Role of the Bacteriophage T7 and T4 Single-stranded DNA-binding Proteins in the Formation of Joint Molecules and DNA Helicase-catalyzed Polar Branch Migration*

(Received for publication, September 17, 1996, and in revised form, December 10, 1996)

Daochun Kong‡, Nancy G. Nossal§, and Charles C. Richardson‡¶

From the ‡Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115 and the §Laboratory of Molecular and Cellular Biology, NIDDK, National Institutes of Health, Bethesda, Maryland 20892-0830

Bacteriophage T7 gene 2.5 single-stranded DNA-binding protein and gene 4 DNA helicase together promote pairing of two homologous DNA molecules and subsequent branch migration (Kong, C. C. (1996) EMBO J. 15, 2010–2019). In this report, we show that gene 2.5 protein is not required for the initiation or propagation of strand transfer once a joint molecule has been formed between the two DNA partners, a reaction that is mediated by the gene 2.5 protein alone. A mutant gene 2.5 protein, gene 2.5-Δ21C protein, lacking 21 amino acid residues at its C terminus, cannot physically interact with gene 4 protein. Although it does bind to single-stranded DNA and promote the formation of joint molecule via homologous base pairing, subsequent strand transfer by gene 4 helicase is inhibited by the presence of the gene 2.5-Δ21C protein. Bacteriophage T4 gene 32 protein likewise inhibits T7 gene 4 protein-mediated strand transfer, whereas Escherichia coli single-stranded DNA-binding protein does not. The 63-kDa gene 4 protein of phage T7 is also a DNA primase in that it catalyzes the synthesis of oligonucleotides at specific sequences during translocation on single-stranded DNA. We find that neither the rate nor extent of strand transfer is significantly affected by concurrent primer synthesis. The bacteriophage T4 gene 41 helicase has been shown to catalyze polar branch migration after the T4 gene 59 helicase assembly protein loads the helicase onto joint molecules formed by the T4 UvsX and gene 32 proteins (Salinas, F., and Kodadek, T. (1995) Cell 82, 111–119). We find that gene 32 protein alone forms joint molecules between partially single-stranded homologous DNA partners and that subsequent branch migration requires this single-stranded DNA-binding protein in addition to the gene 41 helicase and the gene 59 helicase assembly protein. Similar to the strand transfer reaction, strand displacement DNA synthesis catalyzed by T4 DNA polymerase also requires the presence of gene 32 protein in addition to the gene 41 and 59 proteins.

Single-stranded DNA (ssDNA) binding proteins, a class of ubiquitous proteins, not only are essential in DNA replication (1–3) but also play key roles in DNA recombination and repair (4–11). A number of studies have shown that the ssDNA-binding proteins encoded by Escherichia coli and its phages T4 and T7 exert significant function in maintaining the normal level of DNA recombination in vivo. Mutations that alter E. coli, T4, or T7 ssDNA-binding protein, the products of the ssb gene of E. coli, gene 32 of phage T4, or gene 2.5 of phage T7, can significantly depress DNA recombination frequencies in vivo (5, 6, 10, 11).

Although ssDNA-binding proteins are clearly involved in recombination, it has proven difficult to define their precise roles since they not only bind to DNA but also physically interact with other proteins of DNA metabolism to modulate their reactions. In addition, their involvement in both replication and recombination has made specific assignments of function in vivo speculative. One property, however, their ability to stimulate the annealing of complementary DNA strands (12–15), does provide a function that is essential to the overall process of general recombination. Both the T4 gene 32 and T7 gene 2.5 proteins have been shown to be essential for this early step in recombination (10, 12, 13).

Recombination proteins have been identified from a number of organisms that expose single-stranded regions in order for ssDNA-binding proteins of the type discussed above to facilitate annealing of the complementary sequences (reviewed in Ref. 16). We have shown (15) that the T7 gene 2.5 ssDNA-binding protein and the gene 4 helicase mediate both homologous base pairing and subsequent polar branch migration between a single-stranded circle and a linear duplex with a short single-stranded tail created by 5′ to 3′ digestion by the T7 gene 6 exonuclease (Fig. 1). Strand transfer driven by the T7 gene 4 helicase is coupled to hydrolysis of nucleoside 5′-triphosphate and proceeds at a high rate (>120 nucleotides/s) in a polar 5′ to 3′ direction with respect to the invading strand.

Proteins such as the RecA protein of E. coli (17), the UvsX protein of phage T4 (18–20), and the RAD 51 protein of Saccharomyces cerevisiae (21) mediate homologous base pairing and subsequent strand exchange between ssDNA and a fully duplex partner. The overall process of strand exchange by these RecA-like proteins is stimulated by ssDNA-binding proteins (17–20, 22, 23). Recent experiments with the T4 recombination system (24) showed that the T4 UvsY protein promotes homologous pairing by the UvsX protein but inhibits subsequent UvsX-catalyzed branch migration. Polar branch migration is
proteins could exert its negative effect either by an inability of the gene 2.5-Δ21C protein to load the gene 4 protein onto the joint molecule or to a lack of stimulation during the actual strand transfer. Alternatively, a specific interaction between the two proteins may be required for the gene 4 protein to displace gene 2.5 protein bound to ssDNA. The same explanations can also be extended to the inability of the T4 gene 32 protein to substitute for the T7 gene 2.5 protein in the T7 helicase reaction.

In this report we show that the T7 gene 2.5 protein is not required for polar branch migration catalyzed by the T7 gene 4 helicase. In contrast, T4 gene 32 protein is required both for base pairing and for extensive strand exchange in the T4 gene 41 helicase-mediated reaction. This requirement for T4 gene 32 protein during recombination is in accord with its requirement for extensive strand displacement synthesis by the T4 DNA replication system in vitro (29).

MATERIALS AND METHODS

Proteins—T7 gene 2.5 (15) and T7 gene 2.5-Δ21C (28) were overexpressed, and the proteins were purified to apparent homogeneity (>98% pure) from E. coli cells as described. The 65-kDa gene 4 protein (98% pure), a species of gene 4 protein that has both helicase and primase activities, was prepared by B. Beauchamp (Harvard Medical School) as described (30). T4 gene 41 DNA helicase (31) and gene 59 protein (32) were purified to apparent homogeneity (>98% pure) as described. The purity of proteins was determined by Coomassie Blue staining of the proteins on polyacrylamide gels containing sodium dodecyl sulfate. T4 gene 32 protein, E. coli ssDNA-binding (SSB) protein, T7 gene 6 exonuclease, and restriction enzymes were purchased from U.S. Biochemical Corp.

Nucleic Acids and Nucleotides—M13mp18 circular, ssDNA (33) and M13mp18 RF1 DNA (34) were prepared as described previously. To prepare linear, duplex M13mp18 bearing single-stranded 3′-termini of approximately 100 nucleotides, M13mp18 RF1 DNA was cut by restriction enzyme Sma1 and then hydrolyzed with T7 gene 6 5′ to 3′ exonuclease to a limited extent as described previously (15). All nucleotides were purchased from Pharmacia Biotech Inc. Concentrations of DNA are expressed in nucleotide equivalents.

Preparation of Joint Molecules with T7 Gene 2.5 Protein or T4 Gene 32 Protein—Joint DNA molecules consisting of circular, single-stranded M13 DNA annealed to the single-stranded region of homologous linear, duplex DNA having 3′ single-stranded termini were prepared by incubating circular, single-stranded M13mp18 DNA (10 μM as nucleotide equivalents) and linear, duplex M13mp18 DNA having single-stranded termini (20 μM) in a standard reaction containing 25 mM Tris-HCl (pH 7.5), 5 mM dithiothreitol, 10 mM MgCl2 (15 mM MgCl2 when gene 32 protein was present), and 2 μM T7 gene 2.5 protein or T4 gene 32 protein. The reaction was incubated at 32 °C for 30 min and then stopped by the addition of EDTA, SDS, and proteinase K to 50 mM, 0.5%, and 0.6 mg/ml, respectively. After an additional incubation at 32 °C for 10 min, the reaction mixture was extracted sequentially by phenol, phenol/chloroform, and chloroform to remove the T4 or T7 helicase.

In this report we have used the joint molecule formed between a single-stranded circle and linear duplex with a short single-stranded tail (Fig. 1) to compare the roles of the T7 and T4 helicases and ssDNA-binding proteins in polar branch migration. We show that the T4 helicase in the presence of T4 gene 32 protein and gene 59 protein catalyzes strand transfer in a manner similar to that previously described for the T7 helicase (15), thus allowing us to examine the role of the gene 32 protein in reactions devoid of the T4 UvX and UvXY proteins.

One role of gene 2.5 protein in the overall T7 reaction is to promote the formation of the joint molecule, a requisite intermediate for strand transfer by the helicase. In this role the gene 2.5 protein can be distinguished from the T4 or E. coli ssDNA-binding proteins in that its affinity (Kd = 1–4 × 10^6/M) for ssDNA (25) is approximately 50-fold lower than that of the latter two proteins and it facilitates the renaturation of ssDNA much more efficiently provided that either Mg^{2+} or a relatively high concentration of NaCl is present. In the simplified T4 reaction described above, the gene 32 protein and gene 59 protein are required to form the joint molecule and load the helicase. An unresolved question with regard to both systems, one addressed by the current study, is whether or not the ssDNA-binding proteins play a direct role in the strand transfer reaction per se. For example, both the T7 gene 4 and T4 gene 41 proteins share a requirement for a relatively short single-stranded 5′-tail to initiate translocation and display helicase activity on a duplex DNA molecule (26, 27). It is likely that there is sufficient nonprotein branch migration at the junction between single-stranded and duplex DNA to provide for this requirement, but it is not known if an ssDNA-binding protein is required to stabilize this partially unwound structure. Likewise, it is not known if helicase interactions with ssDNA-binding protein on the invading strand are required or if the binding of ssDNA-binding protein to the single-stranded region created behind the translocating helicase is essential to maintain unidirectional strand transfer. Our earlier finding (15) that a mutant gene 2.5 protein, gene 2.5-Δ21C protein, could not support strand transfer by the T7 gene 4 protein is most likely due to its inability to physically interact with the gene 4 protein (28). The lack of an interaction between the two proteins could exert its negative effect either by an inability of the gene 2.5-Δ21C protein to load the gene 4 protein onto the joint molecule or to a lack of stimulation during the actual strand transfer.

In some reactions catalyzed by the T4 gene 32, 41, and 59 proteins, the formation of joint molecules and strand transfer were carried out in
tions of various DNA species are indicated. Gene 2.5 protein promotes the pairing between circular, single-stranded M13 DNA molecule and homologous linear, duplex M13 DNA bearing single-stranded 3‘ termini of approximately 100 nucleotides. Joint molecules were formed by incubating reactions at 32 °C for 30 min as described under “Materials and Methods.” Subsequently, the solution was extracted with phenol, phenol/chloroform, and chloroform to remove gene 2.5 protein. Complete strand exchange catalyzed by T7 helicase results in the production of circular, dsDNA containing a gap of approximately 100 nucleotides and linear, single-stranded DNA. B, strand transfer promoted by gene 4 protein. ssDNA-binding protein-free joint molecules consisting of circular, single-stranded M13 DNA and homologous linear, duplex DNA were prepared by incubating with gene 2.5 protein as described above (lane 1). Strand transfer reactions were carried out in the presence of protein 4 protein alone (lane 2) or gene 2.5 and 4 proteins together (lane 3) as described under “Materials and Methods.” The products were analyzed by agarose gel electrophoresis, and the positions of various DNA species are indicated. gp, gene protein.

two steps. In the first step, joint molecules were formed in a reaction with only gene 32 protein, using the conditions described above. In the second step, 0.2 μM each (monomer) of the gene 41 and 59 proteins and 5 mM ATP were added to initiate the reaction. Reactions were then incubated at 32 °C for 20 min, and the products were analyzed by agarose gel electrophoresis to detect the product of strand transfer.

**DNA Replication—Strand displacement DNA synthesis by the T4 DNA replication system at 37 °C was carried out, and the products were analyzed by alkaline-agarose gel electrophoresis, as described (32). The primer-template was M13mp2 annealed to an 84-base oligonucleotide in which only the 3′-end is complementary, leaving a 50-base tail (32).

**Agarose Gel Electrophoresis—0.8% agarose gel electrophoresis was used to detect the joint molecules or products of strand transfer. Electrophoresis was carried out in TAE (0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0) buffer at 0.6 V/cm for 15 h. After electrophoresis, the gel was stained in TAE buffer containing 0.5 μg/ml ethidium bromide, and DNA bands were illuminated by ultraviolet light and photographed.

**RESULTS**

In our previous report (15) we showed that T7 gene 2.5 protein promotes the formation of joint molecules consisting of circular, single-stranded M13 DNA annealed to homologous linear duplex DNA bearing single-stranded termini. In the presence of T7 gene 4 helicase, strand transfer proceeds at a rate of >120 nucleotides/s in a polar 5‘-to-3‘ direction with respect to the invading strand, resulting in the production of circular duplex M13 DNA (15). Inasmuch as no strand transfer occurred when T4 gene 32 protein was substituted for T7 gene 2.5 protein or T4 gene 41 helicase for T7 gene 4 helicase, it seemed likely that specific protein interactions were important. This belief was strengthened by the observation that gene 2.5-Δ21C protein, an altered protein that does not physically interact with gene 4 protein, and gene 4 protein together did not mediate strand transfer.

**T7 Gene 4 Protein Alone Is Able to Promote Strand Transfer—**To more precisely define the role of gene 2.5 protein in the T7 helicase-mediated strand transfer, we prepared joint molecules free of gene 2.5 protein. With such a DNA substrate, the ability of gene 4 protein alone to catalyze strand transfer can be examined. As shown in Fig. 2A, joint molecules were prepared by incubating circular, single-stranded M13 DNA molecules and homologous linear, duplex M13 DNA bearing single-stranded 3‘-termini with gene 2.5 protein. Gene 2.5 protein was then removed by extraction with phenol, and the protein-free joint molecules were used as substrate for T7 gene 4 helicase.

When joint DNA molecules were incubated with gene 4 and 2.5 proteins, circular, duplex DNA molecules, the product of complete strand transfer, were observed (Fig. 2B, lane 3). As shown in Fig. 2B, lane 2, gene 4 protein alone mediates strand transfer equally well as the combination of gene 2.5 and 4 proteins. Thus, it appears that gene 2.5 protein is not required for either loading the gene 4 protein onto the proper strand or for the subsequent strand transfer reaction.

**Effect of Other ssDNA-binding Proteins on T7 Helicase-mediated Strand Transfer**—The above finding that gene 2.5 protein is not required for strand transfer implies strongly that the lack of strand transfer previously observed (15) with the combination of gene 4 protein and mutant gene 2.5-Δ21C protein reflects an inhibitory property of this protein. To examine this point, increasing amounts of gene 2.5-Δ21C protein were added to a reaction containing joint molecules and gene 4 protein (Fig. 3A). As the amount of the mutant gene 2.5 protein increases, there is a progressive decrease in strand transfer as measured by the decrease in circular, duplex DNA product. Since gene 2.5 protein binds to ssDNA with a stoichiometry of 7 nucleotides bound monomer of gene 2.5 protein (25) the ssDNA in the joint molecule should be completely coated at a concentration of 1 μM. At this concentration of gene 2.5-Δ21C protein, essentially no strand transfer occurs (Fig. 3A, lane 6). Gene 2.5-Δ21C binds
to ssDNA with the same affinity of wild-type protein but does not physically interact with gene 4 protein (28), thus suggesting that the inhibition arises from an inability of the gene 4 protein to displace the bound gene 2.5 protein in a step requiring a specific interaction.

In a similar manner, we have also examined the effect of the ssDNA-binding proteins of E. coli and phage T4 on gene 4 protein-mediated strand transfer (Fig. 3B). At a concentration of T4 gene 32 protein sufficient to coat all of the ssDNA, there is a complete inhibition of strand transfer (lane 3), whereas a similar concentration of E. coli SSB protein was without effect (lane 4). Presumably, the T7 gene 4 protein has evolved such that it can tolerate the relatively abundant host SSB protein present upon phage infection although the host protein cannot replace the gene 2.5 protein in DNA replication (3).

Strand Transfer Catalyzed by T4 Gene 32, 41, and 59 Proteins—The UvsX protein, the bacteriophage T4 RecA analog, catalyzes strand exchange, albeit at a relatively slow rate (18–20). However, Salinas and Kodadek (24) recently demonstrated that the T4 gene 41 helicase can drive polar branch migration at a high rate in a multiprotein complex consisting of the products of the UvsX, UvsY, gene 32, gene 41, and gene 59 proteins. Thus, heteroduplex formation in vivo is most likely mediated by the T4 helicase system. In these studies they showed that joint molecules formed by the action of the UvsX and gene 32 proteins and then deproteinized were substrates for the gene 41 and 59 proteins but that strand transfer was inefficient.

We have used the same joint molecule described above for the T7 helicase-mediated strand transfer reaction to examine the basic requirements for the actual strand transfer reaction and to examine the protein specificity of the reaction. Like the T7 gene 2.5 protein, stoichiometric amounts of the T4 gene 32 protein promote the formation of stable joint molecules consisting of circular, single-stranded M13 DNA annealed to a complementary region of linear, duplex M13 DNA bearing single-stranded 3’-termini (Fig. 4A). After formation, the preparation of joint molecules was deproteinized and used as substrate for the T4 helicase-mediated strand transfer reaction. As shown in Fig. 4B, lane 2, no strand transfer occurs in the presence of T4 gene 32 protein and gene 41 protein. However, if gene 59 protein is also present (lane 3), then strand transfer occurs, reflecting the requirement of gene 59 protein to load the helicase onto gene 32 protein-coated DNA (32, 35–37). This result differs strikingly from that found with the T7 helicase where the presence of its ssDNA-binding protein, gene 2.5 protein, does not impede loading of the T7 gene 4 protein, and hence a loading factor such as the T4 gene 59 protein is not required.

Although gene 59 protein is clearly necessary for loading in the presence of gene 32 protein, the question remains as to whether or not gene 41 protein by itself, or with gene 59 protein, can drive strand transfer in the absence of gene 32 protein. As shown in Fig. 4C, in the absence of gene 32 protein, the gene 41 protein is much less efficient in mediating strand exchange in the absence (lane 2) or presence (lane 3) of gene 59 protein. Clearly, with the DNA substrate used here, T4 gene 41 protein requires both gene 32 protein and gene 59 protein for it to mediate strand transfer (lane 4). Strand transfer catalyzed by gene 32, 41, and 59 proteins requires nucleoside triphosphate hydrolysis, proceeds from 5’ to 3’ with regard to the invading strand, and also takes place in physiological concentrations of salts (200–400 mM potassium glutamate) (data not shown).

Protein Specificity of Strand Transfer with Gene 32, 59, and 41 Proteins—The above results show that efficient strand exchange requires the presence of T4 gene 32, 41, and 59 proteins. A physical interaction among the three proteins appears necessary, since no strand transfer occurred in the absence of gene 59 protein. To confirm that there is a specific interaction among gene 32, 41, and 59 proteins, we performed reactions with T7 gene 2.5 or 4 proteins replacing T4 gene 32 or 41 proteins, respectively. As shown in Fig. 5A, lane 1, T7 gene 4 protein cannot replace T4 gene 41 protein. When gene 32 protein was replaced by gene 2.5 protein, strand transfer was significantly inhibited as shown (Fig. 5B, lane 3). Strand transfer does not occur in the presence of T7 gene 2.5 and T4 gene 41 proteins.

The results confirm that specific interactions among gene 32, 41, and 59 proteins are essential for strand transfer.

FIG. 4. Strand transfer promoted by T4 gene 32, 41, and 59 proteins. A, stoichiometric amounts of gene 32 protein are required for joint molecule formation. Reactions for joint molecule formation contained 10 μM circular, single-stranded M13 DNA, 20 μM linear, duplex M13 DNA having single-stranded 3’-termini of approximately 100 nucleotides, and increasing amounts of gene 32 protein from 0.5 μM to 2.5 μM so that the molar ratio of gene 32 protein to nucleotides of circular ssDNA increased from 1:20 to 1:4. Reactions were carried out as described under “Materials and Methods” and analyzed by agarose gel electrophoresis. B, T4 gene 59 and 41 proteins are required for strand transfer. Joint molecules were first prepared in the presence of gene 32 protein as described under “Materials and Methods.” Strand transfer was initiated by the addition of gene 41 and 59 proteins and ATP as described under “Materials and Methods.” After incubation at 32 °C for 20 min, reaction samples were analyzed by agarose gel electrophoresis. Lane 1, gene 32 and 59 proteins; lane 2, gene 32 and 41 proteins; lane 3, gene 32, 41, and 59 proteins. C, gene 32, 41, and 59 proteins are required for efficient strand transfer. Joint molecules prepared with gene 32 protein were deproteinized and then used in the reaction. Strand transfer reactions were performed in the presence of gene 41 protein, gene 41 and 59 proteins, or gene 32, 41, and 59 proteins together according to “Materials and Methods.” Lane 1, control joint molecules; lane 2, gene 41 protein alone; lane 3, gene 41 and 59 proteins; lane 4, gene 32, 41, and 59 proteins. gp, gene protein.
Proteins to reactions was carried out as described in the legend to Fig. 4B. Replacement of gene 41 protein by gene 2.5 protein significantly inhibits strand initiation of strand transfer reactions as described in Fig. 4B. A, replacement of gene 41 protein by gene 4 protein abolishes strand transfer. Formation of joint molecules was first mediated by gene 32 protein. The subsequent addition of gene 59 and 41 proteins (lane 1) or gene 59 and 41 proteins (lane 2) initiated strand transfer reactions as described in Fig. 4B. B, replacement of gene 32 protein by gene 2.5 protein significantly inhibits strand exchange. Joint molecules were formed in the presence of gene 2.5 or 32 protein. Subsequent strand transfer after the addition of gene 41 and 59 proteins to reactions was carried out as described in the legend to Fig. 5A. lane 1, gp32, 59, and 41; lane 2, gp 2.5 and 41; lane 3, gp 2.5, 59, and 41 proteins. gp, gene protein.

FIG. 5. Protein specificity of strand transfer mediated by T4 gene 32, 41, and 59 proteins. Protein specificity of strand transfer was investigated by replacing the T4 gene 32 and 41 proteins by the T7 gene 2.5 and 4 proteins, respectively. A, replacement of gene 41 protein by gene 4 protein abolishes strand transfer. Formation of joint molecules was first mediated by gene 32 protein. The subsequent addition of gene 59 and 41 proteins (lane 1) or gene 59 and 41 proteins (lane 2) initiated strand transfer reactions as described in Fig. 4B. B, replacement of gene 32 protein by gene 2.5 protein significantly inhibits strand exchange. Joint molecules were formed in the presence of gene 2.5 or 32 protein. Subsequent strand transfer after the addition of gene 41 and 59 proteins to reactions was carried out as described in the legend to Fig. 5A. lane 1, gp32, 59, and 41; lane 2, gp 2.5 and 41; lane 3, gp 2.5, 59, and 41 proteins. gp, gene protein.

FIG. 6. T4 gene 32 protein is required for strand displacement DNA synthesis. The complete replication reaction contained T4 gene 41 helicase, gene 59 helicase assembly protein, gene 32 protein, gene 61 primase, T4 DNA polymerase, and the genes 44/82 and 45 polymerase accessory proteins. Products labeled with [32P]dCTP are displayed on a 0.6% alkaline-agarose gel. Reaction 1, gp59 omitted; reaction 2, complete; reaction 3, gp 32 omitted. kb, kilobase pairs; gp, gene protein.

FIG. 7. Effect of primer syntheses on strand transfer. A, strand transfer reactions contained deproteinized joint molecules (20 μM), 63-kDa gene 4 protein (0.2 μM), 50 mM potassium glutamate, and ATP or CTP (5 mM). Reactions were incubated at 32°C for 20 min and then stopped and analyzed by agarose gel electrophoresis. lane 1, gene 4 protein and ATP, lane 2, gene 4 protein, ATP, and CTP; lane 3, gene 4 and 2.5 proteins, ATP, and CTP. B, time course of strand exchange with or without synthesis of primers. Joint molecules were first catalyzed by gene 2.5 protein as described under “Materials and Methods.” Strand transfer and primer synthesis were initialized by the addition of 0.2 μM gene 4 protein, 5 mM ATP and CTP as indicated, 50 mM potassium glutamate, and MgCl2 raised to 15 mM. The time at which gene 4 protein was added is designed as 0 min. Incubation was continued at 32°C, aliquots were removed at the time indicated, and the reaction stopped by the addition of EDTA, SDS, and proteinase K. After an additional 10 min of incubation to allow digestion of proteins by proteinase K, reaction samples were analyzed by agarose gel electrophoresis. gp, gene protein.

DNA-binding Protein in Helicase-mediated Branch Migration

Gene 4 of phage T7 encodes two co-linear proteins, a 56-kDa protein that has only helicase activity and a 63-kDa protein that has both helicase and primase activity (30); the 63-kDa gene 4 protein is the molecular weight species used in the current studies. The 56-kDa protein also catalyzes strand transfer (15). The 63-kDa gene 4 protein catalyzes the synthesis of tetrabinucleotides (pppACAC and pppACAC) in a template-mediated reaction at specific pentanucleotide recognition sites as it translocates 5’ to 3’ on DNA. In vivo (40) and in vitro (30), these oligoribonucleotides are used as primers for T7 DNA polymerase to initiate lagging strand DNA synthesis. Debyser et al. (41) showed that primer synthesis on the lagging strand decreased the rate of leading strand DNA synthesis, presumably due to pausing of the 63-kDa gene 4 protein as it synthesized a primer and hence reduced its effectiveness as a helicase.

To see if concurrent primer synthesis by the T7 gene 4 protein affects strand transfer in a manner similar to that observed with DNA synthesis on a preformed replication fork, we have measured strand transfer in the presence of ATP and CTP or ATP alone, the ribonucleotide precursors for oligoribonucleotide synthesis. In addition, potassium glutamate was included in the reaction, since it significantly stimulates the synthesis of oligoribonucleotides by the 63-kDa gene 4 protein.3 As shown in Fig. 7, conditions for primer synthesis had no significant effect on either the extent (panel A) or the rate (panel B) of reaction. In addition, the presence of T7 gene 2.5 protein that stimulates oligoribonucleotide synthesis by the gene 4 protein (30) had no effect either (Fig. 7A, lane 3). Analysis of the product of the primase reaction by 25% polyacrylamide gel electrophoresis confirmed that dimer, trimer, tetramer, and pentamer oligonucleotide were efficiently synthesized and that the presence of gene 2.5 protein greatly stimulated synthesis of these oligonucleotides in the reactions where ATP and CTP were present (data not shown).

3 B. B. Beauchamp and C. C. Richardson, unpublished results.
In a previous report (15) we reported that two proteins encoded by bacteriophage T7 work together to mediate DNA strand transfer. Stoichiometric amounts of gene 2.5 ssDNA-binding protein promoted the annealing of complementary DNA strands to form a joint molecule, the intermediate in DNA recombination. Subsequently, the T7 gene 4 helicase, in an energy-requiring reaction, mediated a polar exchange of one strand of the duplex portion of the joint molecule for a ssDNA partner. In bacteriophage T4, Salinas and Kodadek (24) showed that the gene 41 helicase of bacteriophage T4 was responsible for strand transfer in a multiprotein complex consisting of the products of the gene UvsX, UvsY, 32, 59, and 41 of the phage T4. Although these two studies demonstrated the novel role of DNA helicases in general recombination, several aspects of the overall reactions could not be compared directly due to differences in the formation and structure of the intermediate joint molecules. In the T7 system the formation of joint molecules between a single-stranded circle and a linear duplex with a single-stranded tail was accomplished by the gene 2.5 protein, a protein that enhances greatly the annealing of complementary DNA strands (15). 4 In the T4 system, the UvsX, UvsY, and gene 32 proteins, which carry out a search for homology and may also stabilize the resulting joint molecule (reviewed in Ref. 16), were used to form joint molecules between a single-stranded circle and a fully duplex linear DNA (24). In the present study we have prepared joint molecules by incubating the appropriate complementary single-stranded circular and tailed duplex DNA molecules in the presence of either the T4 or T7 ssDNA-binding protein, and then isolated the joint molecules free of protein. With this DNA intermediate, we have addressed the relative roles of ssDNA-binding protein and helicase in the actual process of strand transfer with both the T4 and T7 proteins. Inasmuch as these same proteins are also involved in reactions at the replication fork, the information obtained is pertinent to the roles of these proteins in DNA replication.

We find that T7 gene 4 helicase mediates strand transfer within preformed joint molecules equally well in the presence or absence of gene 2.5 protein. The ability of T7 helicase to mediate strand transfer in the absence of gene 2.5 protein demonstrates that the gene 4 protein can load onto the strand to be displaced without accessory proteins (Fig. 8). Since the gene 4 protein requires at least 17 nucleotides of ssDNA to which it can bind to initiate 5' to 3' translocation (26), it seems likely that this structure must arise as a result of branch migration at the single-stranded duplex junction. This result also necessitated a reexamination of our earlier observation that a truncated form of gene 2.5 protein, gene 2.5-Δ21C protein, could not substitute for the gene 2.5 protein in the overall strand transfer reaction although it mediated the formation of joint molecules as well as did the wild type gene 2.5 protein (15). Gene 2.5-Δ21C protein, lacking 21 amino acid residues at its C terminus, binds to ssDNA normally but cannot physically interact with itself to form dimers or with the T7 DNA polymerase or gene 4 protein to stimulate their activities (28). Rather than the physical interaction between gene 2.5 protein and gene 4 protein playing a positive role in enhancing helicase activity, our results suggested that the interaction may be necessary in order for the two proteins to exist together on ssDNA. This interpretation appears to be correct, since we find that gene 2.5-Δ21C protein is quite inhibitory to the T7 helicase-mediated strand transfer. Therefore, we believe that the gene 2.5 protein bound to ssDNA must physically interact, through its acidic C-terminal region, with gene 4 protein in order for the helicase to displace it and hence load onto ssDNA. In this regard, the T4 gene 32 protein also has an acidic C-terminal region that mediates specific interaction with many T4 proteins (42, 43). The fact that gene 32 protein is also inhibitory to the T7 helicase-mediated reaction illustrates the specificity of the C-terminal interaction. Interestingly, E. coli SSB protein has no effect on the T7 helicase-mediated reaction, perhaps not a surprising result since T7 is faced with a relatively large intracellular pool of this protein upon infection of its host. In any case, the inability of gene 4 protein to load onto ssDNA in the presence of T7 gene 2.5-Δ21C protein provides a molecular basis for the essential nature of the C-terminal region of the protein (28). However, the interaction of the C-terminal region of the gene 2.5 protein with other T7 proteins may serve other functions, since in the case of T7 DNA polymerase the T7 gene 2.5-Δ21C protein does not stimulate the polymerase reaction as does wild-type protein, but neither does it inhibit the reaction (28).

The replication system of bacteriophage T4 is more complex than that of bacteriophage T7, and the helicase-mediated recombination system proves no exception. In contrast to the results obtained with T7 gene 4 protein, T4 gene 41 helicase could not mediate strand transfer within a preformed joint molecule. This result is not unanticipated, however, since T4 gene 41 protein is known to require an accessory protein, T4 gene 59 protein, for efficient loading onto DNA (32, 35–37, 44, 45). In fact, gene 32 protein-bound DNA is practically inaccessible to gene 41 protein, necessitating the presence of gene 59 protein. Salinas and Kodadek (24) reported that T4 gene 41 protein and gene 59 protein together mediated strand transfer on deproteinized joint molecules, but the reaction was much less efficient than in the complete multiprotein complex with the T4 gene 32, UvsX, and UvsY proteins. In the present study, we observe little if any complete strand transfer by the two proteins alone, a difference that most probably reflects the fact that in our studies strand transfer must proceed for 7.1 kilobase pairs, whereas in the studies by Salinas and Kodadek (24) strand transfer was scored after only 2.2 kilobase pairs of transfer. However, when T4 gene 32 protein is present along with gene 59 protein and gene 41 helicase, rapid and extensive strand transfer occurs. Thus, while the T4 UvsX and UvsY proteins are required to form joint molecules if one of the DNA partners is totally duplex, the T4 gene 41 helicase can drive polar branch migration on joint molecules made without the UvsX and UvsY recombination proteins. Since gene 59 protein has been shown to bind to both the gene 32 and 41 proteins (35–37), it seems likely that gene 41 protein is loaded onto the joint molecule through interactions of the gene 59 protein with both the gene 32 protein bound to ssDNA and the gene 41 protein (Fig. 8). This interpretation is in agreement with that of Salinas and Kodadek (24), who showed that a mutant gene 32 protein, gene 32-A protein lacking its C-terminal domain, cannot substitute for gene 32 protein in the strand transfer reaction although it binds to ssDNA.

The requirement for T4 gene 32 protein during strand transfer and strand displacement synthesis is likely to be a consequence of its role in stabilizing a DNA structure needed to load the T4 gene 41 helicase onto joint molecules, and DNA replication forks. Both the T7 gene 4 protein and the T4 gene 41 protein are essential for replication, where they serve as the helicase for leading strand synthesis. The structure of the strand transfer intermediate with the helicase assembled on the displaced strand is not unlike that of a replication fork. Polymerization of nucleotides on the leading strand of the fork is replaced by the sequential annealing of complementary DNA.
sequence as the duplex region is processively unwound by the helicase. In both the T7 and T4 DNA replication systems, the helicase binds preferentially to a preformed fork to unwind the DNA ahead of the polymerase. T7 DNA polymerase and the gene 4 helicase together are sufficient to carry out this synthesis efficiently (46). In the T4 system, in contrast, omitting gene 32 protein greatly decreases the fraction of molecule copied at the high rate dependent on the gene 41 helicase (29, 38, 39), even when the helicase is loaded on the preformed fork by the gene 59 protein (Fig. 6). In these reactions, gene 32 protein appears to be affecting the initiation of unwinding rather than the rate of unwinding, because it increases the number of rapidly replicating molecules but does not change the rate at which the leading strand is elongated on DNA with the helicase. A similar conclusion has been reached by Tarumi and Yonesaki (44), who showed that gene 32 protein could stimulate the unwinding of a partial duplex by the gene 41 helicase if gene 59 protein was present to load the helicase on the gene 32 protein-covered DNA. In this view, the binding of gene 32 protein stabilizes a partially unwound DNA structure attractive to the helicase, and gene 59 protein is then essential to load the helicase onto the gene 32 protein-covered DNA. In the absence of the helicase, the binding of gene 32 protein allows strand displacement synthesis at a much slower rate by T4 DNA polymerase and its accessory proteins. The T4 ssDNA-binding protein is required for this reaction, even on templates containing a preformed fork (29).

The joint molecules used in the present study were constructed using the T7 gene 2.5 ssDNA-binding protein to promote the annealing of complementary ssDNA regions between the two partners. The T7 gene 2.5 protein is clearly involved in recombination, since mutations in gene 2.5 lower recombination frequencies (11). T7 gene 2.5 protein differs from the T4 gene 32 protein and E. coli SSB protein in that it is the most efficient in mediating homologous base pairing (25). In phage T7-infected E. coli cells, it is likely that the gene 2.5 protein is responsible for the annealing of homologous regions to form joint molecules, since recombination is not decreased in the absence of the host RecA pathway (48, 49), and thus far no RecA-type protein has been identified in T7 phage. On the other hand, it seems likely that the T4 UvsX protein plays a major role in homologous base pairing in that, in addition to
facilitating the annealing of complementary strands, it can carry out a true search for homology.

The T7 gene 4 protein is unique in that one form of the gene 4 protein, the 63-kDa gene 4 protein, has full primase activity as well as helicase activity residing within the same polypeptide chain (30). Consequently, the T7 63-kDa gene 4 protein is capable of synthesizing oligoribonucleotides as it translocates along the displaced strand. We find, however, that the addition of ATP and CTP to strand transfer reactions containing the T7 gene 63-kDa gene 4 protein, enables primer synthesis but has no measurable effect on the rate or extent of strand transfer. Our inability to detect an effect of primer synthesis on strand transfer may be due to the limited number of primase recognition sites (eight major recognition sites) on the M13 DNA molecule. Nonetheless, in vivo, it would not be surprising to find that RNA-primed DNA synthesis does occur on the recombination intermediates. Such reactions may be important in the known role of recombination in the initiation of DNA replication in T4 phage-infected cells (11, 47).

Acknowledgment—We are grateful to T. Kusakabe for help in the examination of primer synthesis.

REFERENCES

1. Epstein, R. H., Bolle, A., Steinberg, C. M., Kellenberger, E., Boy De la Tour, E., Chevalley, R., Edgar, R. S., Susman, M., Denhardt, G. H., and Lielausis, A. (1963) Cold Spring Harbor Symp. Quant. Biol. 28, 375–384
2. Meyer, R. R., Glassberg, J., and Kornberg, A. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 1702–1705
3. Kim, Y. T., and Richardson, C. C. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 10173–10177
4. Chase, J. W., and Williams, K. (1986) Annu. Rev. Biochem. 55, 103–136
5. Glassberg, J., Meyer, R. R., and Kornberg, A. (1979) J. Bacteriol. 140, 14–19
6. Berger, H., Warren, A. J., and Fry, A. K. (1969) J. Virol. 3, 171–175
7. Wu, J.-R., and Yeh, Y.C. (1973) J. Virol. 12, 758–765
8. Whittier, R. F., and Chase, J. W. (1981) Mol. & Gen. Genet. 183, 341–347
9. Wachsmann, J. T., and Drake, J. W. (1987) Genetics 115, 405–417
10. Araki, H., and Ogawa, H. (1983) Virology 121, 509–515
11. Mosig, G. (1994) in Molecular Biology of Bacteriophage T4 (Karem, J., ed) pp. 54–82, American Society for Microbiology, Washington, D. C.
12. Tomizawa, J.-i, Anraku, N., and Iwama, Y. (1966) J. Mol. Biol. 21, 247–253
13. Alberts, B. M., and Frey, L. (1970) Nature 227, 1313–1318
14. Christiansen, C., and Baldwin, R. L. (1977) J. Mol. Biol. 115, 441–454
15. Kong, D., and Richardson, C. C. (1996) EMBO J. 15, 2010–2019
16. Kowalczykowski, S. C., and Eggleston, A. K. (1994) Annu. Rev. Biochem. 63, 591–1043
17. Radding, C. M. (1982) Annu. Rev. Genet. 16, 405–437
18. Yonesaki, T., and Minagawa, T. (1985) EMBO J. 4, 3231–3237
19. Hinton, D. M., and Nossal, N. G. (1986) J. Biol. Chem. 261, 5663–5673
20. Formosa, T., and Alberts, B. M. (1986) J. Biol. Chem. 261, 6107–6118
21. Sung, P. (1984) Science 265, 1241–1243
22. McIntee, K., Weinstock, G. M., and Lehman, I. R. (1990) Proc. Natl. Acad. Sci. U. S. A. 77, 857–861
23. Kodadek, T. (1990) J. Biol. Chem. 265, 29966–29969
24. Salinas, F., and Kodadek, T. (1995) Cell 82, 111–119
25. Kim, Y. T., Tabor, S., Burtser, C., Griffith, J. D., and Richardson, C. C. (1992) J. Biol. Chem. 267, 15022–15031
26. Matson, S. W., Tabor, S., and Richardson, C. C. (1983) J. Biol. Chem. 258, 14017–14024
27. Richardson, R. W., and Nossal, N. G. (1989) J. Biol. Chem. 264, 4725–4731
28. Kim, Y. T., and Richardson, C. C. (1994) J. Biol. Chem. 269, 5270–5278
29. Nossal, N. G. (1994) in Molecular Biology of Bacteriophage T4 (Karem, J., ed) pp. 43–53, American Society for Microbiology, Washington, D. C.
30. Mendelman, L. V., and Richardson, C. C. (1991) J. Biol. Chem. 266, 22240–22250
31. Hinton, D. M., Silver, L. L., and Nossal, N. G. (1985) J. Biol. Chem. 260, 12851–12857
32. Spacatopoli, P., and Nossal, N. G. (1994) J. Biol. Chem. 269, 447–455
33. Yamamoto, K. R., Alberts, B. M., Benziger, R., Lawhorne, L., and Treiber, G. (1970) Virology 40, 734–744
34. Mesing, J. (1983) Methods Enzymol. 101, 20–78
35. Morrical, S. W., Hempstead, K., and Morrical, M. D. (1994) J. Biol. Chem. 269, 33069–33081
36. Barry, J., and Alberts, B. (1994) J. Biol. Chem. 269, 33049–33062
37. Yonesaki, T. (1994) J. Biol. Chem. 269, 1284–1289
38. Cha, T.-A., and Alberts, B. M. (1989) J. Biol. Chem. 264, 12220–12225
39. Richardson, R. W., Ellis, R. L., and Nossal, N. G. (1990) UCLA Symp. Mol. Cell. Biol. 127, 249–259
40. Sugimoto, K., Miyasaka, T., Fujiyama, A., Kohara, Y., and Okazaki, T. (1988) MGG 211, 406–406
41. Debyser, Z., Tabor, S., and Richardson, C. C. (1994) Cell 77, 157–166
42. Kraas, K. R., Green, L. S., and Gold, L. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 4014–4018
43. Jiang, H., Giedroc, D., and Kodadek, T. (1993) J. Biol. Chem. 268, 7904–7911
44. Maruo, T., and Yonesaki, T. (1995) J. Biol. Chem. 270, 2614–2619
45. Raney, K. D., Carver, T. E., and Benkovic, S. J. (1996) J. Biol. Chem. 271, 14074–14081
46. Nakai, H., and Richardson, C. C. (1986) J. Biol. Chem. 261, 15217–15224
47. Kreuzer, K. N., and Morrical, S. W. (1994) in Molecular Biology of Bacteriophage T4 (Karem, J., ed) pp. 28–42, American Society for Microbiology, Washington, D. C.
48. Poehling, A., and Knippers, R. (1974) Mol. & Gen. Genet. 134, 173–180
49. Kerr, C., and Sadowski, P. D. (1975) Virology 65, 281–285