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The Homologous Recombination System of Phage λ

PAIRING ACTIVITIES OF β PROTEIN*

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The *red* genes of phage λ specify two proteins, exonuclease and β protein, which are essential for its general genetic recombination in recA⁻ cells. These proteins seem to occur *in vivo* as an equimolar complex. In addition, β protein forms a complex with another polypeptide, probably of phage origin, of M_r 70,000. The 70-kDa protein appears to be neither a precursor nor an aggregated form of either exonuclease or β protein, since antibodies directed against the latter two proteins failed to react with 70-kDa protein on Ouchterlony double diffusion analysis. β protein promotes Mg^{2+}-dependent renaturation of complementary strands (Kmiec, E., and Holloman, W. K. (1981) *J. Biol. Chem.* 256, 12636–12639). To look for other pairing activities of β protein, we developed methods of purification to free it of associated exonuclease. Exonuclease-free β protein appeared unable to cause the pairing of a single strand with duplex DNA; however, like *Escherichia coli* single strand binding protein (SSB), β protein stimulated formation of joint molecules by recA protein from linear duplex DNA and homologous circular single strands. Like recA protein, but unlike SSB, β protein promoted the joining of the complementary single-stranded ends of phage λ DNA. β protein specifically protected single-stranded DNA from digestion by pancreatic DNase. The half-time for renaturation catalyzed by β protein was independent of DNA concentration, unlike renaturation promoted by SSB and spontaneous renaturation, which are second order reactions. Thus, β protein resembles recA protein in its ability to bring single-stranded DNA molecules together and resembles SSB in its ability to reduce secondary structure in single-stranded DNA.

Bacteriophage λ has two genes *exo* and *bet* that enable it to carry out homologous recombination in the absence of *Escherichia coli* recA protein and the recombination pathways that depend upon recA. The pathway that depends on the functions of *exo* and *bet* is called the Red pathway. The product of the *exo* gene is an exonuclease of M_r 24,000 which degrades double-stranded DNA processively from the 5′ end releasing 5′ mononucleotides (Radding and Shreffler, 1966; Little, 1967; Shulman *et al.*, 1970). The enzyme cannot initiate action at a nick in duplex DNA nor digest single-stranded DNA, but rapidly cleaves oligonucleotides (Carter and Radding, 1971; Sriprakash *et al.*, 1975). As a consequence of these properties, λ exonuclease abetted by branch migration can cleanly trim a redundant single-stranded branch from DNA to leave a nick that can be sealed by DNA ligase (Cassuto *et al.*, 1971). This set of events, which has been termed strand assimilation, may be imagined to play a role in the maturation of imperfectly spliced intermediates in recombination. The product of the *bet* gene is a protein of M_r 28,000 that promotes the renaturation of complementary single strands and interacts with λ exonuclease (Kmiec and Holloman, 1981; Radding, 1971). β protein presumably plays a role in pairing events that utilize the products of exonuclease action or that create substrates for it, or both.

The product of the *E. coli* recA gene which is essential for recombination of *E. coli* is an ATPase of M_r 38,000 that requires single-stranded DNA as a cofactor. It carries out two kinds of pairing reaction, the renaturation of complementary single strands and the pairing of single-stranded or partially single-stranded DNA with duplex DNA, which is termed strand invasion (Weinstock *et al.*, 1979; Shibata *et al.*, 1979; McEntee *et al.*, 1979). Strand invasion consists of both homologous pairing and strand exchange. *In vitro*, the exonuclease and β protein of the phage λ Red system do not utilize ATP; and *in vivo* λ recombination does not require recA protein if both exonuclease and β protein are present. Thus, the λ Red system provides the opportunity to study *in vitro* components of an apparently different alternate pathway of homologous recombination.

Attempts to study the function of partially purified β protein have been complicated by the presence of associated residual exonuclease activity. Purification of β protein to near homogeneity and removal of contaminating exonuclease activity have permitted the further study of β protein activities reported here and have revealed the association of β protein with another protein of M_r 70,000.

EXPERIMENTAL PROCEDURES

Bacteria, Enzymes, and DNA—The growth and induction of *E. coli* lysogen K12 W_{3350} (λ_{TH1}) which carries a defective prophage have been described previously (Radding, 1966). Phage P22 DNA (2.6×10^4 cpm/nmol) was prepared essentially as described by Botstein (1968). Circular single-stranded and circular duplex DNA from phage M13 and φX174 were prepared as described by Cunningham *et al.* (1980). Linear M13 or φX174 duplex DNA was alkali denatured as described by Bryant and Lehman (1985). Single-stranded P22 DNA was prepared by boiling at 90 °C in 10 mM Tris-HCl (pH 7.5), 1 mM EDTA for 5 min in a Eppendorf centrifuge tube followed by quick chilling in ice water. Phage λDNA (λcI 857S7) and endonuclease *Eco*RI were obtained from New England Biolabs. *E. coli* recA protein was purified to homogeneity, and its concentration was determined as described by Tsang *et al.* (1985). *E. coli* SSB[1] was the generous gift of John

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[1] The abbreviations used are: SSB, single strand binding protein; SDS, sodium dodecyl sulfate; ATPγS, adenosine 5′-*O*-(thiotriphosphate).

Chase (Dept. of Molecular Biology, Albert Einstein College of Medicine, New York). λDNA was digested with *Eco*RI essentially as described by the supplier, and the specific fragments were isolated from low melting agarose gels (0.7%) after electrophoresis as described by Maniatis *et al.* (1982). Sepharose and Sephadex gels were obtained from Pharmacia.

Buffers—Buffer A was 0.02 M Tris-HCl (pH 7.5), 0.1 M NaCl, 2 mM EDTA, 5 mM 2-mercaptoethanol, and 10% (v/v) glycerol. Buffer B was 0.02 M potassium phosphate (pH 6.8), 2 mM EDTA, 5 mM 2-mercaptoethanol, and 10% (v/v) glycerol.

Purification of β Protein and 70-kDa Protein—All steps were performed at 4 °C. The procedure used was essentially as described before (Radding, 1971) up to chromatography on DEAE-cellulose step. An extract was prepared from cells of a lysogen induced by ultraviolet light. Packed cells (100 g) in 500 ml of 0.01 M Tris-HCl (pH 7.5) were sheared with glass beads (200 g) in a Sorvall Omni-Mixer. The suspension was centrifuged at 13,000 × *g* for 10 min, and the supernatant (500 ml) was stirred with 50 ml of 5% streptomycin sulfate over a period of 15 min. The dense white precipitate obtained after centrifugation was extracted with 1 liter of buffer containing 0.05 M potassium phosphate (pH 6.8), 5 mM 2-mercaptoethanol, and 1 mM EDTA; the volume was subsequently made up to 1600 ml with the buffer used for extraction. To the above suspension, 184 ml of 20% dextran sulfate 500, 516 ml of 30% polyethylene glycol 6000, and 672 g of NaCl were added with continuous stirring over a period of 2 h. The mixture was allowed to separate into phases overnight. The upper phase was dialyzed against 5 changes of a buffer containing 0.01 M Tris-HCl (pH 7.0), 5 mM 2-mercaptoethanol, and 1 mM EDTA. A white precipitate that formed was collected by centrifugation at 13,000 × *g* for 10 min, suspended in 60 ml of Buffer A, and allowed to remain on ice for 2 h. The supernatant obtained by centrifugation at 13,000 × *g* for 10 min was dialyzed overnight against 6 liters of Buffer A.

The dialyzed protein fraction was applied at a flow rate of 15 ml/h to a column of DEAE-cellulose (2.8 × 40 cm) which had been equilibrated with Buffer A. After application of the dialyzed protein, the column was washed with Buffer A containing 0.15 M NaCl until the eluate contained no material that absorbed light at 280 nm. The bound proteins were eluted by increasing the salt concentration to 0.3 M in Buffer A. Exonuclease activity was eluted in a single symmetrical peak of protein. The fractions containing exonuclease activity were combined and dialyzed against three changes of 2 liters of Buffer B. The dialyzed fraction was applied to a column of phosphocellulose (2.8 × 40 cm) which had been equilibrated with Buffer B. The protein fraction was loaded on the column at a flow rate of 1 ml/min. The column was washed with 250 ml of Buffer B and then eluted with a linear gradient from 0.02 to 0.5 M PO_4 in Buffer B (250 ml each). Chromatography on phosphocellulose resulted in three peaks of protein corresponding, respectively, to β protein, exonuclease, and a complex of β protein and exonuclease (Radding, 1971). However, we found substantial amounts of β protein in the flowthrough from phosphocellulose as evidenced by Ouchterlony immunodiffusion analysis (results not given). Attempts to readsorb β protein on a second column of phosphocellulose were unsuccessful, indicating that we were not overloading the first column. When analyzed on a SDS-polyacrylamide gel, two major polypeptide bands corresponding to a M_r of 28,000 and 70,000 were evident. The procedure developed for the isolation of the 28–70-kDa complex and its complete resolution into 28-kDa protein (β protein) and 70-kDa protein comprises a series of steps which are outlined in Fig. 1. Briefly, we precipitated the protein in the passthrough with $(NH_4)_2SO_4$ at 60% saturation at 0 °C over a period of 1 h. The precipitate was collected by centrifugation at 23,000 × *g* for 15 min. The pellet was resuspended in 4 ml of buffer A containing 0.5 M NaCl and applied on a Sephacryl S-200 column (1.8 × 120 cm). The column was eluted with the above buffer at a flow rate of 15 ml/h. The fractions containing 28–70-kDa complex were combined, dialyzed against 0.02 M Tris-HCl (pH 7.5), 5 mM 2-mercaptoethanol, 1 mM EDTA, and 10% glycerol. The resolution of 28–70-kDa complex into 28-kDa protein and 70-kDa protein was achieved by chromatography on a column of Sephacryl S-200 in the presence of 2 M NaCl (see "Results" and Fig. 1). From 100 g of cells, we obtained 7.2 mg of β protein in the free form from chromatography on phosphocellulose and 4.8 mg after dissociation and separation of a complex of β protein-70-kDa protein in the presence of 2 M NaCl by gel filtration on Sephacryl S-200.

Assay for Reannealing of Complementary Single-stranded DNA—Renaturation of complementary single strands was assayed by the conversion of denatured DNA to a form resistant to digestion by S_1 nuclease, essentially according to Weinstock *et al.* (1979). The reaction mixtures (50 μl) contained 10 mM potassium phosphate buffer (pH 6.0), 10 mM $MgCl_2$, and 3.3 nmol of heat-denatured [^{3}H]DNA (2.6×10^4 cpm/nmol). Mixtures containing the indicated concentrations of β protein were incubated at 37 °C for 20 min. At the end of the incubation, 0.45 ml of S_1 nuclease assay buffer containing 0.2 M sodium acetate buffer (pH 4.5), 0.1 M NaCl, and 2 mM $ZnSo_4$ was added, followed by 5 units of S_1 nuclease. Incubation at 37 °C was continued for 30 min before the addition of 250 μl of carrier calf thymus DNA (1 mg/ml) and 250 μl of cold 1.75% percholoric acid, and the tubes were transferred to an ice bath. After 10 min on ice, mixtures were centrifuged at 4 °C for 15 min at 15,000 × *g* in an Eppendorf centrifuge. An aliquot from each supernatant was removed for scintillation counting in Liquiscint fluor.

Assay for Joining of the Ends of λDNA—This assay is based on the complementarity of the single-stranded ends of λDNA (Wu and Taylor, 1971; Sanger *et al.*, 1982). To monitor efficiently the joining of ends by base pairing, we cleaved the phage DNA with *Eco*RI which generates 6 fragments, the largest and smallest being the terminal fragments containing 12 unpaired protruding nucleotides. The reaction was done for β protein at pH 6.0 in 0.02 M potassium phosphate buffer containing 10 mM $MgCl_2$ and 5 mM 2-mercaptoethanol; or the reaction for recA protein contained 0.02 M Tris-HCl at pH 7.5, 10 mM $MgCl_2$, and 5 mM 2-mercaptoethanol plus DNA. After incubation at 37 °C for 15 min, EDTA was added to 25 mM followed by SDS to 0.1% and proteinase K to 0.2 mg/ml. Incubation was continued at 37 °C for another 15 min. Glycerol was added to 5% along with loading dye and buffer, and individual samples were loaded on a 0.7% agarose gel and subjected to electrophoresis in 0.089 M Tris/borate containing 2 mM EDTA (pH 8.3) for 16 h at 40 mA. After electrophoresis, the gel was soaked in solution containing ethidium bromide (0.5 μg/ml) for 30 min and photographed.

Other Methods—Protein concentrations were determined by the method of Lowry *et al.* (1951), using bovine serum albumin as standard. Polyacrylamide gel electrophoresis was by the Laemmli (1970) procedure. The assay for joint molecules is based on the method of Beattie *et al.* (1977) and was described by Muniyappa *et al.* (1984). Exonuclease activity was assayed as described by Radding (1966). ATPase activity was assayed as described by Shibata *et al.* (1981). Antibodies to β protein and λ exonuclease were produced in rabbits as detailed elsewhere (Radding and Shreffler, 1966).

RESULTS

Further Purification of β-Protein and Discovery of an Associated Protein of M_r 70,000

β protein, purified as described previously (Radding, 1971), contains residual amounts of exonuclease activity and small amounts of a protein of M_r 70,000. We found that substantial amounts of β protein could be recovered in the passthrough of a phosphocellulose column, which was the last step of the earlier protocol for purification (Fig. 1). We precipitated the proteins in the passthrough with $(NH_4)_2SO_4$ at 60% saturation, and the redissolved proteins were chromatographed on

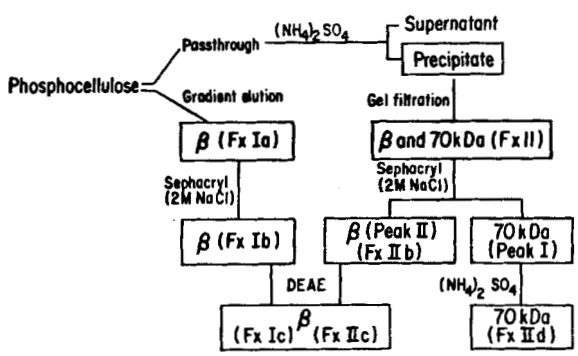

FIG. 1. **Fractionation scheme for purification of β protein and 70-kDa protein.** See "Experimental Procedures" for details.

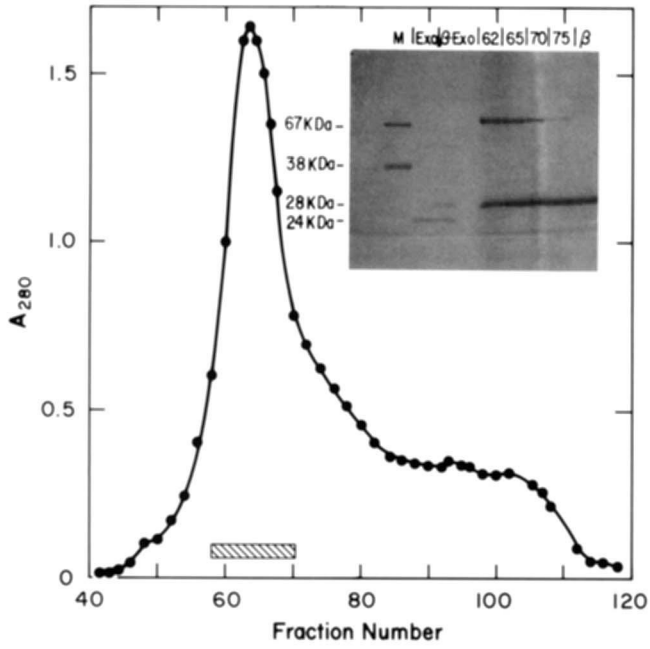

FIG. 2. **Identification of a complex of β protein and a protein of M_r 70,000.** Protein in the flowthrough from phosphocellulose column was precipitated by the addition of $(NH_4)_2SO_4$ at 60% saturation with continuous stirring at 4 °C. The precipitate was collected by centrifugation at 23,000 × *g* for 15 min at 4 °C. The pellet was resuspended in 4 ml of buffer containing 20 mM Tris-HCl, 10 mM 2-mercaptoethanol, 1 mM EDTA, 0.5 M NaCl, and 10% (v/v) glycerol (pH 7.5) and was applied to a column of Sephacryl S-200 (1.8 × 120 cm). The column was eluted with the above buffer, and fractions of 1.9 ml were collected at a flow rate of 15 ml/h. *Inset,* SDS-polyacrylamide gel electrophoresis of 12 μg protein from each of fractions 62, 65, 70, 75 in the peak and 5 μg each of markers (*M*), bovine serum albumin, and recA protein. *Exo,* authentic exonuclease, 5 μg; *β*-Exo, the complex of exonuclease and β protein, 10 μg; and *β*, authentic protein, 5 μg.

a column of Sephacryl S-200 in the presence of 0.5 M NaCl and 10% glycerol. When the fractions in the protein peak (Fig. 2) were analyzed by SDS-polyacrylamide gel electrophoresis, two major protein components were evident, β protein and a protein of M_r 70,000 ± 2,000 (*inset* of Fig. 2). Rechromatography of some of the peak fractions from the Sephacryl S-200 column under identical conditions yielded the same pattern of elution, with both proteins contained in a single peak. However, when we increased the concentration of NaCl to 2 M, in the absence of glycerol, the two proteins resolved into two distinct peaks on a Sephacryl S-200 column, the first peak containing the 70-kDa protein, and the second containing β protein (data not shown). Under denaturing conditions on SDS-polyacrylamide gels, the protein from the second peak migrated as a single molecular species and exhibited a M_r of 28,000, when compared to a set of molecular weight standards. Migration was the same as that of authentic β protein. Further evidence that the protein in the second peak was β protein was provided by its ability to reanneal complementary single strands and its cross-reaction in an Ouchterlony test by antibodies to authentic β protein (data not given). The protein from the first peak consisted largely of a single polypeptide chain of M_r 70,000 ± 2,000. The 70-kDa protein appeared to be neither a precursor nor an aggregated form of either exonuclease or β protein since the antibodies directed against these proteins failed to react with 70-kDa protein on Ouchterlony double diffusion analysis (data not given).

Gel filtration on Sephacryl S-200 and chromatography on DEAE-cellulose were used to purify β protein that was retained by phosphocellulose, as well as β protein present in the passthrough, associated with 70-kDa protein (Fig. 1).

Previous observations have shown that β protein forms a relatively weak complex with λ exonuclease (Radding, 1971). Current observations suggest that β protein forms a more stable complex with the 70-kDa protein. Exonuclease and β protein were partially separated by 0.16 M phosphate buffer (pH 6.8) in 10% glycerol, whereas β protein and 70-kDa protein were resolved by concentrations of NaCl above 1.5 M in the absence of glycerol.

Characterization of 70-kDa Protein

The 70-kDa protein was not associated with β protein when the latter was purified from strains in which the gene for β protein or the genes for both β protein and λ exonuclease were cloned in an inducible plasmid,[2] which suggests that the 70-kDa protein may be the product of phage λ rather than *E. coli*.[3] When the M_r of native 70-kDa protein was determined by gel filtration on Sephadex G-100 column, the elution volume of 70-kDa protein closely coincided with that of bovine serum albumin, indicating a M_r of 70,000 ± 2,000. Since this was also the M_r obtained from SDS-polyacrylamide gels we conclude that the native protein is a monomer of M_r 70,000 ± 2,000. When equimolar amounts of 70-kDa protein and β protein were incubated together at 37 °C for 30 min in 0.02 M potassium phosphate (pH 6.8) and chromatographed on a column of Sephacryl S-200 in the absence of NaCl, both proteins eluted in the void volume, as a single complex of higher molecular weight (data not shown). Preliminary observations have suggested that various amounts of the complex of β protein-70-kDa protein promoted renaturation less than half as well as comparable amounts of β protein alone (see "Renaturation of Complementary Single Strands," below). Further studies on the physical and functional properties of the complex are in progress.

Only 5% of ATP (50 μM) was hydrolyzed by 70-kDa protein after 30 min at 37 °C. Nuclease activity of 70-kDa protein was undetectable when assayed using M13 single-stranded or duplex [^{3}H]DNA at pH 6.0 or 7.5. Less than 1.0% of the initial radioactivity became acid soluble. Agarose gel electrophoresis also confirmed the absence of significant DNA nicking or degradation activity when M13 superhelical DNA was incubated with 70-kDa protein for 30 min at 37 °C.

Characterization of β Protein

β protein from phosphocellulose chromatography was subjected to further purification by gel filtration on a column of Sephacryl S-200 in the presence of 2 M NaCl, which removed any detectable amounts of associated exonuclease or 70-kDa protein. Fraction Ib (Fig. 1) thus obtained contained at least 95% β protein and produced no detectable acid-soluble radioactivity when incubated with [^{3}H]P22 double-stranded DNA for 1 h at 37 °C. β protein obtained from the dissociation of 70-kDa protein-β protein complex (Fraction IIb, Fig. 1) and β protein that had been retained by phosphocellulose chro-

[2] K. Muniyappa and C. M. Radding, manuscript in preparation.

[3] Since induction of β protein in a lysogen involves brief intermittent exposures to UV light, which also is known to induce host dnaK protein of M_r 70,000, a "heat shock protein" (Neidhardt and Van Bogelen, 1981), we tested for the ability of polyclonal antibodies to dnaK protein to recognize 70-kDa protein. When analyzed on Ouchterlony double diffusion analysis, authentic dnaK protein showed cross-reactivity with the antibodies but 70-kDa protein did not. Polyclonal antibodies to dnaK protein and the authentic antigen were kindly provided by C. Georgepoulos of the University of Utah.

matography (Fraction Ia, Fig. 1) were indistinguishable by several criteria. When reacted with antibodies to β protein, both fractions produced a single confluent precipitin line on Ouchterlony double diffusion plates and showed identical mobility on SDS-polyacrylamide gels. Both promoted reannealing of complementary single strands to the same extent.

Activities of Purified β Protein

Specific Protection of Single-stranded DNA—Since β protein promotes the renaturation of complementary single strands (Kmiec and Holloman, 1981), one would expect it to bind preferentially to single-stranded DNA. At pH 6.0 and molar concentrations of β protein approaching that of the nucleotide residues, β protein protected 80% of M13 viral single-stranded DNA from digestion to acid-soluble nucleotides by DNase I, whereas only 20% of double-stranded DNA remained acid precipitable (Fig. 3). Consistent with its ability to renature complementary single strands, β protein initially protected single-stranded DNA obtained by denaturing φX174 duplex DNA, but this protection was lost in 5 min at 37 °C as renaturation proceeded (data not shown).

Potentiation of Joint Molecule Formation by recA Protein—Secondary structure in single-stranded DNA impedes the binding of recA protein and thereby reduces its ability to pair a single strand with duplex DNA. This negative effect of secondary structure manifests itself as a sharp temperature threshold below which the rate of formation of joint molecules by recA protein is negligible. Factors that diminish secondary structure in single-stranded DNA, including helix-destabilizing proteins such as *E. coli* SSB, lower the temperature threshold by 5–6 °C. We reported previously that β protein can lower this temperature threshold by 2–3 °C (Muniyappa *et al.*, 1984).

Under any conditions that we have yet examined, β protein alone did not promote significant formation of D-loops, but rather acted only as an auxiliary factor for recA protein. At 30 °C, β protein promoted the formation of joint molecules by subsaturating amounts of recA protein, namely 1 molecule of recA protein/7.6 nucleotide residues of single-stranded DNA (Fig. 4*A*). In reaction mixtures containing recA protein and β protein, a 17% reduction in the amount of recA protein sharply reduced the formation of joint molecules. β protein itself was required in stoichiometric amounts, about 1 molecule of β protein/4 nucleotide residues (Fig. 4*A*). Potentiation of the recA reaction by helix-destabilizing proteins similarly requires stoichiometric amounts of the auxiliary protein, although the final complex formed on single-stranded DNA consists largely of recA protein (Tsang *et al.*, 1985). Controls showed that β protein was free of exonuclease activity and that the signal measured by the D-loop assay depended upon the presence of homologous single-stranded DNA (Fig. 4*B*). In addition, by gel electrophoresis we confirmed that in the presence of β protein, recA protein at 30 °C produced nicked circular duplex DNA as the final product from the pairing of a circular single strand with linear duplex DNA. In the absence of β protein at 30 °C, recA protein produced no nicked circular duplex DNA (data not shown).

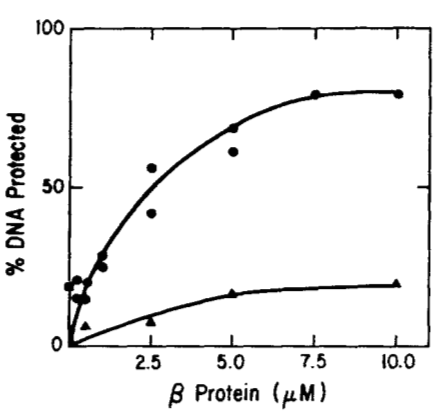

FIG. 3. **Protection of M13 circular single-stranded DNA from DNase I by phage λ β protein.** Ten μM [^{3}H]DNA was incubated with the indicated amounts of β protein in a reaction mixture (25 μl) containing 0.02 M potassium phosphate buffer (pH 6.0) and 10 mM Mg^{2+} at 37 °C for 10 min. At the end of 10 min of incubation, DNase I was added to a final concentration of 10 μg/ml, and incubation was continued at 37 °C for another 5 min. The reaction was terminated by the addition of 100 μl of cold 1.75% perchloric acid and 100 μl of denatured calf thymus DNA (0.85 mg/ml). Reaction mixtures were left on ice for 30 min before centrifugation at 15,600 rpm for 10 min in an Eppendorf centrifuge. An aliquot was taken for the determination of acid-soluble radioactivity (Williams *et al.*, 1981). ▲, linear duplex DNA; ●, circular single-stranded DNA; ■, circular single-stranded DNA in the absence of β protein.

Joining of the Complementary Ends of λ DNA—Whereas *E. coli* SSB promoted renaturation of complementary single strands at acid pH or at neutral pH in the presence of polyamines, it did not promote the joining of the complementary ends of λDNA (Christiansen and Baldwin, 1977). To monitor the pairing of complementary ends, we cleaved full length λDNA with *Eco*RI which generates 6 fragments, the smallest (*f*) and the largest (*a*) being the terminal ends of the λ genome containing 12 unpaired complementary nucleotides. We incubated the cleaved λ DNA with β protein or other proteins and subjected the products to electrophoresis after digesting with proteinase K. Joining of the complementary ends was shown by a decrease in the intensities of the small *f* band and the larger *a* band from the right and left ends of λDNA, respectively, and by the formation of a new band containing the combined *a* and *f* molecules (Fig. 5). Although the formation of a new band was clearly evident when the mixture of six fragments was incubated with β protein (Fig. 5, *Panel A*, *lanes c* and *d*), we isolated the individual fragments to establish that the smallest and the largest fragments in the *Eco*RI digest of λDNA were in fact contributing to the formation of a new band. When the isolated *a* and *f* fragments were incubated with increasing amounts of β protein, a new band appeared as both *a* and *f* diminished (Fig. 5, *Panel B*, *lanes f*, *g*, and *h*), but not when fragment *a* was incubated with the mixture of fragments *b*, *c*, *d*, and *e* (Fig. 5, *Panel B*, *lane i*). When a similar incubation was done with *a* and *f* fragments in the absence of β protein there was little or no formation of a new band. As expected from the observations of Christiansen and Baldwin (1977), we did not observe the formation of a new band by *E. coli* SSB at the concentrations tested, either when incubated with *a* and *f* fragments or with fragment *a* plus the mixture of *b*, *c*, *d*, and *e* fragments (Fig. 5, *Panel B*, *lanes j* and *k*). Indeed, it appeared that SSB inhibited the small degree of spontaneous joining that occurs in the absence of any protein (Fig. 5, *Panel B*, compare *lanes e* and *j*). In agreement with these results when the assay for end joining was done with β protein in the presence of SSB, the reaction was inhibited suggesting that the negative effect may not be due to the lack of SSB binding to the ends of λDNA but may be due to its inability to promote the reaction. As a further control, we tested the ability of recA protein to join the complementary ends of λDNA. In the presence of either ATP or ATPγS, recA protein promoted the joining of λ ends at pH 7.5, whereas SSB did not (data not shown).

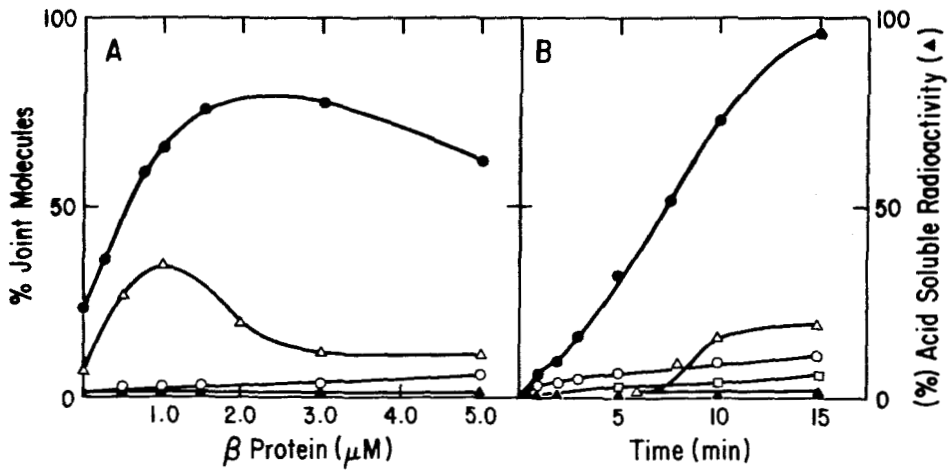

FIG. 4. **Effect of β protein on the formation of joint molecules by recA protein.** *A*, effect of various concentrations of protein. Reaction mixtures containing recA protein, at the indicated concentration, in an ATP-regeneration system were incubated at 30 °C for 15 min. The concentrations of M13 linear duplex [^{3}H]DNA (linearized by using restriction endonuclease *HpaI*) and M13 circular single strands were 3 and 8 μM, respectively. RecA protein was at 1.06 μM (●) and 0.88 μM (△). ○, heterologous control, 8 μM φX174 circular single strands in place of M13 and 1.06 μM recA protein. ▲, acid-soluble radioactivity in a reaction mixture that contained M13 single- and double-stranded DNA, 1.06 μM recA protein, and at the indicated concentration of β protein. The maximum acid-souble radioactivity at 5 μM protein was 1.2% of the input [^{3}H]DNA. *B*, time course. The concentrations of M13 linear duplex [^{3}H]DNA, single-stranded DNA, and incubation conditions are as in *A*. Aliquots (10 μl) were directly added into 0.3 ml of 25 mM EDTA on ice and after 5 min, diluted with 3 ml of SSC (10 ×) and filtered through nitrocellulose filters. ●, 1.06 μM recA protein plus 3 μM β protein; △, 1.06 μM recA protein alone; □, 3 μM β protein alone; ○, heterologous control, 8 μM φX174 circular single strands in place of M13; and ▲, acid-soluble radioactivity in a reaction mixture that contained M13 single- and double-stranded DNA plus 1.06 μM recA protein and 3 μM β protein.

Renaturation of Complementary Single Strands

We confirmed the observation of Kmiec and Holloman (1981), who discovered that β protein promotes the renaturation of complementary single strands. We observed that saturating amounts of β protein purified through phosphocellulose renatured complementary single-stranded DNA to a maximal extent of 50%. However, the same preparation of β protein purified further by passage over DEAE-cellulose (Fraction Ic, Fig. 1) renatured more than 80% of the P22 DNA in 30 min (data not shown). When we studied the kinetics of renaturation of denatured φX174 duplex DNA, higher amounts of β protein were required to obtain maximal extent of renaturation, and the reason for this difference is unclear.

The kinetics of renaturation of complementary single strands by recA protein is first order (Bryant and Lehman, 1985). To examine the order of the renaturation reaction promoted by β protein, we varied the concentration of single-stranded DNA and held the ratio of β protein to DNA at 1 molecule of protein/6 nucleotide residues. Under these conditions, the half-time of the reaction was independent of the concentration of DNA (Fig. 6*A*, *inset*). As a control we did a similar experiment with SSB (Fig. 6*B*). Consistent with an earlier report (Christiansen and Baldwin, 1977), the reaction promoted by SSB appeared to be second order, as shown by a plot of the half-time as a function of the reciprocal of the initial concentration of DNA (Fig. 6*B*, *inset*).

DISCUSSION

In recent years a number of different proteins have been shown to promote the pairing of homologous DNA molecules (Cox and Lehman, 1981; Hubscher *et al.*, 1980, Radding, 1982, for review). In all cases but one, the activity described has been limited to the renaturation of complementary single strands. The prokaryotic and eukaryotic homologs of recA protein promote in addition the pairing of a single strand with duplex DNA, a reaction that is clearly important for genetic recombination (Radding, 1982; Kmiec and Holloman, 1982; Kenne and Ljungquist, 1984). Acting on Z-DNA, the rec-1 enzyme of *Ustilago maydis* also pairs fully duplex molecules (Kmiec *et al.*, 1985). Although the mechanisms of these reactions appear to be varied and are not understood in all cases, two distinct mechanisms have thus far been identified. The helix-destabilizing proteins appear to act by destabilizing secondary structure in single strands and removing a kinetic barrier to intermolecular base pairing. Accordingly, these reactions are second order (Wetmur and Davidson, 1968). RecA protein and rec-1 protein, on the other hand, appear to act by a synaptic mechanism, *i.e.* they first bring DNA molecules together without respect to homology and thereby facilitate subsequent homologous pairing (Radding, 1982). Accordingly, such reactions are first order (Bryant and Lehman, 1985). By destabilizing secondary structure, helix-destabilizing proteins can favor the pairing of a single strand with duplex DNA by the recA synaptic protein (Muniyappa *et al.*, 1984). Our observations on β protein show that it acts in some respects like a helix-destabilizing protein and in some respects like a synaptic protein. β protein by itself did not promote the pairing of a single strand with duplex DNA but was able to promote the recA protein reaction probably by destabilizing secondary structure in single-stranded DNA. On the other hand, β protein joined the ends of λDNA, like recA protein and unlike SSB. More significantly perhaps, β protein promoted renaturation of complementary single strands by an apparent first order reaction, suggesting that it plays a synaptic role.

Recombination promoted by the λ Red pathway, of which exonuclease and β protein are a part, occurs by a break-copy mechanism in which DNA replication plays an essential role. When replication is severely blocked by phage and host mutations, Red recombination is abolished except for exchanges

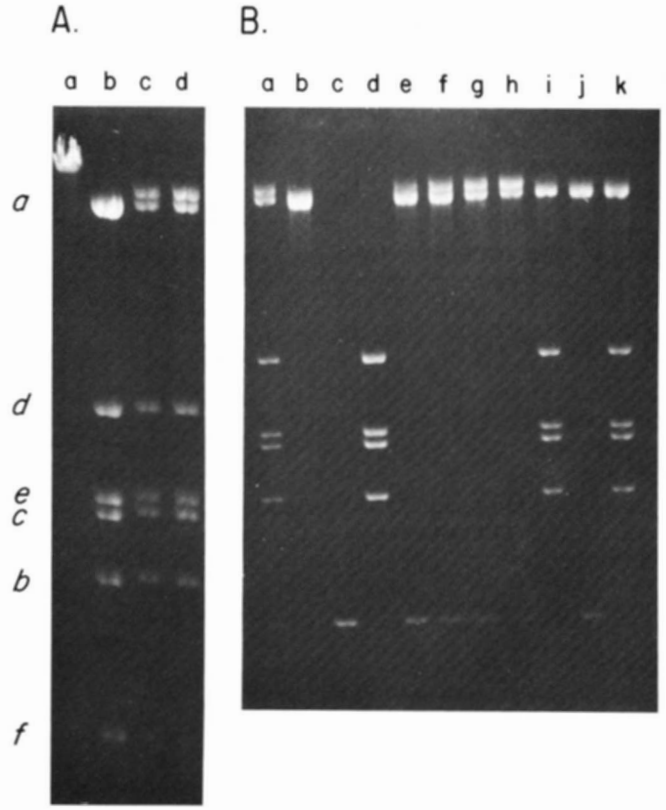

FIG. 5. **Joining of complementary ends phage λ DNA by β protein and SSB.** *Eco*RI-digested λDNA and β protein were incubated at the indicated concentrations in a buffer containing 0.01 M potassium phosphate and 10 mM Mg^{2+} (pH 6.0) in a final volume of 30 μl at 37 °C for 15 min. The reaction was terminated by the addition of SDS and EDTA to 0.1% and 25 mM, respectively. Protein was digested by proteinase K at 200 μg/ml for 15 min at 37 °C. The reaction mixture was loaded on 0.7% agarose gel after the addition of loading dye. Electrophoresis was done in a buffer containing 0.089 M Tris, 0.089 M boric acid, 2 mM EDTA (pH 8.3), and 0.5 μg/ml ethidium bromide for 16 h at 40 V. *Panel A: lane a,* undigested full length linear λDNA (8 μM); *lane b,* *Eco*RI-digested λDNA (12 μM); the individual bands (*a–f*) are identified according to Thomas and Davis (1975); *lane c,* same as *lane b,* but incubated in the presence of 6 μM β protein; *lane d,* same as *lane c,* but contained 12 μM β protein. *Panel B: lane a,* 8 μM *Eco*RI-digested λDNA and 6 μM β protein; *lane b,* isolated fragment *a* (8 μM); *lane c,* isolated fragment *f* (8 μM); *lane d,* fragments *b–e* (8 μM); *lane e,* fragments *a* and *f* (8 μM each) incubated in the absence of β protein; *lane f,* same as *e,* but incubated in the presence of 3 μM β protein; *lane g,* same as *f,* but contained 6 μM β protein; *lane h,* same as *g* but contained 12 μM β protein; *lane i,* 12 μM DNA of fragments *a* and *b–e* plus 12 μM β protein; *lane j,* 8 μM each fragments *a* and *f,* plus 8.5 μM SSB; *lane k,* 12 μM DNA of fragments *a* and *b–e* plus 17 μM SSB.

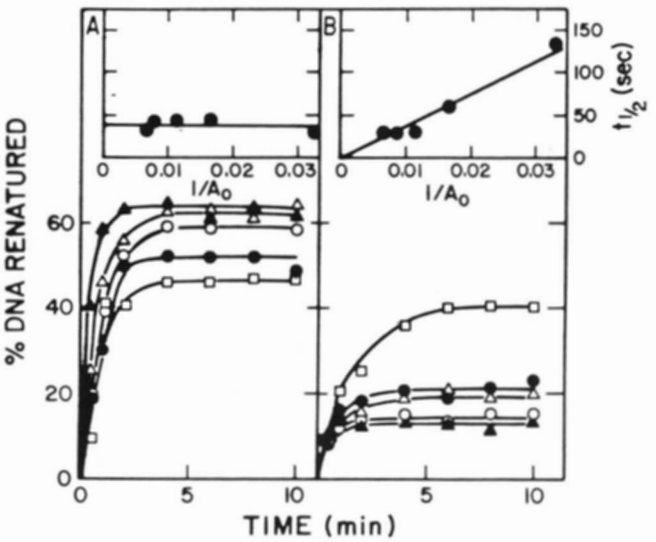

FIG. 6. **Renaturation of denatured φX174 duplex DNA by β protein and SSB.** Renaturation reactions were done at 37 °C with the indicated concentrations of DNA and protein. The reaction mixtures (100 μl) contained, for β protein: 0.02 M potassium phosphate buffer (pH 6.0), 10 mM $MgCl_2$; and for SSB: 0.02 M Tris-acetate buffer (pH 5.5) plus 10 mM $MgCl_2$. At the indicated time intervals aliquots of 10 μl were added to an Eppendorf tube containing 1 μl of 10% SDS to quench the reaction. The mixtures were diluted with 120 μl of a buffer containing 0.05 M sodium acetate (pH 4.5), 0.1 M NaCl, and 1 mM $ZnSO_4$. The DNA was digested with 50 units of S_1 nuclease for 30 min at 37 °C. The reaction was terminated by the addition of 100 μl of 1.75% perchloric acid (cold) and 100 μl (0.85 mg/ml) of heat-denatured calf thymus DNA. They were kept on ice for 30 min and filtered on GF/C filters (Bryant and Lehman, 1985). The filters were washed with 5 ml of cold 1.75% perchloric acid followed by 1 ml of 95% ethanol. The filters were dried, and the bound radioactivity was quantitated by scintillation counting in Betafluor. Renaturation was promoted by: *A,* β protein; and *B,* SSB. The *insets* represent the plot of $t_{1/2}$ *versus* the initial concentration of DNA. The ratio of protein to DNA was: β protein (1:6); SSB (1:10), and the concentration of denatured duplex [^{3}H]DNA was: □, 30 μM; ●, 60 μM; ○, 90 μM; △, 120 μM; and ▲, 150 μM.

near the right end of the λ chromosome which require little new DNA synthesis (Stahl *et al.*, 1974; McMilin *et al.*, 1974; White and Fox, 1975). In complementary fashion, the Red genes play a role in λ replication, although it is not an essential role. Red mutants synthesize DNA more slowly and tend to make shorter concatemeric forms than wild type (Skalka, 1974). The effects of λ replication and recombination can be rationalized by supposing that the Red system promotes the formation of new replication forks by the invasion of duplex DNA by a single-stranded 3′ terminus of a broken DNA molecule (Skalka, 1974; Stahl *et al.*, 1985). Thus far, however, studies of β protein *in vitro* have failed to demonstrate an ability to form D-loops.

The data presented here show that β protein can accelerate another step that is essential in the vegetative growth of λ, namely the pairing of its complementary single-stranded ends, a reaction that is also promoted by recA protein, but not by *E. coli* SSB. Skalka has reported that the burst size of λ phage in a $recA^-B^-$ strain of *E. coli* is reduced by two-thirds when the phage is bet^- (Skalka, 1974). It is possible that in the absence of either β or RecA protein, the circularization of λDNA which is essential for vegetative growth is delayed or reduced. To our knowledge, there are no published data on the rate of circularization of λDNA after infection of a $recA^-$ host by $\lambda\beta^+$ *versus* $\lambda\beta^-$. Other evidence that is consistent with this hypothesis is provided by studies of *Salmonella* phage P22 which requires an essential recombination function (erf) to circularize P22 DNA (Botstein and Matz, 1970; Weaver and Levine, 1977). The product of the *erf* gene is a single strand binding protein of M_r 23,000, which under certain conditions at least can promote renaturation of complementary single strands (Poteete and Fenton, 1983). Moreover, Poteete (1982) has shown that the bet^+ gene alone introduced on a plasmid can complement a P22 erf^- mutant. One should recall, however, that the ends of mature P22 DNA are redundant and double stranded. Thus, if the β gene acts by promoting renaturation of complementary strands, another enzyme may act to make the ends of P22 DNA single stranded. Recent observations of Conley and Saunders (1984) on transformation of *E. coli* with pBR322 DNA linearized at a unique *Sal*I site are consistent with an alternative role of recA protein in promoting the pairing of complementary ends of λDNA.

SalI generates ends with 4 unpaired nucleotides; modification of these ends either by filling them in or by dephosphorylation decreased the frequency of transformation and at the same time increased the frequency of adjacent deletions. Mutations in recA also reduced the transformation frequency.

Particularly given the importance of replication in λ recombination, a further understanding of the enzymatic basis of the Red pathways requires the discovery and elucidation of other proteins and enzymes. Previous studies from this laboratory have shown that the β protein occurs as a complex with exonuclease. The procedure formerly used to purify β protein contained residual amounts of exonuclease and a protein of M_r 70,000. As described here, during the separation of residual amounts of exonuclease activity from β protein on a gel filtration column we found a complex which after dissociation resolved into β protein and a protein of M_r 70,000 whose function remains unknown. By several criteria it appeared that β protein and 70-kDa protein form a specific and stable complex. Exonuclease forms a complex with β protein that binds to phosphocellulose and is dissociated by 0.16 M PO_4 in the presence of glycerol. By contrast, the complex of β protein and 70-kDa protein does not adsorb to phosphocellulose and is resolved into its individual components only by 2 M NaCl in the absence of glycerol. The identity and function of the 70-kDa protein are being studied.

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