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Single-stranded DNA binding protein and DNA helicase of bacteriophage T7 mediate homologous DNA strand exchange

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Two proteins encoded by bacteriophage T7, the gene 2.5 single-stranded DNA binding protein and the gene 4 helicase, mediate homologous DNA strand exchange. Gene 2.5 protein stimulates homologous base pairing of two DNA molecules containing complementary single-stranded regions. The formation of a joint molecule consisting of circular, single-stranded M13 DNA, annealed to homologous linear, duplex DNA having 3′- or 5′-single-stranded termini of ~100 nucleotides requires stoichiometric amounts of gene 2.5 protein. In the presence of 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. Strand transfer is coupled to the hydrolysis of a nucleoside 5′-triphosphate. The reaction is dependent on specific interactions between gene 2.5 protein and gene 4 protein.

Keywords: DNA replication/gene 2.5 protein/gene 4 protein/recombination

Introduction

The process of general recombination is defined by the exchange of DNA sequences between homologous chromosomes. Essential to the overall process is (i) the identification of homologous regions between two participating DNA molecules, (ii) the annealing of the homologous regions to form a joint molecule, and (iii) the subsequent branch migration to increase the length of the heteroduplex to form a recombinant molecule. In recent years a number of proteins from a variety of sources have been identified that mediate one or more of the above steps (Kowalczykowski and Eggleston, 1994).

The ability of three of these proteins, the RecA protein of *Escherichia coli* (Radding, 1982), the UvsX protein encoded by bacteriophage T4 (Formosa and Alberts, 1986) and the RAD 51 protein of *Saccharomyces cerevisiae* (Sung, 1994), to mediate the overall process of strand exchange has made them a paradigm for general recombination. However, in many organisms, a RecA-type protein has not been identified. Instead, a number of proteins have now been described that mediate strand transfer, provided that homologous base pairing has occurred between the two participating molecules (Kowalczykowski and Eggleston, 1994). Additional proteins are required to expose single-stranded regions and to facilitate annealing. This process does not involve a real search for homology, in contrast to that mediated by the RecA-type proteins. In general, these strand exchange proteins do not require ATP and the extent of strand transfer is often not extensive. These proteins include the RecT protein of *E.coli* (Hall and Kolodner, 1994), the β protein encoded by phage λ (Muniyappa and Radding, 1986) and the *S.cerevisiae* Sep1/STPβ protein (Kolodner *et al.*, 1987; Dykstra *et al.*, 1990).

The unique property of the RecA-type proteins is their ability to form a pre-synaptic complex and to carry out a search for homology within a duplex molecule (Radding, 1982). However, other proteins can mediate some of the subsequent steps in recombination equally as well as the RecA-type protein. For example, the single-stranded DNA (ssDNA) binding proteins, of which the *E.coli* SSB protein and the T4 gene 32 protein are examples, stimulate the annealing of complementary DNA strands (Chase and Williams, 1986), and *in vivo* studies have shown that both are involved in recombination (Tomizawa *et al.*, 1966; Berger *et al.*, 1969; Glassberg *et al.*, 1979; Mosig *et al.*, 1979). Under some conditions these proteins stimulate strand exchange mediated by the RecA (McEntee *et al.*, 1980; Radding, 1982) and UvsX (Formosa and Alberts, 1986; Kodadek, 1990) proteins, but they can also inhibit strand transfer (Kodadek, 1990).

Branch migration catalyzed by the RecA and UvsX proteins is extremely slow, proceeding at rates of ~4 (Cox and Lehman, 1981; Kahn *et al.*, 1981) and 15 (Kodadek *et al.*, 1988) bp/s, respectively. Again, another class of replication proteins, the DNA helicases, catalyze a similar reaction as they translocate 5′ to 3′ on a DNA strand and unwind DNA at a replication fork (Matson and Kaiser-Rogers, 1990). However, the rate of unwinding is considerably faster than that observed with the RecA-type proteins; when coupled to DNA synthesis the bacteriophage T7 helicase unwinds DNA at a rate of 300 bp/s (Lechner and Richardson, 1983).

Recombination in cells infected with bacteriophage T7 is high, approaching 20–40% between mutations widely separated on the T7 genetic map (Studier, 1969). Phage T7 encodes the majority of its replication proteins (Studier, 1972; Richardson, 1983) and the same appears to be the case for the proteins involved in recombination, since recombination frequencies are not decreased in the absence of the host RecA pathway (Powling and Knippers, 1974; Kerr and Sadowski, 1975). Studies with mutants of phage T7 have implicated most of the replication proteins: recombination is decreased by mutations in genes 2.5 (ssDNA binding protein), 3 (endonuclease), 4 (helicase/primase), 5 (DNA polymerase) and 6 (exonuclease) (Powling and Knippers, 1974; Kerr and Sakowski, 1975; Araki and Ogawa, 1981). Two of these proteins, the gene 2.5 ssDNA binding protein and the gene 4 helicase are promising candidates for mediating DNA exchange.

The gene 2.5 protein is an essential protein for T7 DNA replication (Kim and Richardson, 1993). It binds to ssDNA (binding constant of 1.2×10^6/M) and physically interacts via an acidic C-terminal domain (Kim and Richardson, 1994) with T7 DNA polymerase and the gene 4 protein to stimulate their activities (Nakai and Richardson, 1988; Mendelman and Richardson, 1991; Kim *et al.*, 1992b; Kim and Richardson, 1994). Sadowski *et al.* (1980) have suggested that gene 2.5 protein is responsible for the renaturation activity induced by infection of *E.coli* with phage T7 and, in agreement with this interpretation, we have found that purified gene 2.5 protein facilitates homologous base pairing. In this regard, the gene 2.5 protein joins a relatively large family of ATP-independent proteins that mediate homologous pairing (Kowalczykowski and Eggleston, 1994). When compared with other prokaryotic proteins known to be involved in recombination, such as *E.coli* RecA protein, *E.coli* SSB protein and T4 gene 32 protein, the gene 2.5 protein is considerably more efficient in mediating homologous base pairing (Kim *et al.*, 1992a; S.Tabor and C.C.Richardson, unpublished results). Here we show that the gene 2.5 protein mediates the formation of joint molecules in which a linear, duplex DNA molecule is stably annealed through a single-stranded region to circular ssDNA.

The T7 helicase, the product of gene 4, catalyzes the unwinding of duplex DNA in a reaction coupled to the hydrolysis of a nucleoside 5′-triphosphate (Matson *et al.*, 1983). Situated at the replication fork, the gene 4 protein translocates 5′ to 3′ on the lagging strand, unwinding the DNA so that leading strand synthesis can continue through the duplex DNA (Lechner and Richardson, 1983; Debyser *et al.*, 1994). The active form of gene 4 protein is a hexamer (Patel and Hingorani, 1993; Egelman *et al.*, 1995; Notarnicola *et al.*, 1995). Here we show that the T7 helicase can catalyze the unwinding of linear, duplex DNA present in a joint molecule with the concomitant annealing of one strand to the complementary circular, single-stranded partner. As expected from the unidirectional movement of the helicase, the strand transfer also proceeds in a polar manner. While we were preparing this manuscript, Salinas and Kodadek (1995) published studies showing that the helicase encoded by gene 41 of bacteriophage T4 catalyzes polar branch migration in a similar fashion in a multiprotein complex of which the UvsX protein is a component.

Results

Gene 2.5 protein promotes the formation of joint molecules

The T7 gene 2.5 protein binds specifically to ssDNA, stimulates the renaturation of complementary ssDNA (Kim *et al.*, 1992a; S.Tabor and C.C.Richardson, unpublished results) and is involved in recombination *in vivo* (Araki and Ogawa, 1980; F.W.Studier, unpublished results). Consequently, a likely role for the gene 2.5 protein is to facilitate homologous base pairing between complementary single-stranded regions in duplex DNA to generate a joint molecule (Kowalczykowski and Eggleston, 1994).

As shown schematically in Figure 1A, DNA substrates for the formation of joint molecules consist of circular, single-stranded M13 DNA and of linear, duplex M13 DNA having 3′-single-stranded termini of ~100 nucleotides. The latter substrate was prepared by incubation of linear, duplex M13 DNA with T7 gene 6 protein, a 5′ to 3′-exonuclease (Kerr and Sadowski, 1972). When these two substrates are incubated with gene 2.5 protein, a major portion of the two DNAs form stable joint molecules (Figure 1B). The formation of the joint molecule is dependent on the presence of gene 2.5 protein. In the experiment shown in Figure 1B, the duplex DNA molecules having single-stranded 3′ termini generated by

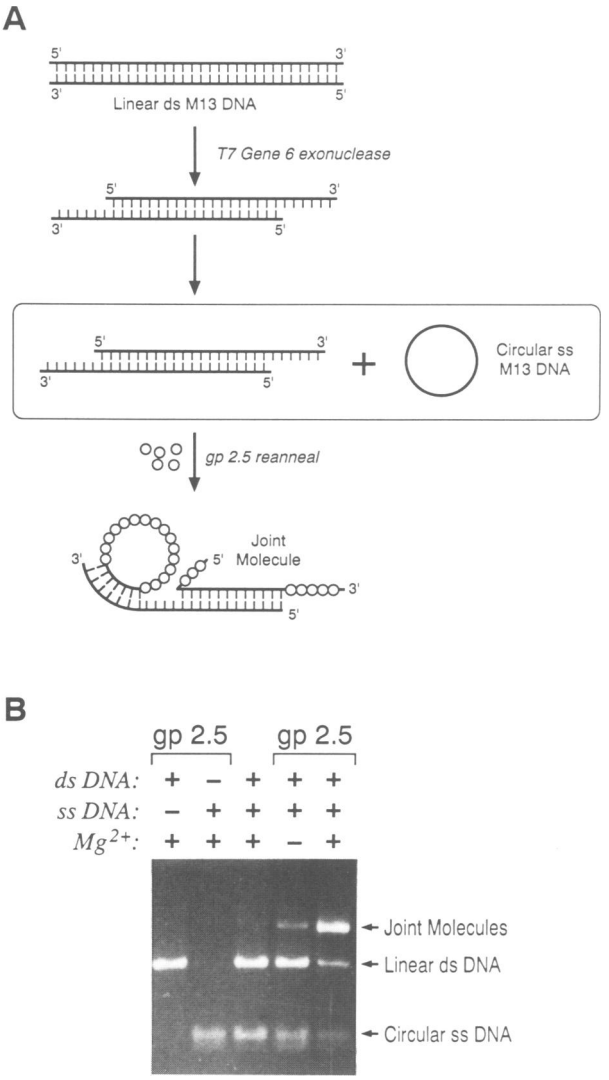

Fig. 1. Gene 2.5 protein promotes the formation of joint molecules. (**A**) Schematic diagram showing the formation of joint molecules. The DNA substrates for the formation of joint molecules are circular ssDNA and linear, duplex DNA bearing 3′-single-stranded termini. The latter are prepared by incubation with the 5′ to 3′ T7 gene 6 exonuclease. In the presence of gene 2.5 protein, the complementary single-stranded regions of the two molecules anneal to form a joint molecule. (**B**) Formation of joint molecules by gene 2.5 protein. The complete reaction contained 10 μM circular, single-stranded M13DNA, 10 μM linear duplex M13 DNA having single-stranded 3′ termini of ~100 nucleotides, 10 mM Mg^{2+} and 2 μM gene 2.5 protein. The reaction mixture was incubated at 32°C for 30 min and the products analyzed by agarose gel electrophoresis. Mg^{2+}, ssDNA, linear, duplex DNA and gene 2.5 protein were present (+) or absent (−) from the reaction mixture as indicated.

Table I. Requirements for the formation of joint molecules

Reaction mixture	Joint molecules formed (%)
Complete	100
–Gene 2.5 protein	3
–$MgCl_2$	15
–$MgCl_2$, + $CaCl_2$	20
+50–400 mM potassium glutamate	100
+10–80 mM NaCl	100
+5 mM ATP	100
–Gene 2.5 protein +gene 2.5-Δ21C protein (2 μM)	100
1 min incubation	30

The complete reaction mixture contained 25 mM Tris–HCl (pH 7.5), 5 mM DTT, 10 mM $MgCl_2$, 10 μM M13mp18 circular, single-stranded DNA, 10 μM linear, duplex M13mp18 DNA having 3′ single-stranded termini and 2 μM T7 gene 2.5 protein. A value of 100% is equivalent to 70% of the linear, duplex DNA converted to joint molecules.

incubation with T7 gene 6 exonuclease were used. However, identical results were obtained with DNA molecules having single-stranded 5′ termini generated by *E.coli* exonuclease III (Figure 5A). No joint molecules were obtained with linear, duplex DNA having either blunt ends or a four base overhang (data not shown).

The formation of joint molecules proceeds rapidly; after 1 min ~30% of the joint molecules obtained after 30 min of incubation were produced (Table I). Mg^{2+} significantly stimulates the reaction (Figure 1B), but the addition of ATP has no effect (Table I). Joint molecules are formed equally well in the presence of 50–400 mM potassium glutamate or 10–80 mM sodium chloride (Table I), suggesting that the reaction proceeds within physiological limits.

Formation of joint molecules requires stoichiometric amounts of gene 2.5 protein

We have examined the formation of joint molecules at various ratios of gene 2.5 protein to ssDNA to determine if the protein is required in stoichiometric amounts. As shown in Figure 2, no joint molecules were obtained when the molar ratio of gene 2.5 protein to nucleotides of circular ssDNA was <0.05. Only at a ratio of gene 2.5 protein to nucleotides of ssDNA of 0.1 or higher are joint molecules formed rapidly. The ratio of gene 2.5 protein to linear, duplex DNA is not critical since joint molecules are formed from a ratio of 0.3:0.01.

Gene 2.5 protein binds to ssDNA with a stoichiometry of seven nucleotides bound per monomer of gene 2.5 protein (Kim *et al.*, 1992a). The results presented in Figure 2 show that gene 2.5 protein promotes homologous DNA pairing only when ssDNA is covered completely by gene 2.5 protein.

Gene 2.5 protein alone is unable to mediate branch migration

Although gene 2.5 protein stimulates the formation of joint molecules between circular ssDNA and homologous linear, duplex DNA having short single-stranded termini, we did not detect the products of complete strand exchange, circular, duplex DNA containing a short gap and a displaced linear ssDNA (Figures 1 and 2). However, since agarose gel analysis of the reaction products is limited to the detection of branch movement of >7 kb, we have

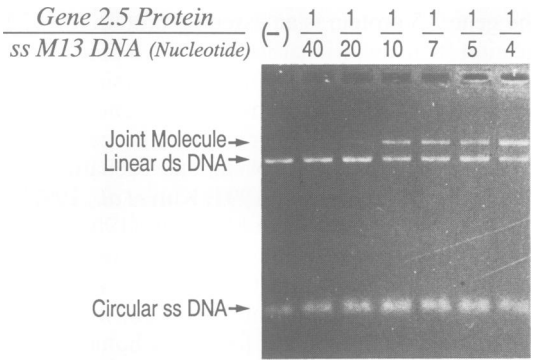

Fig. 2. Stoichiometry of gene 2.5 protein in the formation of joint molecules. Reactions contained 10 μM circular single-stranded M13 DNA, 10 μM linear, duplex M13 DNA having single-stranded 3′ termini and increasing concentrations of gene 2.5 protein from 0.25 μM to 2.5 μM. The molar ratio of gene 2.5 protein to nucleotides of circular ssDNA increased from 1/40 to 1/4. After incubation at 32°C for 30 min, the reaction mixtures were analyzed by agarose gel electrophoresis.

used the procedure of Kodadek and Alberts (1987) to detect branch migration.

As shown in Figure 3, the linear duplex M13 DNA has a *Bgl*II site and an *Eco*105I site located 688 and 2270 bp from the annealed left end, respectively. In order to follow the fate of the duplex DNA, its 3′ termini were labeled with ^{32}P. If branch migration progressed for ~600 bp and passed the *Bgl*II site, the displaced ssDNA should escape the cut by *Bgl*II and appear at the length (7.1 kb) of the linear DNA used as substrate (lane M) in a denaturing agarose gel. If the branch does not migrate pass the *Bgl*II site, *Bgl*II cleavage will generate two fragments of 688 and 6561 bp. As shown in Figure 3, branch migration did not reach the *Bgl*II site even after 30 min of incubation since no full-length DNA appeared after incubation with *Bgl*II. The same experiment was carried out with *Eco*105I restriction endonuclease and again no full-length DNA appeared after incubation with *Eco*105I (data not shown). We conclude that gene 2.5 protein does not mediate branch migration within the sensitivity of 600 bp of the assay.

Gene 2.5 protein and gene 4 protein together mediate branch movement

The high frequency of genetic recombination in T7-infected cells (Studier, 1969) and its independence of the host RecA pathway (Powling and Knipper, 1974; Kerr and Sadowski, 1975) suggest that T7 encodes its own recombination proteins. Among the T7-encoded proteins, the most likely candidate to mediate strand exchange is the gene 4 helicase. The T7 helicase catalyzes the unwinding of duplex DNA either alone (Matson *et al.*, 1983) or in conjunction with leading strand synthesis at a replication fork (Lechner and Richardson, 1983). In addition, it interacts physically with the gene 2.5 protein (Kim and Richardson, 1994).

In the presence of gene 4 protein, the joint molecules already formed in the presence of gene 2.5 protein are converted rapidly to circular, duplex M13 molecules and linear, single-stranded M13 DNA (Figure 4A), the expected products of complete strand exchange. The conversion is rapid, with ~30% of the conversion occurring within the first minute. If the gene 4 protein was present

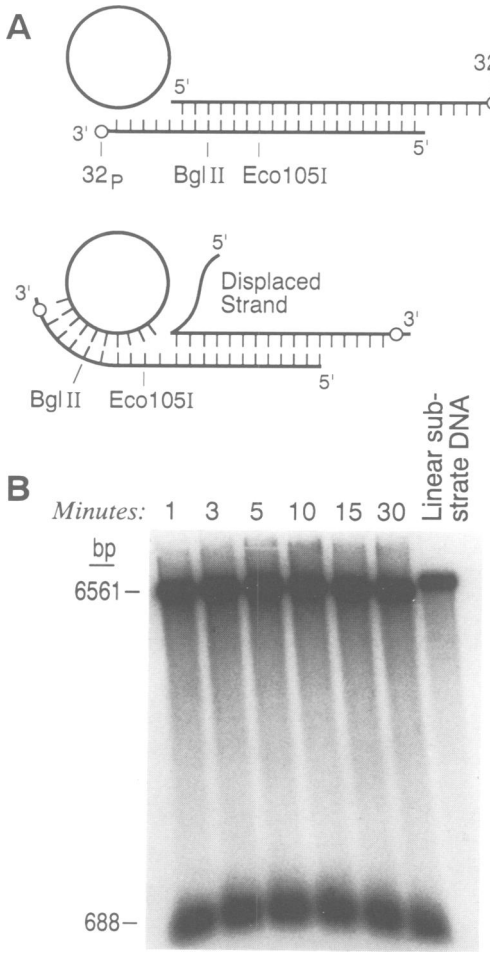

Fig. 3. Assay for branch migration. (**A**) Schematic drawing for the branch migration assay. In order to detect branch migration in the joint molecules, unique restriction sites were used to detect strand transfer through these sites. The linear, duplex M13 DNA contains a *Bgl*II and an *Eco*105I site located 688 and 2270 bp from the annealed 3′ end of the linear, duplex DNA. If branch migration proceeds through either site, then the displaced strand will not be cleaved by the respective restriction enzyme, resulting in the appearance of a full-length (7.1 kb) single-stranded M13 molecule. Otherwise, the molecule will be cleaved and fragments of 688 (2270) and 6561 (4979) nucleotides would appear after cleavage at the *Bgl*II (*Eco*105I) site. To follow the fate of the displaced strand, the linear, duplex DNA was labeled with ^{32}P at the 3′ termini. (**B**) Gene 2.5 protein does not mediate branch migration. The reaction mixture contained 10 μM circular, single-stranded M13 DNA, 10 μM linear, duplex M13 DNA having single-stranded 3′-^{32}P-termini and 2 μM gene 2.5 protein added at 0 min. The reaction was incubated at 32°C. Aliquots were removed at the times indicated and incubated with excess *Bgl*II for 1 min at 37°C. Samples were analyzed in alkaline agarose gel eletrophoresis as described in Materials and methods and an autoradiograph of the gel are shown. Lane M contains the linear DNA substrate used above that is ~100 nucleotides shorter (7.1 kb) than full-length M13 DNA. The positions of ssDNA bands with sizes of 6561 and 688 nucleotides are indicated.

at the beginning of the reaction, along with gene 2.5 protein, strand exchange also occurred but the amount of strand exchange was reduced by 40%.

Gene 4 of T7 encodes a 56 and a 63 kDa protein, the former being expressed from an internal ribsome binding site and start codon located 189 bases from the 5′ end of the gene 4 open reading frame (Dunn and Studier, 1983). Both the 56 and 63 kDa proteins catalyze ssDNA-dependent hydrolysis of ATP or dTTP, translocate unidirectionally on ssDNA and have helicase activity (Bernstein and Richardson, 1988a,b). The 63 kDa gene 4 protein also has primase activity by virture of a zinc motif located in its amino-terminus (Mendelman *et al.*, 1994).

As shown in Figure 4B, both the 56 and 63 kDa gene 4 proteins mediate branch migration to the same extent. Furthermore, the branch movement is dependent on hydrolysis of a nucleoside 5′-triphosphate. Circular duplex DNA is not generated in the absence of dTTP. The non-hydrolyzable nucleoside 5′-triphosphate analog, β,γ-methylene dTTP, an inhibitor of translocation and helicase activity of gene 4 protein (Matson *et al.*, 1983) cannot replace dTTP, suggesting that hydrolysis of dTTP or ATP is required (Figure 4C). In most of the reactions in the present study, we have used an ATP regeneration system to avoid the depletion of NTP and accumulation of NDP, the product of hydrolysis of NTP. Strand exchange proceeds equally well in the presence of 200–300 mM potassium glutamate.

From the results presented in Figure 4A, we calculate the rate of branch migration mediated by the gene 2.5 and 4 proteins to be at least 120 bases/s. This rate is considerably faster than the four bases/s reported for the *E.coli* RecA protein (Cox and Lehman, 1981; Kahn *et al.*, 1981) and 15 bases/s for the T4 UvsX protein (Kodadek *et al.*, 1988). In the experiment shown in Figure 4, the molar ratio of gene 4 protein hexamer to circular ssDNA molecule was 20 to 1. If the concentration of gene 4 protein was reduced 10-fold to give a ratio of hexamers of gene 4 protein to DNA molecules of 2, the rate for formation of circular, duplex DNA was reduced, but by 30 min the extent of the reaction was identical to that obtained in Figure 4A (data not shown). Therefore, the gene 4 protein appears to be acting catalytically.

Strand exchange mediated by gene 2.5 and gene 4 proteins has a 5′ to 3′ polarity with regard to the invading strand

In view of the unidirectional 5′ to 3′ translocation of the gene 4 helicase on ssDNA (Tabor and Richardson, 1981; Matson *et al.*, 1983), it seemed likely that this property of the gene 4 helicase would dictate the polarity of the strand exchange. In order to examine the polarity of strand exchange, we prepared two linear duplex DNA substrates, one having single-stranded 3′ termini and another having single-stranded 5′ termini. The single-stranded 3′ termini were generated by treatment of linear, duplex M13 DNA with T7 gene 6 exonuclease and the single-stranded 5′ termini were generated by treatment with *E.coli* exonuclease III. The single-stranded region in each substrate was ~100 nucleotides.

Gene 2.5 protein promotes the annealing of each form of the linear, duplex DNAs to circular, single-stranded M13 DNA with high efficiency to form joint molecules (Figure 5A and B, lane 1). Upon addition of gene 4 protein, however, strand exchange occurs only with the joint molecule prepared with linear, duplex DNA having single-stranded 3′ termini (Figure 5A, lane 2) and not with that prepared with linear, duplex DNA having single-stranded 5′ termini (Figure 5B, lane 2). We conclude that the gene 4 helicase mediates strand exchange in a 5′ to 3′ direction with regard to the invading strand.

Interestingly, the amount of joint DNA constructed from duplex molecules bearing single-stranded 5′ termini

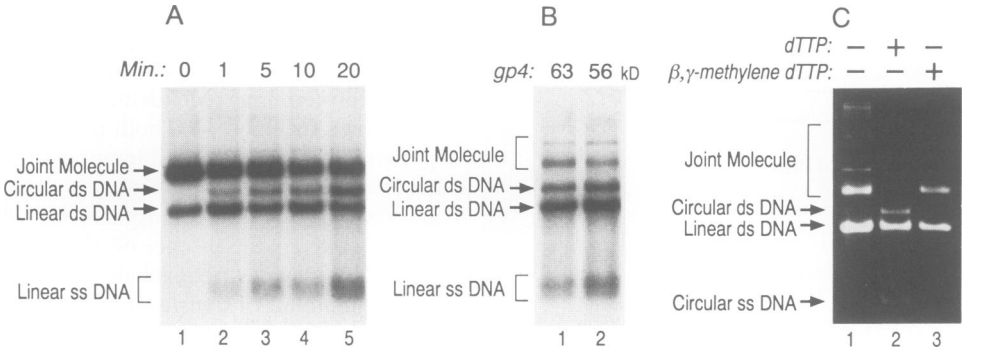

Fig. 4. Gene 2.5 and gene 4 proteins mediate strand exchange. (**A**) Time course of strand exchange mediated by gene 2.5 and gene 4 proteins. Reaction mixtures contained 10 μM circular, single-stranded M13 DNA, 10 μM linear, duplex M13 DNA bearing single-stranded 3′-^{32}P-labeled termini and 2 μM gene 2.5 protein. After incubation at 32°C for 20 min to form joint molecules, 63 kDa gene 4 protein was added to 0.2 μM, ATP to 5 mM, creatine phosphate to 10 mM, creatine phosphokinase to 5 U/ml and $MgCl_2$ to raise its concentration to 15 mM. The time at which gene 4 protein was added is designated as 0 min. Incubation was continued at 32°C and aliquots were removed at the times indicated and analyzed by agarose gel eletrophoresis as described in Materials and methods. An autoradiograph of the gel is shown here. The positions of circular, duplex DNA and displaced linear ssDNA are indicated. (**B**) Both the 56 and 63 kDa gene 4 proteins mediate strand exchange. Reaction mixtures and analysis of products were as described in (A) except that 56 kDa gene 4 protein was substituted for 63 kDa gene 4 protein as indicated. (**C**) Nucleoside 5′-triphosphate hydrolysis is required for strand exchange. The reaction mixtures for the formation of joint molecules by gene 2.5 protein were as described in (A). Strand exchange was initiated by addition of the 63 kDa gene 4 protein to 0.2 μM, $MgCl_2$ to raise its concentration to 15 mM and dTTP or β, γ-methelene dTTP, as indicated, to 5 mM. After incubation at 32°C for 20 min, the products were analyzed by agarose gel electrophoresis. An autograph of the gel is shown here.

decreased significantly during incubation with gene 4 protein. This joint molecule (Figure 5B) is a substrate for the T7 helicase translocating 5′ to 3′ on the circular M13 DNA since it bears a short region of unpaired 3′ nucleotides at the beginning of the fully dupex region (Matson *et al.*, 1993). Consequently, helicase action will unwind the short annealed region and reduce the amount of joint molecules. In contrast, for the joint molecule depicted in Figure 5A to be a substrate for unwinding would require that the 3′ end of the annealed segment have a displaced single-stranded tail. A more intriguing question is how the gene 4 helicase loads onto the displaced strand, an essential step for strand exchange (see Discussion).

Protein specificity of the strand exchange reaction

In order to determine if the physical interaction of gene 2.5 protein with gene 4 protein is important in the strand exchange reaction, we have used a genetically altered gene 2.5 protein, gene 2.5-Δ21C protein that lacks 21 amino acids at its C-terminus (Kim and Richardson, 1994). Gene 2.5-Δ21C protein cannot interact physically with gene 4 protein as does the wild-type gene 2.5 protein (Kim and Richardson, 1994).

The gene 2.5-Δ21C protein retains its ability to bind to ssDNA (Kim and Richardson, 1994) and we find that it promotes homologous base pairing as well as does the wild-type gene 2.5 protein (Table I and Figure 6A, lanes 3 and 4). However, gene 4 protein is unable to mediate strand exchange in a gene 2.5-Δ21C protein-coated joint molecule as judged by the lack of appearance of circular duplex DNA molecules (Figure 6A, lane 4). Likewise, the phage T4 helicase, the gene 41 protein that also translocates 5′ to 3′ (Richardson and Nossal, 1989), is unable to replace the gene 4 protein in the strand exchange reaction (Figure 6B, lane 3).

In order to increase the sensitivity of measurement of branch migration mediated by the gene 2.5-Δ21C and gene 4 proteins or gene 2.5 and T4 gene 41 proteins, we

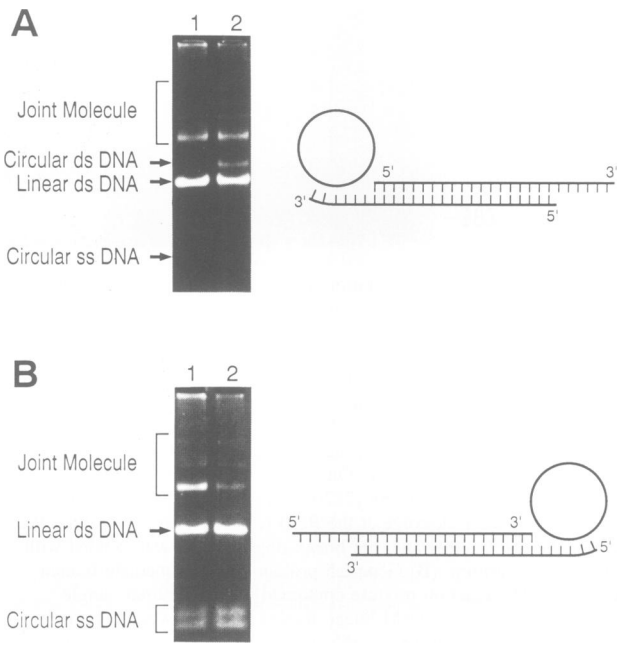

Fig. 5. Branch migration mediated by gene 2.5 and 4 proteins exhibits 5′ to 3′ polarity with regard to the invading strand. Strand exchange reactions were carried out as described in Materials and methods. Circular, single-stranded M13 DNA was present in all reactions along with one of two linear, duplex M13 DNA substrates. One linear, duplex molecule bears single-stranded 3′ termini (**A**) and the other single-stranded 5′ termini (**B**). The preparation of the linear, duplex DNA is described in Materials and methods. After incubation for 20 min at 32°C in the presence of gene 2.5 protein, an aliquot was analyzed by agarose gel electrophoresis (lane 1). The 63 kDa gene 4 protein was then added to 0.2 μM along with dTTP to 5 mM and incubation was continued for 20 min at 32°C. The products were analyzed by agarose gel electrophoresis.

employed the same restriction enzyme assay described in the experiment shown in Figure 3. As shown in Figure 6C, no detectable branch migration occurred in reactions containing either the gene 2.5-Δ21C protein (lanes 5 and

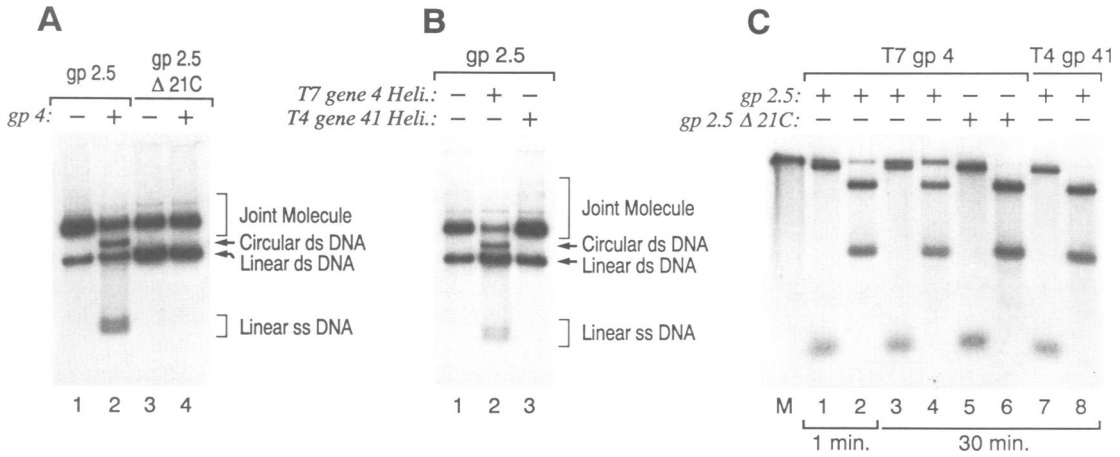

Fig. 6. Protein specificity of strand exchange. The protein specificity of strand exchange was examined by replacing gene 2.5 protein with a truncated gene 2.5 protein, gene 2.5-Δ21C protein (A and C) and by replacing gene 4 protein with the phage T4 gene 41 helicase (B and C). (**A**) Gene 2.5-Δ21C protein functions in homologous base pairing but not in strand exchange. Assays for the formation of joint molecules were carried out as described in Materials and methods with 2 μM gene 2.5 protein or gene 2.5-Δ21C protein as indicated. Assays for branch migration were carried out as described in Materials and methods with 0.2 μM 63 kDa gene 4 protein and either gene 2.5 protein or gene 2.5-Δ21C protein as indicated. Linear, duplex M13 DNA bearing single-stranded 3′-^{32}P-labeled termini was used in all reactions. The products of the reactions were analyzed by agarose gel electrophoresis and the autoradiograph of the gel is shown. (**B**) Phage T4 gene 41 helicase cannot substitute for T7 gene 4 helicase. Assays for the formation of joint molecules by gene 2.5 protein and for strand exchange were carried out as described in (A). 0.2 μM T7 gene 4 protein (63 kDa) or T4 gene 41 helicase was present as indicated. (**C**) Extent of branch migration. Strand exchange reactions were carried out with gene 2.5 or gene 2.5-Δ21C protein and gene 4 or T4 gene 41 proteins as indicated. Assays were as described above in (A) and (B) using linear, duplex DNA bearing single-stranded 3′-^{32}P-termini and circular, single-stranded M13 DNA. At the times indicated, a 10 μl aliquot was removed and incubated with *Bgl*II (lanes 1, 3, 5 and 7) or *Eco*105I (lanes 2, 4, 6 and 8) as described in the legend to Figure 3. An autoradiograph of the products of the reaction after alkaline agarose gel electrophoresis is shown. The time at which T7 gene 4 or T4 gene 41 protein was added is designated as 0 min. Lane M contains the full-length linear ssDNA band from denatured linear, duplex DNA bearing single-stranded 3′-^{32}P-termini.

6) or the T4 gene 41 helicase (lanes 7 and 8). No strand movement proceeded even as far as 600 bp, as indicated by the lack of full-length of displaced ssDNA after cleavage by *Bgl*II after 30 min of incubation (Figure 6C). In contrast, in reactions containing wild-type gene 2.5 protein and gene 4 protein, branch migration proceeded through the *Bgl*II and *Eco*105I sites located 688 and 2270 bp away at both 1 min (lanes 1 and 2) and 30 min (lanes 3 and 4) (Figure 6C). Clearly, a physical interaction between the gene 2.5 and 4 proteins is essential for strand exchange (see Discussion).

Discussion

Two proteins encoded by bacteriophage T7 work together to mediate DNA strand exchange (Figure 7). The gene 2.5 ssDNA binding protein promotes the annealing of complementary DNA strands. Consequently, a joint molecule consisting of circular ssDNA stably annealed to the single-stranded terminus of a homologous duplex DNA molecule is formed in the presence of stoichiometric amounts of gene 2.5 protein. Subsequently, the T7 gene 4 helicase, in a reaction requiring the energy of nucleotide hydrolysis, can mediate exchange of one strand of the duplex molecule for the circular ssDNA partner. The rate of strand exchange proceeds at >120 nucleotides/s with a 5′ to 3′ polarity with regard to the invading strand.

In vivo studies have shown that, in addition to their well known roles in DNA replication, *E.coli* SSB protein (Glassberg *et al.*, 1979), T4 gene 32 protein (Tomizawa *et al.*, 1966; Berger *et al.*, 1969; Mosig *et al.*, 1979) and T7 gene 2.5 protein (Araki and Ogawa, 1981) are also involved in recombination. However, the multiple reactions in which these proteins participate have made it difficult to dissect their contributions to recombination and replication since they may serve identical roles in both processes. For example, the ability of the gene 2.5 protein to bind to ssDNA is certain to be essential for its role at the replication fork and in mediating renaturation of ssDNA. Likewise, the ability of the gene 2.5 protein to interact physically with the T7 DNA polymerase and helicase underlies its ability to coordinate reactions during DNA synthesis and strand exchange. Consequently, it is unlikely that mutations will be found that affect one process without affecting the other. However, in the case of the T7 gene 2.5 protein, earlier studies have provided evidence for a direct role of gene 2.5 protein in recombination, a role distinct from its role in DNA replication (Araki and Ogawa, 1981). In these studies, an amber mutation was identified in gene 2.5 protein that gave rise to a truncated protein that was defective in both replication and recombination. However, although DNA synthesis was restored to levels of 60–70% of that observed with wild-type gene 2.5 protein, genetic recombination remained defective. Moreover, a novel assay recently has provided direct evidence for the role of the gene 2.5 protein in recombination *in vivo* in the absence of DNA replication (F.W.Studier, personal communication). T7 phage carrying amber mutations in gene 2.5 cannot grow on non-permissive strains of *E.coli*, presumably as a result of their defect in DNA replication. Furthermore, the T7 phage infection cannot be rescued by the presence of a plasmid harboring a non-complementing portion of gene 2.5, a portion of gene 2.5 which, however, can rescue gene 2.5 by recombination to eliminate the amber mutation. In contrast, similar experiments with gene 5, the DNA polymerase gene, show that recombination between the plasmid gene 5 and the defective phage gene occur

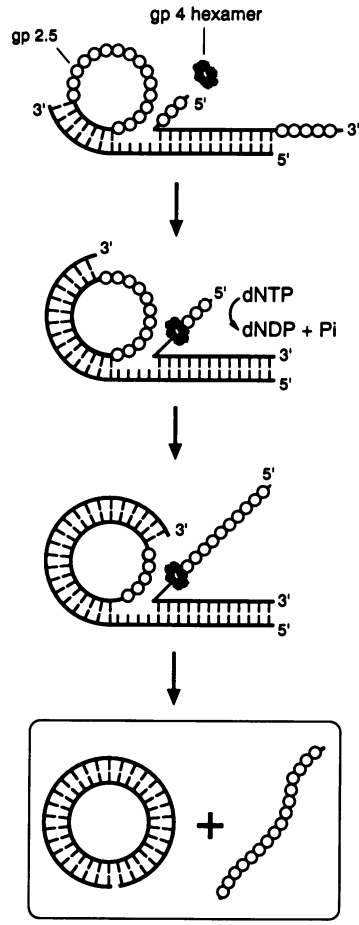

Fig. 7. A model for the mechanism of branch migration mediated by gene 2.5 protein and gene 4 helicase. Gene 2.5 protein coats the single-stranded portions of the two partner molecules and promotes homologous base pairing to form a stable joint molecule. Limited branch migration, perhaps assisted by the gene 2.5 protein, creates a 5′-single-stranded tail on the joint molecule. The hexamer of gene 4 protein, in the presence of NTP and assisted by gene 2.5 protein, binds to the 5′-single-stranded tail and translocates 5′ to 3′ using the energy of hydrolysis of NTP. The continued movement of the gene 4 protein enables it to function as a helicase to unwind the duplex DNA. Consequently, one strand of the duplex is displaced while the other, aided by the gene 2.5 protein, processively anneals to the M13 circular DNA as complementary bases are exposed.

normally. The data indicate that recombination in the gene 2.5-defective cells is at least 1000-fold less efficient than when gene 2.5 protein is present (F.W.Studier, personal communication).

The ability of ssDNA binding proteins to stimulate the renaturation of complementary strands of DNA (Chase and Williams, 1986) is clearly important to recombination. In this regard, the gene 2.5 protein can be distinguished from SSB protein and gene 32 protein in that it facilitates the renaturation of ssDNA much more efficiently provided that either Mg^{2+} or a relatively high concentration of NaCl is present (S.Tabor and C.C.Richardson, personal communication). The stimulation of base pairing requires stoichiometric amounts of gene 2.5 protein, an amount determined by the amount of ssDNA. Homologous base pairing reaches a maximum at approximately one monomer of gene 2.5 protein per 10 nucleotides of ssDNA, in agreement with earlier studies that show the monomer to cover seven nucleotides.

The mechanism by which gene 2.5 protein stimulates homologous base pairing so efficiently is not clear. It is not simply due to the elimination of secondary structure of ssDNA since the gene 2.5 protein is much less effective in this regard than is the *E.coli* SSB protein or the gene 32 protein. Although gene 2.5 protein binds specifically to ssDNA, its affinity (K_{assoc} = 1–4×10^6/M) is considerably less than that of SSB or gene 32 protein for ssDNA (K_{assoc} = 1×10^8/M) (Kim *et al.*, 1992a). The affinity of gene 2.5 protein for ssDNA is virtually identical to that of the *E.coli* RecA protein (Menetski and Kowalczykowski, 1985). However, unlike renaturation promoted by RecA protein, that mediated by gene 2.5 protein is not stimulated by ATP; gene 2.5 protein likewise does not promote binding of ssDNA to duplex DNA. Electron micrographs of gene 2.5 protein–DNA complexes show the ssDNA as highly compact with indications of intrastrand interactions (Kim *et al.*, 1992a). The fact that gene 2.5 protein exists in solution as a dimer (Kim *et al.*, 1992a) raises the possibility that protein–protein interactions between two gene 2.5 protein-coated ssDNA molecules nucleate the homologous base pairing reaction. However this appears unlikely in view of our results using a truncated gene 2.5 protein. Gene 2.5-Δ21C cannot form dimers, yet binding to ssDNA is normal (Kim and Richardson, 1994) and, as shown in the present study, it promotes the formation of joint molecules as well as does wild-type protein.

The strand exchange reaction mediated by the gene 4 helicase is in accord with the known properties of the T7 DNA helicase in DNA replication (Figure 7). First, the reaction is dependent on the presence of an NTP and is inhibited by the presence of a non-hydrolyzable NTP analog such as β,γ-methylene dTTP (Matson *et al.*, 1983). Second, the polarity of the strand transfer is 5′ to 3′, as is the unidirectional movement of the gene 4 protein on ssDNA (Tabor and Richardson, 1981; Matson *et al.*, 1983). Third, the rate of strand exchange of at least 120 nucleotides/s compares favorably with the rate of 300 nucleotides/s observed for its movement at the replication fork (Lechner and Richardson, 1983). The rate of strand exchange is particularly impressive when compared with the rates of ~4 and 15 nucleotides/s measured for the RecA and UvsX proteins, respectively (Cox and Lehman, 1981; Kahn *et al.*, 1981; Kodadek *et al.*, 1988).

The polarity of the gene 2.5 protein- and gene 4 protein-mediated strand exchange determines that a joint molecule made by circular ssDNA annealed to a 3′-single-stranded terminus of the homologous duplex molecule will be a proper substrate. Duplex DNA molecules with 5′-single-stranded termini do not participate in the exchange reaction and in fact joint molecules containing this species are destroyed by the action of the helicase. This latter result is anticipated since the 5′ to 3′ translocation of the helicase on the circular molecule will allow it to invade and separate the short duplex region through which the two participating molecules are bound. Of course, a helicase that translocates 3′ to 5′, of which there are several examples (Matson and Kaiser-Rogers, 1990), should mediate strand exchange with this DNA substrate but with an opposite polarity of exchange. We have used the T7 gene 6 exonuclease to generate the proper 3′-single-stranded termini for the gene 4 protein. The gene 6 exonuclease has been implicated in recombination in T7

phage-infected cells (Powling and Knippers, 1974; Kerr and Sadowski, 1975; Araki and Ogawa, 1981) and, from the current studies, it is apparent that its specificity of hydrolysis makes it likely that it, along with the gene 2.5 protein and helicase, are components of the T7 recombination machinery.

An interesting process is the mechanism by which the gene 4 protein loads onto the displaced strand of the linear, duplex DNA of the joint molecule to initiate unwinding. Gene 4 protein requires a single strand of DNA of at least 17 nucleotides to which it can bind in order to initiate 5′ to 3′ translocation (Matson *et al.*, 1983). In addition, there is a requirement for a short (>6 nucleotides) single-stranded 3′ tail. A similar requirement has also been found for the *E.coli* DnaB helicase (Lebowitz and McMacken, 1986) and the T4 gene 41 helicase (Richardson and Nossal, 1989). It is likely that there is sufficient non-protein branch migration at the single-strand–duplex junction to provide a single-stranded tail of at least 17 nucleotides to which the gene 4 protein can bind and initiate translocation (see Figure 7). Whether the gene 2.5 protein is essential for this reaction is not yet known. The fact that extensive strand exchange does not occur with the gene 2.5-Δ21C protein could be due to one of two mechanisms. The gene 2.5-Δ21C protein does not interact physically with the gene 4 protein (Kim and Richardson, 1994) although it does promote the formation of joint molecules. Consequently, the lack of interaction between the two proteins could reflect a specific role of the gene 2.5 protein in loading the gene 4 protein onto the DNA. Alternatively, a specific interaction of the two proteins may be required for the gene 4 protein to displace gene 2.5 protein bound to ssDNA. At any rate, our results underline the need for a specific interaction between the two proteins. The T4 gene 41 protein cannot substitute for the T7 gene 4 protein and the T4 ssDNA binding protein cannot substitute for the gene 2.5 protein (data not shown).

Once the gene 4 protein has assembled on the displaced strand, the resulting complex is not unlike that of a replication fork. At a replication fork the T7 DNA polymerase catalyzes the polymerization of nucleotides on the leading strand as the helicase bound to the lagging strand unwinds the duplex DNA, resulting in a displaced strand. In the model shown in Figure 7, base pairs are formed on the analogous leading strand, not by nucleotide polymerization, but rather by annealing of the complementary strands as the helicase unwinds the DNA, again with the resulting displacement of the analogous lagging strand. The exact contribution of base pair formation to the continuous and extensive unwinding is not known, nor is the role of gene 2.5 protein as it undoubtedly assembles on the displaced strand.

Do DNA helicases play a major role in recombination, particularly in strand exchange, in other systems? While we were carrying out these studies, Salinas and Kodadek (1995) showed that the gene 41 helicase of bacteriophage T4, not the UvsX protein, is responsible for the branch migration observed in a multiprotein complex. In the T4 system, homologous base pairing to form a joint molecule is carried out by the UvsX–UvsY protein complex and ssDNA is required in only one of the two partners. Once the gene 41 protein has been loaded onto the displaced strand by the gene 59 protein, the subsequent helicase-mediated strand exchange is strikingly similar to that reported here for the T7 gene 2.5 protein–gene 4 protein system.

Another, perhaps less analogous, role for helicases in recombination, comes from recent studies in which the RuvA DNA binding protein and the RuvB helicase have been shown to be involved in the movement of Holliday junctions (Iwasaki *et al.*, 1992; Tsaneva *et al.*, 1992, 1993). Salinas and Kodadek (1995) have suggested that helicases may also play a major role in other recombination systems, including the yeast *RAD51* recombination system and other eukaryotic systems involving *RAD51*-like genes, and we will not elaborate further on them here. We feel that it is equally important to re-examine a number of strand exchange reactions that have been described, including those mediated by such well characterized proteins as the RecA protein.

It is likely that bacteriophage T7 with its usual economy of proteins has mustered several of the proteins of DNA replication for its recombination system. The gene 6 exonuclease is also involved in the breakdown of host DNA (Center *et al.*, 1970) after infection and the removal of RNA primers from the 5′ termini of Okazaki fragments (Shinozaki and Okazaki, 1977). Gene 4 protein provides both helicase and primase activity at the replication fork. Gene 2.5 protein is essential for T7 DNA replication (Kim and Richardson, 1993) and appears to have been designed uniquely as a ssDNA binding protein to play roles in both replication and recombination since it exhibits functions of both the SSB and gene 32 protein as well as of RecA protein. What other proteins are likely to be involved in recombination? The gene 3 endonuclease of T7 is known to resolve structures in DNA that resemble Holliday junctions (de Massey *et al.*, 1987; Dickie *et al.*, 1987). Lacking so far is a RecA-type protein that can carry out a true search for homology within a duplex DNA molecule. Although such a protein may exist in T7-infected cells, the case may equally be that sufficient single-stranded regions are generated at the replication fork during the rapid replication of the T7 chromosome to allow for recombination mediated by gene 2.5 single-stranded DNA binding protein and gene 4 helicase.

Materials and methods

Proteins

T7 gene 2.5 protein was purified to apparent homogeneity from an *E.coli* strain overproducing the protein as described by Kim *et al.* (1992a). The preparation of T7 gene 2.5-Δ21C protein has been described (Kim and Richardson, 1994). The 56 and 63 kDa gene 4 proteins were purified from cells overproducing each by B.Beauchamp (Harvard Medical School) as described (Mendelman and Richardson, 1991). Most of the experiments carried out in this study have used a 63 kDa gene 4 protein that has Met at position 64 replaced by Gly but which has all of the catalytic properties of the wild-type protein (Mendelman *et al.*, 1993). The 63 kDa gene 4 protein also has primase activity in the presence of rATP and rCTP (Mendelman *et al.*, 1993). T4 gene 41 helicase was kindly provided by Dr Nancy Nossal (National Institutes of Health). T7 gene 6 exonuclease, *E.coli* exonuclease III, terminal deoxynucleotidyl transferase and restriction enzymes were purchased from United States Biochemical.

Nucleic acids

Circular, single-stranded M13mp18 DNA was purchased from United States Biochemical. M13mp18 RFI DNA was prepared as described by

Messing (1983). Linear, duplex M13 DNA was prepared by incubation of circular duplex M13 DNA with restriction enzymes *Sma*I or *Eco*RI. Linear duplex M13 DNA molecules with single-stranded 3′ termini were prepared by incubation of *Sma*I-linearized DNA with T7 gene 6 exonuclease by a minor modification of the procedure of Johnson and Kolodner (1991). The reaction mixture (100 μl) contained 15 μg of DNA, 100 mM KCl, 5 mM dithiothreitol (DTT), 5 mM $MgCl_2$, 50 mM Tris–HCl (pH 8.0) and 15 U of T7 gene 6 exonuclease. After incubation at 12°C for 50 s, the reaction was stopped by the addition of 3 μl of 0.5 M EDTA and 100 μl of Tris–HCl buffer-equilibrated phenol and then immediately extracted with phenol–chloroform and chloroform. The DNA was precipitated with ethanol, and then dissolved in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5). Linear duplex M13 DNA molecules with single-stranded 5′ termini were prepared by incubation of *Eco*RI-linearized M13 DNA with *E.coli* exonuclease III (Richardson *et al.*, 1964). The reaction conditions were the same as described for hydrolysis with gene 6 exonuclease, except that 10 μg of DNA was digested by 200 U of *E.coli* exonuclease III at 23°C for 3 min. The average length of single-stranded 5′ or 3′ termini was determined by examining whether the exonuclease-treated DNAs could be cut by restriction enzymes *Hin*dIII and *Pvu*I whose sites are located 35 (51) and 158 (174) bp away from the *Sma*I (*Eco*RI) site, respectively. Since the majority (70%) of the M13 DNA molecules lost the *Hin*dIII site but retained the *Pvu*I site, the average length of single-stranded 5′ or 3′ termini is ~100 nucleotides. 3′-^{32}P-labeled linear, duplex DNA having single-stranded 3′ termini was prepared with [α-^{32}P]ddATP and terminal nucleotidyl transferase as described (Tu and Cohen, 1980). DNA concentrations are expressed in nucleotide equivalents.

Formation of joint molecules with T7 gene 2.5 protein

The assay for the formation of joint molecules measures the formation of a stable complex of linear, duplex M13 DNA bearing single-stranded termini and circular, single-stranded M13 DNA. The standard reaction, unless stated otherwise, contained 25 mM Tris–HCl (pH 7.5), 5 mM DTT, 10 mM $MgCl_2$, 10 μM circular single-stranded M13DNA, 10 μM linear duplex DNA having 3′- or 5′-single-stranded termini and 2 μM gene 2.5 or gene 2.5-Δ21C protein. After incubation at 32°C for 30 min, the reaction was stopped by the addition of SDS to 0.5%, EDTA to 50 mM and proteinase K to 0.6 mg/ml and followed by incubation at 32°C for an additional 15 min. Samples were electrophoresed through a 0.8% agarose gel in Tris-acetate-EDTA (TAE) buffer at 0.5 V/cm for 15 h. After electrophoresis, gels were stained in TAE buffer containing 0.5 μg/ml ethidium bromide and DNA bands were illuminated under UV light and photographed.

Strand exchange reaction mediated by gene 2.5 protein and gene 4 protein

The assay for strand exchange was carried out in two steps (i) the formation of joint molecules by gene 2.5 protein, followed by (ii) the addition of gene 4 protein to catalyze strand exchange. (i) The formation of joint molecules was carried out as described above except that the time of incubation was 20 min in order to allow for maximum formation of joint molecules. (ii) After the formation of the joint molecules, the second stage of the reaction was initiated by the addition of gene 4 protein to 0.2 μM, ATP to 5 mM, creatine phosphate to 10 mM, creatine phosphokinase to 5 U/ml and $MgCl_2$ to raise its concentration to 15 mM. After 20 min of incubation at 32°C, the reaction was stopped and analyzed by electrophoresis as described above for the analysis of joint molecules. If ^{32}P-labeled linear duplex DNA was used, the gel was further dried and autoradiographed. A nucleoside 5′-triphosphate (5 mM ATP or dTTP) or an ATP regeneration system was present as indicated in the experiments.

Assay of branch migration

The extent of branch migration was measured by determining if the branch passes the unique sites of the *Bgl*II or *Eco*105I restriction enzymes in the linear, duplex DNA (Kodadek and Alberts, 1987). In all reactions, 3′-^{32}P-labeled linear duplex DNA having single-stranded 3′ termini was used. The assay for measuring the extent of branch migration mediated by only gene 2.5 protein was carried out as described for the formation of joint molecules with gene 2.5 protein. The assays for measuring the extent of branch migration mediated by gene 2.5 and 4 proteins, gene 2.5-Δ21C and gene 4 proteins, or gene 2.5 and T4 gene 41 proteins were carried out as described for the strand exchange reaction with gene 2.5 and gene 4 proteins. The time at which gene 4 protein or T4 gene 41 protein was added is designated as 0 min. Aliquots (10 μl) were removed at the times indicated, mixed with *Eco*105I (18 units) or *Bgl*II (18 units), incubated at 37°C for 1 min, and quenched by addition of NaOH to 30 mM and EDTA to 30 mM. When *Bgl*II was used, 100 mM NaCl was added to the digestion reaction. A control reaction showed that 1 min is sufficient for the DNA cleavage. Samples were electrophoresed through 1.2% alkaline agarose gel at 0.6 V/cm for 15 h in buffer of 30 mM NaOH, 1 mM EDTA. After electrophoresis, the gel was neutralized in TAE buffer, dried on Whatman chromatography paper and autoradiographed.

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