In vitro, Escherichia coli RecA protein acts upon gapped and partially homologous linear duplex DNA to generate recombination products linked by Holliday junctions. When strand exchange reactions are supplemented with purified RecBCD enzyme, we observe the formation of products that resemble "patch" recombinants. This formation of "splice" recombinant products was not observed. The individual subunits, RecB, RecC, or RecD, had no effect on RecA protein-mediated strand exchange nor on the Holliday junctions formed in the reaction. Analysis of the way in which patch products arise indicates exonucleolytic digestion of the linear arms of the recombination intermediates (structures) by RecBCD enzyme. We find no evidence for specific resolution events at the site of the Holliday junction by RecBCD enzyme using these DNA substrates.

RecBCD enzyme is a multifunctional enzyme involved in genetic recombination, DNA repair, cell viability, and the degradation of foreign DNA in Escherichia coli. The protein, which is made up of three polypeptide subunits encoded by the recB, recC, and recD genes, possesses numerous activities including (i) ATP-dependent double- and single-stranded exonuclease; (ii) ATP-stimulated single-stranded endonuclease; (iii) a unidirectional DNA helicase; and (iv) a site-specific endonuclease that cuts at recombination hot spots (chi sites) as the protein unwinds DNA. The activities of RecBCD enzyme have been the subject of a number of recent reviews (1-3).

The role of RecBCD enzyme in genetic recombination is at present unclear, and it has been hypothesized to act at either early or late (or both) steps in the recombination process. In one "initiation" model for RecBCD action, it was proposed that the enzyme unwinds DNA from a double-stranded end and introduces nicks into chi sequences present within the unwound loop. The resulting single-stranded tail may then be utilized by RecA protein which catalyzes strand invasion into a homologous duplex DNA molecule (4). This model, which is consistent with the activities of RecBCD protein, recently gained support from experiments in which purified RecA and RecBCD proteins were used to generate joint molecules in vitro (5, 6).

In contrast, several lines of evidence are consistent with a role for RecBCD enzyme in the later stages of recombination, after RecA-mediated strand exchange and presumably at the level of resolution of the Holliday junction intermediate. (i) The generation of detectable intermediates in bacterial conjugation experiments did not require functional RecBCD enzyme while production of viable recombinants was drastically reduced (7). (ii) Faulds et al. (8) speculated that RecBCD enzyme plays a role in Holliday junction resolution during chi-stimulated recombination of bacteriophage λ. (iii) Rosenberg (9) challenged the prediction that chi-stimulated initiation of recombination in λ yields recombinant patches on the λ l chain that ends 3' at λ's right, by demonstrating chain bias for the λ r chain. (iv) This chain bias was independent of the orientation of chi, arguing against a role for RecBCD enzyme in initiation (10). (v) The isolation of a mutant (recB2153) that is recombination deficient, while retaining all known in vitro activities of RecBCD protein, might be considered to provide indirect support for this hypothetical role in the resolution of Holliday junctions (11).

Because of the complex nuclease properties of RecBCD enzyme, there has been interest in the action of RecBCD on recombination intermediates in vitro. For example, the ability of RecBCD enzyme to nick DNA at D-loops (12) or at model Holliday junction structures (13) has been investigated. Unfortunately, the experiments have been largely inconclusive. Using cruciform DNA structures that provide a convenient approach to the junction from two opposing linear termini. However, during recombination of bacteriophage λ, approach from a single direction is sufficient for RecBCD-dependent recombination (14).

In recent studies, we developed a system in which recombination intermediates could be made and resolved in vitro (15). We utilized the homologous pairing and strand exchange properties of RecA protein to produce structures in which two duplex molecules were linked by a Holliday junction. These structures were used to detect an activity from fractionated E. coli extracts which resolved the intermediates into recombinant products. Resolution occurred by specific endonucleolytic cleavage at the site of the Holliday junction (16). More recent studies have shown that mutants defective in ruvC lack the Holliday junction resolution activity (17), and purified RuvC protein is capable of resolving Holliday junctions in vitro.1

The phenotypic properties of ruv mutants are consistent with a defect in Holliday junction resolution (18, 19). The ruv locus contains three genes, designated ruvA, ruvB, and ruvC, a mutation in any of which confers a sensitivity to UV light, ionizing irradiation, and mitomycin C (20-22). The ruvA and

1 H. Dunderdale and S. C. West, unpublished observations.
ruwB genes are part of the inducible SOS system of DNA repair and are regulated by LexA protein (22-24). Cells carrying mutations in ruw are recombination deficient in a recBC sbcBC or recBC sbcA background although ruw single mutants show only slight recombination deficiency (18, 25).

Recent observation that the recombination-defective phenotype exhibited by ruw mutants is seen only in recBC sbcBC or recBC sbcA genetic backgrounds may indicate two alternative pathways for resolution: one utilizing Ruv proteins, the other RecBCD enzyme. The present experiments therefore ask whether RecBCD nuclease is capable of resolving Holliday junctions made during a defined in vitro recombination reaction and thus whether E. coli might possess more than one enzyme or activity capable of resolution.

MATERIALS AND METHODS

**Enzymes**—RecA protein of E. coli was purified to homogeneity by modification of a previously published procedure (26). Concentrations of RecA protein in the text refer to mol of monomeric protein. RecBCD holoenzyme and the individual RecB, RecC, and RecD subunits were purified from overexpression vectors as described elsewhere (27). Units of activity are defined according to Eichler and Lehman (28). T4 endonuclease VII, a gift of Dr. B. Kemper (University of Köln, Federal Republic of Germany), was purified as described (29). Restriction endonucleases were obtained from New England Biolabs, and Klenow fragment of DNA polymerase I of E. coli was from Bethesda Research Laboratories.

**DNA Substrates**—Circular duplex DNA with a defined gap of 162 nucleotides in the (−) strand (gDNA) was prepared by annealing circular single-stranded ϕX174 (+) DNA with a denatured 5,224-base-pair PstI-Aval linear duplex fragment. Annealing and purification of gDNA were essentially as described (30).

The partially homologous duplex DNA was prepared by PstI linearization of the plasmid pCt10 (15). It was 3′ end labeled with αP using terminal transferase and [α-32P]dideoxy-ATP. All DNA concentrations refer to mol of nucleotide residues.

**Strand Exchange and Resolution Reactions**—Complexes between RecA protein and gDNA were formed in 20 mM Tris-Cl (pH 7.5), 10 mM MgCl₂, 2 mM dithiothreitol, 100 μg/ml bovine serum albumin, and 3 mM ATP for 5 min. Strand exchange was initiated by the addition of PstI-linearized pCJ10 DNA. All incubations were at 37°C. Unless otherwise indicated, reactions were stopped by the addition of EDTA and sodium dodecyl sulfate to 10 mM and 0.5% (w/v) respectively, followed by extraction with phenol/chloroform.

To detect resolution, aliquots were taken from strand exchange reactions (after 10 min of incubation) and supplemented with T4 endonuclease VII, RecBCD holoenzyme, or its individual subunits. Incubation was then continued as described in the figure legends. Recombinant products were analyzed by agarose gel electrophoresis or concentrated by ethanol precipitation and analyzed by denaturing polyacrylamide gel electrophoresis.

**Gel Electrophoresis**—DNA samples were analyzed on 0.7% (w/v) agarose gels using 40 mM Tris-acetate (pH 8.0) and 1 mM EDTA as the buffer system. Denaturing 5% (w/v) polyacrylamide gels contained 7 M urea and used 89 mM Tris base, 89 mM boric acid, and 2 mM EDTA as the buffer system. Denaturing gels were fixed in 10% (v/v) methanol and 10% (v/v) acetic acid prior to drying. Gels were dried and the DNA visualized by autoradiography using Kodak XAR films.

**RESULTS**

In previous studies, we used the strand exchange/resolution system shown in Fig. 1 to demonstrate Holliday junction resolution by T4 endonuclease VII (15), T7 endonuclease I (31), and a newly identified ruwC-dependent nuclease from fractionated E. coli cell extracts (16, 17). In brief, RecA protein acts upon gapped circular and linear duplex DNA to generate Holliday junctions. Because of the chimeric nature of the linear duplex DNA, RecA-mediated strand exchange proceeds in polar fashion until the progress of the Holliday junction is blocked by heterologous DNA sequences (15). Addition of a resolvase enzyme to the stalled in vitro reaction leads to resolution by cleavage of the Holliday junction (Fig. 1, center).

The products of resolution, nicked circular and linear duplex DNA (cutting at a and c) or dimeric linear duplex DNA (cutting at b and d), are representative of "patch" and "splice" recombinant products, respectively. The substrates and products of the reaction can be identified easily by agarose gel electrophoresis.

The effect of addition of either T4 endonuclease VII or RecBCD enzyme to the RecA-mediated strand exchange reaction between gapped circular and chimeric linear duplex DNA is shown in Fig. 2. As described previously, T4 endonuclease VII produced two αP-labeled products: linear dimer and nicked circular DNA (lane b) that are representative of the resolution reaction (15). Neither product was observed when T4 endonuclease VII was omitted (lane a). In this case, we observed the presence of a minor species of slow migrating DNA molecules, typical of strand exchange intermediates that have failed to branch migrate after deproteinization. One additional band observed in Fig. 2, lane b, which comigrated with a linear ϕX174 DNA marker, was a product of secondary processing of the nicked circular product by T4 endonuclease VII. It was produced only in reactions containing excess T4 endonuclease VII (data not shown).

When varying amounts of RecBCD holoenzyme were added to strand exchange reactions, we failed to observe the formation of linear dimer DNA (Fig. 2, lanes c–i). However, in
reactions that contained 0.5 unit or more of RecBCD enzyme, $^{32}$P-labeled nicked circular DNA was produced. At 5 units of RecBCD and more, the end label was lost from the linear duplex substrate DNA. Thus, T4 endonuclease VII produced patch (nicked circular DNA) and splice (linear dimer duplex) recombinant products whereas the action of RecBCD led to the formation of the patch product only. The above reactions were carried out in a RecA buffer containing 20 mM Tris-HCl (pH 7.5), 10 mM MgCl$_2$, 2 mM dithiothreitolf, 100 $\mu$g/ml bovine serum albumin, and 3 mM ATP. Although our choice of reaction conditions was limited to those in which RecA protein was active, we did observe the formation of similar products over a range of buffer conditions (RecA buffer adjusted to 25 mM MgCl$_2$, 2 mM ATP; 6 mM MgCl$_2$, 3 mM ATP; or 4 mM MgCl$_2$, 3 mM ATP, 4.4% polyethylene glycol 8000). In further attempts to minimize the nonspecific nuclease activity of RecBCD enzyme we also used E. coli SSB protein (32). In all cases we observed the formation of nicked circular DNA products only (data not shown).

To determine whether any of the individual subunits of RecBCD enzyme could act upon the Holliday structures generated in this in vitro assay, strand exchange reactions were supplemented with purified RecB, RecC, or partially purified RecD protein (27). The single subunits were found to have no effect on strand exchange, and the products observed in the presence of the RecB (Fig. 3, lane c), RecC (lane d), and RecD (lane e) subunits were similar to those observed in their absence (Fig. 2, lane a). In control reactions from which gDNA was omitted, the presence of RecB, RecC, or RecD proteins had no effect (Fig. 3, lanes h-j). However, in the absence of gDNA, we observed elevated levels of nuclease activity by both T4 endonuclease VII (lane f) and the RecBCD holoenzyme (lane g) resulting in a loss of the linear substrate DNA (in the complete reaction, it is possible that the linear duplex DNA was protected from nonspecific nuclease digestion because of its interaction with the aggregated RecA-gDNA nucleoprotein filaments). The results presented in Fig. 3 therefore indicate that the formation of labeled nicked circular duplex DNA was dependent upon strand exchange and RecBCD holoenzyme.

There are two ways in which the RecBCD enzyme could process the recombination intermediates to form nicked circular products only. (i) RecBCD could resolve the Holliday junction in a reaction analogous to that observed with T4 endonuclease VII. Because of its circular nature, the nicked circular product would be immune to subsequent exonucleolytic digestion whereas the other resolution product, a linear dimer, would be digested. (ii) RecBCD could promote exonuclease digestion of the $\alpha$-structure made by RecA protein (see Fig. 1, center) after entry at the linear termini. Digestion, which would remove the arms of the $\alpha$-structure, would be expected to progress from right to left as drawn, since the left arm has a single-stranded overhang that would not permit access to the nuclease (3, 33).
formation of an intense band approximately 330 nucleotides in length (Fig. 4, lane a). The individual RecB, RecC, and RecD subunits did not induce any DNA cleavage about the Holliday junction (lanes c-e). However, the nucleolytic activity of RecBCD holoenzyme led to the formation of a disperse series of fragments (lane b). These were not targeted to the junction point and in general were shorter than the specific resolution fragments produced by T4 endonuclease VII. In control reactions from which gDNA was omitted we failed to observe specific resolution by T4 endonuclease VII (Fig. 4, lane f), and only nonspecific cleavage of the 3' 32P-labeled linear duplex substrate was observed. Similarly, we failed to observe any specific fragments produced by RecBCD holoenzyme, RecB, RecC, or RecD proteins (lanes g-j).

The formation of the disperse series of fragments in the RecA/RecBCD reaction of Fig. 4, lane b, argues against a specific resolution event. In addition, we failed to detect the small region of exchange (approximately 165 nucleotides) present in the linear product following cleavage at a-c of Fig. 1 (data not shown). These results favor the notion that the RecBCD exonuclease activity progressively removes the right arm of the recombination intermediate, cleaves at the crossover, and finally degrades the entire linear duplex DNA. This interpretation is supported by the experiment of Fig. 5 in which we assayed in more detail for the formation of linear dimer DNA (the product of resolution at positions b and d of Fig. 1). In this experiment the concentrations of RecBCD enzyme were carefully controlled so as to give rise to the formation of nicked circular DNA without leading to complete degradation of the linear duplex DNA (Fig. 5, lanes c-f). Under these conditions we were again unable to detect the formation of linear dimer DNA.

**DISCUSSION**

Many models for genetic recombination and the recombinational repair of DNA after DNA damage invoke the formation and resolution of an intermediate that contains a crossover point, or Holliday junction (34-37). Several enzymes or activities have been isolated which cleave Holliday junctions in vitro (38-42), the best studied examples being T4 endonuclease VII and T7 endonuclease I. However, both these enzymes have a wide substrate specificity and act upon four-armed X-structures, three-armed Y-structures, heteroduplex loops, mismatched bases, and cruciforms that are extruded from supercoiled DNA (39, 43-49). It is therefore likely that their major role in the phage life cycle is an involvement in the resolution of branched structures and the repair of DNA before packaging.

Studies of the resolvase activities described above have focused upon their interaction with, and resolution of, synthetic DNA substrates that model Holliday junctions. Typically these consist of substrates produced by annealing partially complementary single strands that form an X-structure (50). As models for Holliday junctions they have advantages and disadvantages. Whereas the structure of the four strands at the crossover point may be similar to that of a Holliday junction, these structures lack sequence homology. The presence of homology is necessary for branch migration (which can occur at great speed) and as such a Holliday junction is a dynamic structure (51). Moreover, synthetic junctions are protein free, and it is not clear at present whether resolution of a Holliday structure might be influenced by enzymes involved in their formation.

In recent studies we utilized the strand exchange abilities of RecA protein to generate Holliday structures efficiently in vitro. Junctions generated in this reaction served as substrate for either T4 endonuclease VII (15) or T7 endonuclease I (31) and were resolved into patch and splice products characteristic of recombinant DNA molecules. In addition, the assay was developed to detect a resolution activity from fractionated *E. coli* extracts (16). This activity was initially purified from recB sbcBC endA cells that were defective in a number of cellular nucleases including the RecBCD enzyme. In recent
studies, resolution activity was shown to be dependent on the product of the ruwC gene (17). Although ruw mutants are UV and mitomycin C sensitive (20) they are only recombinant deficient in combination with recBC sbcA or recBC sbcBC genetic backgrounds (15, 52). The ruwA and ruwB genes are induced by UV damage, indicating a recombinational repair role for the ruw-encoded Holliday junction nuclease activity (22-24). These findings may indicate two alternative pathways for resolution: one utilizing an inducible resolvase and the second utilizing a constitutive resolvase. The recombinational defect observed with recB sbcA ruw or recB sbcBC ruw mutants would indicate that RecBCD enzyme is a candidate for an alternative resolvase.

In the experiments described in this paper, we have determined whether RecBCD enzyme, or the individual subunits, are capable of resolving recombination intermediates containing Holliday junctions. In previous studies, Taylor and Smith asked a similar question using a synthetic X-junction as DNA substrate (13). They showed that RecBCD enzyme could cleave the substrate to produce recombinant-type products but only if allowed to approach the junction from two accessible linear termini. This need to approach the junction from more than one direction is inconsistent with the formation of recombination DNA during RecBCD-dependent recombination between χ and adv (14), leading Taylor and Smith (13) to conclude that intracellular RecBCD cannot cleave preexisting Holliday junctions although it might cleave those in whose formation it participates.

Rather than use synthetic junctions, we chose to investigate the action of RecBCD enzyme on Holliday junctions generated by RecA protein, a reaction that more truly mimics the in vivo situation. In these experiments we observed the formation of one product (nicked circular DNA) which is similar in structure to the patch recombiant product formed in the same system by T4 endonuclease VII, T7 endonuclease I or the ruw-encoded E. coli nuclease. However, we did not observe the formation of the splice recombiant. As we argued under "Results," it appears likely that this nicked circular product arose by nucleolytic digestion of the α-structure rather than by any specific resolution event at the site of the junction. We conclude that RecBCD is unable to promote the specific resolution of Holliday junctions as provided by this in vitro assay. These results are in agreement with those of Taylor and Smith that RecBCD enzyme gains entrance to an accessible DNA terminus, progresses along the DNA duplex, and cleaves at the junction point.

Results presented elsewhere describe reactions catalyzed by the ruwC-dependent nuclease and indicate a specificity for Holliday junctions that link two homologous DNA molecules (17). If RecBCD protein participates in a late stage during conjugal recombination, its role in generating recombintants after RecA-mediated strand exchange may be by means of exonucleolytic digestion of the donor portion of the recombination intermediate, as suggested in this in vitro assay. Alternatively, RecBCD enzyme may act upon other types of recombination intermediate such as an exchange that involves three rather than four DNA strands. In this regard, RecBCD enzyme has been shown to introduce nicks into D-loop DNA structures (12). Clearly further efforts will be required to determine the precise role of this complex enzyme in genetic recombination.

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