate times before dilution.)

stranded nonhomologous DNA was added showed no effect (Table 4); therefore, the faster average rate of DNA chain growth seen in Figure 8, series B reflects some homology-dependent reaction. We conclude that there is a constant stopping and restarting of DNA synthesis at the high uvsX protein concentration, and that the increased synthesis seen at high DNA concentrations is due to efficient restarts of recently terminated single-stranded DNA chains on a second DNA template. Such restarts are expected to be favored at a high double-stranded DNA concentration, at which the collisions between homologous primer (single-stranded DNA) and template (double-stranded DNA) molecules needed to form new D-loops are more frequent.

In series C and D (Figure 8), the uvsX protein is not present in the diluent, and the uvsX concentration is dropped to a level at which the formation of all new D-loops is blocked (data not shown); therefore, only the continued elongation of those replicating molecules already present

Table 4. At High Concentrations of the uvsX Protein, High Concentrations of Homologous Double-Stranded DNA Are Required to Maintain Vigorous DNA Synthesis after Dilution of Replicating Complexes

Duplex DNA Added to Diluent	dCTP Incorporated (nmol/ml)
M13 double-stranded DNA	1.31
pBR322 double-stranded DNA	0.42
No double-stranded DNA	0.51

Initial reactions were performed as described in Experimental Procedures. After a 6 min incubation at 37°C, the replicating complexes were diluted 1:5 with a reaction mixture of identical composition except that the single-stranded DNA was omitted, and double-stranded DNA was added as noted to maintain the original double-stranded DNA concentration; the uvsX protein concentration was maintained at 100 μg/ml. An aliquot was removed 30 min after dilution and was precipitated as described in Experimental Procedures.

is observed. As expected, whether there is a high or low concentration of double-stranded DNA present after the dilution has very little effect on the size of the products formed (Figure 8, series C and D). The striking result is that, at the low concentration of double-stranded DNA, a decrease in the uvsX protein concentration results in longer DNA products (Figure 8, series A vs. C).

We interpret this experiment as evidence that the uvsX protein plays an active role not only in the initiation of DNA synthesis but also during the subsequent growth of the DNA chain. Apparently, DNA synthesis is not very processive in the presence of high concentrations of the uvsX protein (where "processivity" is the number of times that a single polymerase molecule adds a nucleotide without dissociation), and the growing DNA chain is frequently released from the double-stranded template as a free single strand. New collisions between the growing 3′-OH end and a homologous double-stranded template molecule are therefore required at frequent intervals to restart DNA synthesis. In contrast, at the lower concentration of uvsX protein, DNA synthesis is highly processive, and each DNA chain keeps growing for thousands of nucleotides on the same DNA template molecule.

The uvsX protein, at concentrations up to 200 μg/ml, has no effect on the rate of DNA chain elongation when semiconservative DNA synthesis proceeds from a nick on a circular template, as occurs in the standard reaction catalyzed by the T4 DNA polymerase holoenzyme in the presence of the 32 protein (data not shown). Yet, even 100 μg/ml of uvsX protein profoundly decreases the processivity of the recombination-dependent DNA synthesis reaction (Figure 8). It seems reasonable to conclude that this decrease is caused by an increased rate of migration of the back side of the D-loop at high uvsX protein concentrations, as predicted from the scheme shown in Figure 7.

The Extent of Polymerization in the Recombination-Dependent Reaction Is Increased by Increasing the Rate of DNA Synthesis

In the bubble-migration model, DNA synthesis should halt whenever the uvsX-catalyzed reannealing of the template strands overtakes the moving DNA polymerase molecule,

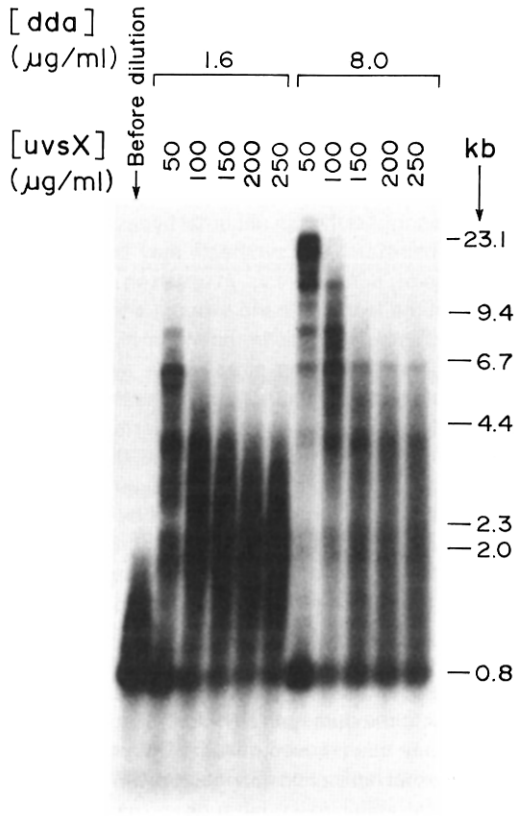

Figure 9. Increasing the Rate of DNA Chain Growth Increases the Processivity of DNA Synthesis

Recombination-dependent DNA synthesis was initiated as described in Experimental Procedures, with the 766 base HaeIII fragment of single-stranded M13 DNA labeled at the 5′ end serving as the primer (dda protein and ligase were omitted). The reaction was allowed to proceed under these conditions for 5 min at 37°C to synchronize chain starts; aliquots were then diluted 5-fold into solutions of identical composition except that the DNAs were omitted, dda protein was added to a final concentration of either 1.6 or 8 μg/ml, and the final concentration of the uvsX protein was varied as indicated. The reaction was continued at 37°C for 10 min and then stopped and analyzed by electrophoresis through an alkaline agarose gel. The product size here directly reflects the processivity of DNA synthesis, since the initiation of new DNA chains is very inefficient after dilution because of the low concentrations of both primer and template DNAs.

thereby causing the growing DNA single strand to dissociate from its template. If this view is correct, increasing the rate of movement of the DNA polymerase should lead to fewer dissociations and thus produce longer DNA product chains. Since high concentrations of the dda protein increase the rate of DNA synthesis in the recombination-dependent DNA synthesis assay, we tested our model by starting DNA synthesis in the absence of the dda protein, and examining the effect on subsequent DNA synthesis of adding different amounts of dda protein. As shown in Figure 9, under conditions where reinitiations are blocked, increasing the rate of polymerization at a low uvsX protein concentration greatly increases the average product length; a similar, but less pronounced, effect is seen at higher uvsX protein concentrations. Thus, the growing DNA chains proceed further before stopping when the rate of DNA polymerase movement is increased.

According to Figure 7, one might expect the large increase in polymerization rate that is caused by a high concentration of the dda protein to prevent all disruption of DNA synthesis caused by high uvsX protein concentrations. However, at 8 μg/ml dda protein in the experiment shown in Figure 9, high uvsX protein concentrations still inhibit DNA synthesis; in fact, the average time that each fork persists seems not to be increased significantly by addition of the dda protein. To explain these results, it seems necessary to propose that some mechanism tends to keep the size of the replication bubble small even at an elevated polymerization rate. For example, the bubble might be bound up in a large protein–DNA complex of defined dimensions, or the dda protein might increase the reannealing rate at the back of the bubble in addition to increasing the polymerization rate. As predicted by the latter hypothesis, the dda protein has been recently found to accelerate the directional strand-exchange reaction that is catalyzed by the uvsX protein (unpublished results of Tom Kodadek, this laboratory).

Discussion

The Linkage of Recombination and Replication Produces a Conservative Mode of DNA Synthesis

We have discovered that a combination of bacteriophage T4 proteins will catalyze a novel type of DNA synthesis in vitro. We interpret our results as support for the bubble-migration DNA synthesis mechanism shown in Figure 7. As in the mechanism proposed in Figure 1, the 3′ end of a single-stranded DNA fragment invades a double-stranded DNA molecule at a complementary sequence, and this 3′ end serves as a primer that allows the single-stranded DNA fragment to be elongated. However, instead of DNA synthesis continuously enlarging the D-loop, the replication complex catalyzes a reannealing of the template strands after synthesis has occurred, releasing the newly formed DNA as a single strand. This is therefore a conservative rather than a semiconservative mode of DNA synthesis. The replication bubble (D-loop) retains a fairly constant size during the course of the reaction, while it is translocated along the template molecule. Therefore, superhelical density does not accumulate ahead of the replication complex on a circular template, and no DNA topoisomerase is required.

The uvsX Protein Actively Dissociates the Growing DNA Strand from Its Template

Using dilutions to lower the template concentration and thus to prevent the reinitiation of DNA synthesis on terminated primer chains, we showed that the processivity of the replication reaction decreases at high concentrations of uvsX protein. Since the uvsX protein does not affect the normal DNA replication fork, we propose that this protein catalyzes the reannealing of the template strands at the back of each replication bubble at a rate that increases with uvsX concentration. At high uvsX protein concentrations the trailing edge of the replication bubble advances

relative to the leading edge, which shrinks and eventually collapses the bubble and causes the release of the nascent DNA chain. This release is only mildly inhibitory if the concentration of double-stranded template is high, since synthesis is rapidly reinitiated, but it is very inhibitory if reinitiation is slowed by diluting this template.

Evidence for a Unique Protein Machine

Protein-affinity chromatography has revealed specific interactions between the uvsX protein and the 32 protein, as well as between both of these proteins and the dda protein (Formosa et al., 1983; Formosa and Alberts, 1984). We interpret the inability of E. coli homologs to substitute for the uvsX and 32 proteins as evidence that the T4 proteins function as components of a discrete multiprotein complex whose parts function together as a "protein machine" (Alberts, 1984).

The Role of Bubble-Migration DNA Synthesis in T4 Bacteriophage Multiplication

Genetic evidence suggests that a recombination-dependent form of DNA replication plays an important role in the life cycle of bacteriophage T4 (Luder and Mosig, 1982; Mosig, 1983). Because mutations in the uvsX gene cause a 3-fold reduction in the total amount of T4 DNA synthesis at late times, a large amount of DNA must be synthesized by a uvsX-dependent mechanism (Bernstein and Wallace, 1983; Dewey and Frankel, 1975; Cunningham and Berger, 1977). We suggest that the uvsX-dependent reaction we have described in vitro models a conservative mode of DNA synthesis that occurs in vivo. If this synthesis generates single-stranded DNA products inside of the cell, these new strands would rapidly reanneal to form double-stranded progeny DNA molecules—if we assume that the single-stranded DNA becomes freed from the uvsX protein and is covered with the gene 32 protein instead (Alberts and Frey, 1971). It is also possible that additional T4 proteins recognize the single-stranded DNA emanating from the migrating bubble, and assemble a mobile RNA primase there. In this case, a special "replication fork" would result that generates one double-stranded progeny molecule by conservative DNA synthesis.

The bubble-migration mechanism is not new—RNA polymerase has long been known to produce RNA transcripts in a reaction that involves a transient base-pairing interaction with the template—but its implications for DNA metabolism have not previously been explored. Some of these implications are therefore briefly discussed below.

Linkage of Recombination and DNA Synthesis during "Translesion" DNA Synthesis

Many bulky lesions in DNA, such as thymine dimers, disrupt DNA replication in vivo, presumably because they cause the replication machinery to stall (Setlow et al., 1963; Moore et al., 1981; Villani et al., 1978). The replication machinery is often able to proceed past such damaged DNA eventually and to continue normal replication without the repair of the lesion. Because E. coli can live with a large number of UV-induced pyrimidine dimers in its genome without a major increase in replication errors (Walker, 1985), any mechanism used for translesion DNA synthesis must produce the strand complementary to the dimer by copying an undamaged region of a second DNA template. Genetic studies in E. coli suggest that this type of DNA synthesis requires genetic recombination functions, including the direct participation of the E. coli recA protein (see Friedberg, 1985).

Our data suggest that an accurate bypass of DNA damage by translesion DNA synthesis may involve a "copy-choice" type of DNA synthesis, in which an encounter with damage on the leading strand triggers a transient switch to a second DNA template. In the model illustrated in Figure 10, whenever leading strand DNA synthesis stalls, spontaneous branch migration displaces the 3′ end of the growing DNA chain and thereby converts it to a single-stranded form (step 1). As a single strand, this 3′ end eventually invades an homologous DNA sequence on another DNA template and becomes extended by bubble-migration DNA synthesis, as previously described. Because this type of synthesis is transient, it releases the 3′ end in an elongated form (step 2). Protein-directed branch migration (which acts to "pin down" the 3′ end and to release the 5′ end for both the E. coli recA protein and the T4 uvsX protein) will then rapidly reanneal the newly synthesized DNA strand back to the damaged DNA template (step 3). Once the 3′ end has been moved past the DNA damage in this way, the normal replication fork restarts, having accurately bypassed the initial lesion (step 4).

The second DNA molecule used transiently as a template to continue DNA synthesis past a lesion, as shown in Figure 10, need not always be exactly identical in sequence to the DNA molecule that was damaged. The proposed mechanism would therefore be expected to alter blocks of DNA sequences occasionally by a gene-conversion process.

The above mechanism seems especially useful for explaining the process known as "multiplicity reactivation," in which the undamaged genetic information from several heavily damaged copies of a bacteriophage genome that coinfect a cell is pooled to produce one good copy (Luria, 1947; Luria and Dulbecco, 1949). According to the mechanism shown in Figure 10, bubble-migration DNA synthesis would automatically cease at any site of DNA damage on a second template, releasing a free single strand and forcing synthesis to switch back to the original template. In this way, a single undamaged DNA molecule could eventually be generated by many cycles of template switching that would have the effect of summing only the good nucleotide sequences present in different DNA molecules. Although the product is genetically a recombinant (as observed), extensive breakage and rejoining of the parental DNA strands is not required.

While differing in detail, several earlier models for translesion DNA synthesis contain features similar to ours (Higgins et al., 1976; Fujiwara and Tatsumi, 1976; Clark and Volkert, 1978). An attractive feature of the Clark and Volkert (1978) model is that the damage bypass occurs only after the undamaged parental DNA strand (the lagging strand) has been copied past the point of damage, which would make the daughter double helix available

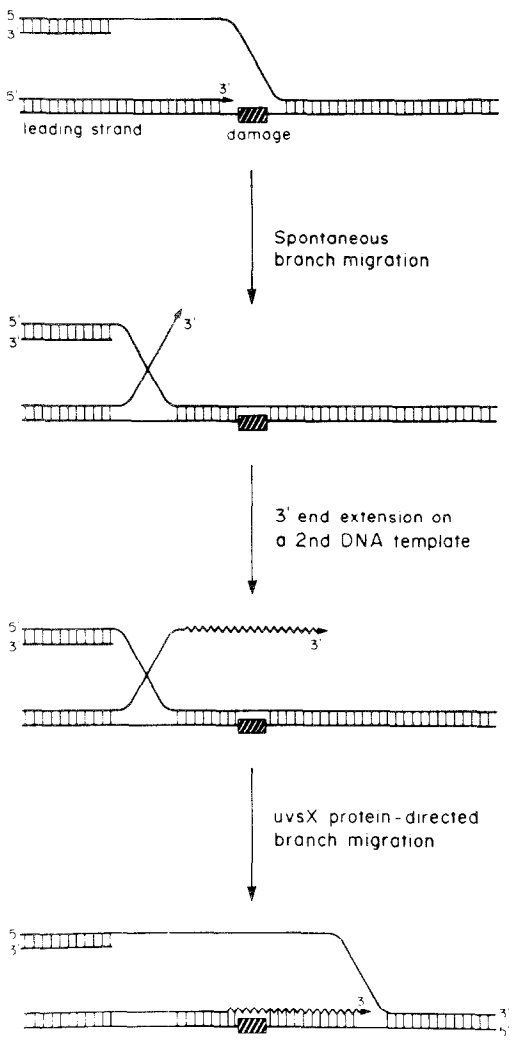

Figure 10. A Model for Translesion DNA Synthesis

DNA damage is encountered by the leading-strand DNA replication complex, causing both leading and lagging strand synthesis to halt. After the 3′ end of the leading strand is displaced as a single strand by spontaneous branch migration, this 3′ end is transiently extended by recombination-dependent synthesis on a second DNA template (as in Figure 7). The model requires that an intact double-stranded copy of the damaged sequence be present in the cell to serve as this second template (not shown). See text for details.

as the homologous template for the transient bubble-migration DNA synthesis that we propose. (Note that in this case leading- and lagging-strand synthesis must become uncoupled, with lagging-strand synthesis temporarily extending ahead of leading-strand synthesis). While probably not important in a T4-infected cell, where multiple copies of each DNA sequence should be present to serve as the template, such a switch to the lagging strand as the template best explains translesion DNA synthesis in higher eukaryotic cells—where finding a homologous chromosome is presumably difficult because of the complexity of the genome and the low copy number of most sequences. Even in a lower eukaryote, it has been reported that the repair of X-ray-induced lesions occurs preferentially from sister chromatids (Fabre et al., 1984; Resnick, 1979).

Recombination-Initiated DNA Synthesis in Double-Strand Break Repair

Evidence from the yeast Saccharomyces cerevisiae suggests that under certain conditions double-strand breaks in DNA are very efficiently repaired, often with concurrent conversion of the original sequence to a new sequence (Orr-Weaver et al., 1981; Szostak et al., 1983; Resnick, 1976).

The mechanism of DNA synthesis illustrated in Figure 7 suggests a simple mode of double-strand break repair, in which the 3′ ends at opposite sides of a break in a double-stranded DNA molecule are independently extended by bubble-migration DNA synthesis on a homologous DNA template that is present in the same cell. Once the 3′ ends are extended far enough, the new single-stranded DNA chains produced, being complementary in sequence, should anneal to form a double helix that fills in the gap in the DNA sequence. Leftover single-stranded "whiskers" would be trimmed by nucleases, leaving only two nicks to be sealed by DNA ligase to effect complete repair of the double-strand break. This model differs from previous ones (see Szostak et al., 1983) in predicting that double-strand-break repair usually proceeds without recombination between flanking markers. While such recombination is sometimes associated with repair events of this type, flanking-marker exchange is avoided during the double-strand break repair process that mediates yeast mating-type switching (Klar and Strathern, 1984), and it is not associated with intrachromosomal gene conversion (Fink and Petes, 1984; Klein and Petes, 1981; Jackson and Fink, 1981; Klein, 1984).

Is Recombination-Initiated DNA Synthesis Involved in Telomere Maintenance?

Telomeres are specialized DNA sequences occurring at the ends of linear eukaryotic chromosomes; they are required to stabilize linear DNA molecules, presumably by providing a means for completely copying their ends (see Blackburn and Szostak, 1984). The mechanism that we have described for 3′-end extension by bubble migration presumably represents at least part of the mechanism that bacteriophage T4 uses to solve its end-replication problem. In eukaryotes a similar mechanism could be used, in which the 3′ end of each linear DNA chromosome would be extended by a recombination-initiated synthesis that uses either another homologous telomere or a repeated DNA sequence in the same telomere as the template. Once the 3′ end is extended in this way, retrograde synthesis could be primed on the resulting single-stranded tail to complete the end of the double helix.

A mechanism of this type is consistent with the observed multiple repeats of DNA sequences at telomeres, with the observed homologies between the telomeres of different chromosomes (see Blackburn and Szostak, 1984), and with the heterodisperse lengths found for the terminal fragments of cloned telomeres (Shampay et al., 1984). Some other recent findings, including the observation of a template-independent terminal addition reaction in Tetrahymena, are less easily explained by such a simple mechanism (Shampay et al., 1984; Pluta et al., 1984;

Greider and Blackburn, 1985). However, it should be recognized that the faithful maintenance of telomeres is likely to require additional components even if the basic scheme is correct.

Experimental Procedures

DNAs

Bacteriophage DNAs were prepared as described elsewhere (Formosa and Alberts, 1986). Specific fragments of single-stranded DNAs were obtained by digesting the single-stranded circular, viral form of the DNA of bacteriophages M13 and φX174 with HaeIII restriction nuclease, purifying each fragment by agarose gel electrophoresis, and then extracting the DNA obtained with a mixture of phenol, chloroform, and 3-methylbutanol (50:50:1), followed by several extractions with diethyl ether (Yang et al., 1979). The DNA fragments were then precipitated with ethanol, collected by centrifugation, washed with 70% ethanol, dried, and resuspended in 10 mM Tris–acetate (pH 7.4) and 1 mM Na_3EDTA.

When M13 single-stranded DNA was treated by the above procedure, single-stranded DNA fragments of the lengths expected for a HaeIII digest of the double-stranded form of M13 DNA were obtained, plus an additional 766 nucleotide fragment that appears to be an unusual product since its sequence contains two HaeIII sites (nucleotide 5414 to nucleotide 6180). This fragment was not appreciably affected by further exposure to HaeIII. It may represent a region of the single-stranded DNA that is relatively free of secondary structure and therefore is very poorly cut by this restriction enzyme (Wells and Neuendorf, 1981). The 766 nucleotide DNA fragment was used for most of the studies reported here because it is a poor template for self-primed DNA synthesis, and it therefore displays a low background of DNA synthesis.

DNAs were labeled at their 5′ ends using T4 polynucleotide kinase (New England Biolabs) and [α-^{32}P]ATP (Amersham) as described elsewhere (Formosa and Alberts, 1986; Maniatis et al., 1982). All DNA concentrations are expressed as the molarity of total nucleotides.

Assay for Recombination-Dependent Initiation of DNA Synthesis

The T4 proteins used were at least 98% homogeneous and were free of detectable endonucleases; they were prepared as described elsewhere (Jongeneel et al., 1984a; Formosa and Alberts, 1986). A typical reaction mixture contained 20 mM Tris–acetate (pH 7.4), 10 mM magnesium acetate, 60 mM potassium acetate, 1 mM dithiothreitol, 100 μg/ml human serum albumin (Sigma), 10 mM creatine phosphate (Sigma), 10 μg/ml phosphocreatine kinase (Sigma), 0.5 mM spermine, 0.3 mM spermidine, 3.8 μg/ml of T4 gene 43 protein, 25 μg/ml of gene 44/62 protein, 25 μg/ml of gene 45 protein, 85 μg/ml of gene 32 protein, 2 μg/ml of dda protein, 100 μg/ml of uvsX protein, and 20 μM supercoiled double-stranded DNA circles (where noted, linear molecules produced by AvaI treatment were substituted for intact circles). Except where noted, reactions also contained 1–2-units/ml of T4 ligase (BRL) to reduce synthesis from nicks present in a minor fraction of the DNA circles. After this mixture was incubated at 37°C for 2 min, the reaction was started by adding 150 μM each of dATP, dGTP, dTTP, and [α-^{32}P]dCTP (spec. act. = 800 cpm/pmol), 2 mM ATP, and 2 μM single-stranded DNA fragments. At various times thereafter, samples were withdrawn, made 25 mM in Na_3EDTA, and held on ice. When all samples were collected, a portion of each was spotted onto a glass-fiber filter disc (Whatman, GF/A, 2.1 cm), which was then immersed in 5% trichloroacetic acid containing one-tenth volume of saturated sodium pyrophosphate at 0°C and was held for a minimum of 10 min to precipitate any DNA synthesized. The discs were then batch-washed three times with 1 M HCl at 4°C and twice with ethanol, dried with a heat lamp, and placed in vials containing scintillation fluid (Liquiscint, National Diagnostics) for counting. The remainder of the reaction mixture was fractionated by agarose gel electrophoresis under denaturing (alkaline) conditions.

Since DNA polymerases can add nucleotides only to preexisting primers, and since no RNA primers can be formed here because of the absence both of RNA primase and of three out of the four normal ribonucleoside triphosphates, DNA synthesis in this system can only occur at a 3′ DNA end. Such a primer can be provided in these reactions either by the single-stranded DNA fragments or by occasional nicks in the double-stranded DNA template. In our standard reactions the DNA synthesis primed at such nicks is suppressed by adding a mixture of spermine, spermidine, and ligase to the reaction (C. V. Jongeneel, personal communication). However, even with this precaution a small amount of synthesis from nicks is observed. The products of this side reaction are readily identified by gel electrophoresis, since adding even a single nucleotide to a nick in a double-stranded circular DNA molecule produces a long DNA molecule: even at very early time points, the smallest product will be just larger than full genome length (6407 bases for M13, 5386 bases for φX174). This behavior is observed when double-stranded DNA is incubated without homologous single-stranded DNA fragments in the assay for recombination-dependent DNA synthesis (Figure 3). However, when homologous single- and double-stranded DNAs are present, the initial products of the reaction are seen to be much smaller than full genome length, which shows that nearly all of the DNA synthesis starts from the 3′ ends of the single-stranded DNA molecules.

Another possible side reaction is the self-priming that can occur on linear single-stranded DNA molecules (Englund, 1971). Synthesis of this type produces a unique product that runs at approximately twice the size of the original single-stranded molecule on an alkaline agarose gel. (Because the DNA polymerase moves rapidly in the presence of 32 protein [Huberman et al., 1971; Huang et al., 1981], little or no intermediate-sized product appears.) In our assays, there is almost no DNA synthesis when the double-stranded DNA is omitted from the reaction (see above), and traces of the product expected from self-priming are barely detectable by agarose gel electrophoresis and autoradiography (Figure 3).

Alkaline Agarose Gel Electrophoresis

Gels containing 0.6% or 0.8% agarose (Seakem ME) in 30 mM NaOH and 2 mM Na_3EDTA were prepared in a horizontal 15 cm × 15 cm gel box containing a well comb and were allowed to harden for several hours. The gels were overlaid with anode solution (30 mM NaOH, 2 mM Na_3EDTA), and the anode compartment was filled with anode solution. The cathode compartment was filled with cathode solution (60 mM NaOH, 2 mM Na_3EDTA). Samples were prepared in a final volume of 10 μl containing final concentrations of 30 mM Na_3EDTA, 10% sucrose, 30 mM NaOH, and 0.04% bromcresol green. After the samples were loaded into the submerged gel wells, excess buffer was removed from both the anode and cathode compartments so that only the two ends of the gel were in contact with buffer. Electrophoresis was performed for about 18 hr at 20 V, which brought the dye to the end of the gel. For staining, the gels were soaked twice for 10 min in 50 mM Tris–HCl (pH 7.4), 10 mM $MgCl_2$, followed by 20 min in 50 mM Tris–HCl (pH 7.4), 2 mM Na_3EDTA, and finally 20 min in the same buffer containing 1 μg/ml of ethidium bromide. Stained gels were photographed with ultraviolet illumination using Polaroid film (Type 55). Gels that were not stained were washed briefly in 50 mM Tris–HCl (pH 7.4), dried under vacuum, and autoradiographed using Kodak XAR-5 film exposed at −70°C with an intensifying screen.

S1 Nuclease Digestions

To determine whether the radioactively labeled products were double- or single-stranded (Table 4), 50 μl of a reaction mixture was added to 10 μl of a 20× buffer (containing 4.4% SDS, 220 mM bicine [pH 9.2]) and was incubated at room temperature for 10 min to dissociate DNA–protein complexes. Then, 140 μl of water was added to this mixture, and the sample was split into two aliquots: one aliquot was boiled for 6 min and then chilled on ice (denatured), and the other was left alone (native). Twenty-five microliters of 5× S1 buffer (0.3 M sodium acetate [pH 4.6], 0.5 M NaCl, 50% [w/v] glycerol, 50 mM $ZnCl_2$) was added to each sample (total volume 125 μl). One 60 μl aliquot of this solution was added to 1 μl of S1 nuclease (PL Biochemicals; about 500 units), and an identical aliquot was added to 1 μl of S1 buffer. Both were incubated at 37°C for 10 min, and two 25 μl aliquots of each reaction mixture were then spotted onto filters and precipitated with trichloracetic acid. Filters containing only unlabeled DNA were used to calculate the background level of nonspecific binding to the filters; values obtained with and without S1 nuclease were used to determine the percentage of undigested material.

Acknowledgments

We thank K. McEntee and J. Griffith for recA and SSB proteins, Tom Kodadek and Doug Koshland for helpful comments, and Kathleen Rañeses for preparation of the manuscript. This research was supported by a grant from the National Institutes of Health (GM24020).

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Received May 28, 1986; revised September 15, 1986.

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