Genetic recombination of bacterial plasmid DNA: Electron microscopic analysis of in vitro intramolecular recombination

(Escherichia coli/recombination-deficient mutants/recA protein/exonuclease V/novobiocin)

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ABSTRACT A tetramer of pMB9 DNA containing a single EcoRI site per tetramer was used to investigate intramolecular recombination in Escherichia coli. When transformed into wild-type E. coli strains, the tetramer was converted into dimers and a small proportion of trimers and monomers. The conversion was blocked in recA strains and recB recC recF strains but not in recB recC strains or recF strands. Extracts of E. coli converted the tetramer into dimers, trimers, and monomers. Figure of 8 molecules and catenanes were minor products. The proportion of recombinant molecules ranged from 7% to 14%. Intramolecular recombination in vitro was blocked in extracts of recA strains and recB recC recF strains but was not significantly blocked in extracts of recB recC strains and recF strains. recA protein restored activity to recA extracts; activity in recB recC recF extracts was restored by purified exonuclease V (recBC nuclease) or a recF protein donor extract. Novobiocin and oxolinic acid inhibited the reaction by 70-80%.

The enzymatic mechanisms of genetic recombination in Escherichia coli and other organisms has been of considerable interest for a number of years. The availability of recombination-deficient mutants (1) and considerable information about the physical and genetic structures of recombination intermediates (2) has made E. coli an attractive organism for these studies. To date, two enzymes involved in general genetic recombination in E. coli have been extensively purified and characterized.

Exonuclease V consists of two subunits and has a number of enzymatic activities (3-6). The enzyme is absent from recB and recC strains (7), which are recombination deficient, and there is evidence that the RecB gene is the structural gene for one of its subunits (8, 9). Although it seems clear that exonuclease V functions directly in general recombination, its enzymatic role is less certain, especially considering several recombination models that suggest different roles for exonuclease V (10, 11) and the finding that the exonuclease activities of the enzyme are inhibited at physiological ATP levels (4). Similar enzymes exist in other organisms (12, 13).

In addition to properties of a regulatory protein (14), the recA protein has a number of enzymatic activities which suggest that it is involved in the initiation of homologous pairing during genetic recombination (15-17) and is consistent with previous observations suggesting that the recA protein functions early in recombination (2).

To study the mechanism of genetic recombination and identify and purify unknown proteins involved in recombination, several investigators have developed in vitro systems that promote various aspects of E. coli recombination. A system that catalyzes bacteriophage λ integrative recombination has been developed (18), and recombination of bacteriophage T7 DNA has been studied in vitro (19). Recently, an E. coli in vitro system that catalyzes intermolecular recombination between circular DNA molecules to form figure of 8 recombination intermediates (10) has been developed (20). The reaction does not require the recA protein and appears to be due to a novel enzymatic activity (21). None of these in vitro recombination systems appears to utilize the E. coli general genetic recombination pathways defined by the recA, recB, recC, and recF mutations (1).

MATERIALS AND METHODS

Chemicals. Nucleoside 5'-triphosphates and NAD were purchased from P-L Biochemicals. Novobiocin was purchased from Sigma. Oxolinic acid was a gift from Charles C. Richardson. Enzymes. Exonuclease V (recBC DNase) was purified through the phosphocellulose step of Eichler and Lehman (4). Homogeneous recA protein was purified by an unpublished procedure similar to that of Weinstock et al. (15). E. coli recA protein was a gift of James Wang.

Bacterial Strains and Plasmids. E. coli AB1157, thr1, leu6, thi1, lacY1, galK2, ara14, xyl5, mtl1, proA2, his4, argE3, str31, tss33, supE44, λ*, λ' (22) and an isogenic set of strains containing different combinations of the recA13, recB21, recC22, and recF143 alleles were generously provided by A. J. Clark. E. coli W3110 recK12 recA1 (23) was used for maintenance and growth of most plasmids. The plasmid pRK1 is a derivative of pMB9 DNA (24) that does not contain any EcoRI sites. The plasmid pRK2 consists of three monomer units of pRK1 DNA and one monomer unit of pMB9 DNA joined in tandem to form a tetramer containing only one EcoRI site.

DNA Isolation. Plasmid-containing strains were grown in L broth or, for radioactive labeling, in Frazier's medium supplemented with 2 μg of thymine and 3.3 μCi (1 Ci = 3.7 × 10^10 becquerels) of [3H]dThd per ml to an OD_{590} of 0.6 and then subjected chloroamphenicol induction for 18 hr (25). DNA was extracted from the cells (25) and purified by centrifugation in CsCl/ethidium bromide density gradients.

Electron Microscopy. DNA samples were usually treated with γ irradiation (≈6000 rads [60 gray]) and mounted for electron microscopy as described (26). Grids were rotary shadowed and shadowed from a fixed angle in order to distinguish between overlaid DNA strands and DNA molecules containing fused junctions (27). The structures of all putative recombinant molecules were verified from length measurements.

RESULTS

Recombination of pRK2 DNA in Vivo. Earlier studies (28, 29) suggested that a tetrameric plasmid DNA molecule such as pRK2 should be converted to monomers, dimers, and trimers in vivo. To test this, pRK2 DNA was transformed into an isogenic set of E. coli strains (30), and the DNA was purified from the resulting transformants and analyzed by electrophoresis (Fig. 1). The DNA bands on the gel were identified by com-
paring their mobilities with the mobilities of standards (lanes A and B and data not shown) and by electron microscopy (data not shown).* The results show that pRK2 DNA was maintained as a circular tetramer in recA strains (lane C) and in recB recC recF strains (lane D) but was converted to circular dimers and some circular trimers in recB recC strains (lane E), in recF strains (lane F), and in wild-type strains (lane G). Monomers were also formed in some experiments. There was variation in the extent of conversion observed in different experiments but extensive conversion (>60%) was always observed. Digestion of these DNA samples with EcoRI showed (data not shown) that all circular tetramers had a single EcoRI site; circular dimers consisted of an equal mixture of EcoRI-resistant dimers and dimers containing one EcoRI site. Approximately 90% of the circular trimers had a single EcoRI and the remainder were resistant to EcoRI. These results are consistent with the dimers and trimers being formed by intramolecular recombination of a pRK2 tetramer.

Intramolecular Recombination of pRK2 DNA in Vivo. To determine if pRK2 DNA would be converted to monomers, dimers, and trimers in vivo, pRK2 [3H]DNA was incubated with an extract of E. coli AB1157, and the reaction products were examined by electron microscopy. Control experiments showed that little substrate DNA (~1–2%) was degraded to acid-soluble material or linear molecules during the incubation and that 80% or more of the DNA remained covalently closed during the reaction (no significant degradation of substrate DNA was observed in any experiments). Various DNA structures were formed during the incubation (Fig. 2). The predominant DNA species was the circular tetramer (Fig. 2A); the major products observed were trimers (Fig. 2A), dimers (Fig. 2A), and monomers (Fig. 2B). The relaxed molecules shown in Fig. 2 were only observed after γ irradiation; most of the substrate and product molecules were supercoiled prior to irradiation (Fig. 2C and D). Observed at lower frequencies were molecules that appeared to be two dimers joined by a fused junction (Fig. 2E), a monomer and trimer joined by a fused junction (Fig. 2F), and catenanes consisting of two dimers (Fig. 2G) or of a monomer and a trimer (Fig. 2H). The molecules containing fused junctions resembled figure of 8 recombination intermediates (10). This was confirmed, in part, by digesting DNA preparations containing figure of 8 molecules with EcoRI to produce α and β forms (10, 27, 32, 93), although analysis of these experiments was complicated by the complex mixture of products formed. Branch migration of the fused junction (10, 32) was not investigated.

The frequency of product molecules observed in different experiments ranged from 7% to 14% of the total DNA molecules. A typical frequency distribution is presented in Table 1. All of the molecular forms discussed above increased during the reaction. The major product molecule was the circular dimer; dimer-related molecules (figure of 8s and catenanes) made up >70% of the total product molecules. Circular trimers, monomers, and catenanes and figure of 8 containing a monomer and a trimer made up the remaining product molecules. In most experiments, trimers were observed at higher frequencies than monomers. Length measurements eliminated the possible overestimation of the frequency of trimers by scoring tetramers as trimers, but systematic underestimation of monomers although unlikely, could occur.

Two other types of DNA molecules have been observed but do not appear to be involved in the recombination reaction. Catenanes of two tetramers made up about 1% of the substrate DNA molecules, and their frequency was not altered during the incubation. Cairns replicative intermediates were formed during the incubation and made up 4–5% of the DNA molecules. Their formation required both rATP and the four dNTPs and was not inhibited in any of the recombination-deficient extracts tested and therefore appears to be unrelated to the recombination reaction (Fig. 3; see below).

A complication in these experiments has been the background of product molecules present in the substrate DNA purified from recA strains (Table 1). Similar observations have been made by others (29). This appears to be due to a recA-independent recombination reaction (20, 21) because it is not affected by recA deletion mutants.

**Reaction Conditions.** The reaction had a pH optimum of 7.4 in KPO₄ buffer, and KPO₄ was required because it inhibited the conversion of the substrate DNA to linear molecules, which occurred in the presence of other buffers. This endonucleolytic

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* The nomenclature for circular DNA molecules of Vinograd et al. (31) is used.

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**Table 1. Frequency distribution of product molecules**

<table>
<thead>
<tr>
<th>Product molecules</th>
<th>Figure of 8</th>
<th>Catenated</th>
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<tbody>
<tr>
<td>Dimer/monomer</td>
<td>0.8</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Trimer/monomer</td>
<td>0.8</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Dimer/monomer</td>
<td>0.6</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Trimer/monomer</td>
<td>0.4</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>n</td>
<td>1008</td>
<td>1000</td>
</tr>
</tbody>
</table>

* Reactions were carried out as described in the legend to Fig. 2.
† Product molecules are illustrated in Fig. 2.
‡ Catenanes containing two tetramers and replicating tetramers were scored as tetramers (Fig. 3).

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**Fig. 1. Electrophoretic analysis of interconversion of plasmid DNA in vivo.** Lanes: A, pRK1 monomer DNA purified from JC929 recA13 and irradiated with 300 rads of γ irradiation to produce some form II monomer; B, pRK1 monomer DNA purified from AB1157 recA; C, pRK2 DNA purified from JC929 recA13; D, pRK2 DNA purified from JC881 recB21 recC22 recF143; E, pRK2 DNA purified from JC519 recB21 recC22; F, pRK2 DNA purified from JC9239 recF143; G, pRK2 DNA purified from AB1157 recA.*

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* The structure of product molecules was verified by length measurement (data not shown). Typical measurements in one experiment showed that monomers were 0.251 (SD ± 0.011) times the length of a tetramer. Similar standard deviations were obtained in most measurements.
activity was not endonuclease I because it was not inhibited by tRNA (34) nor did it appear to be the E. coli K-12 restriction endonuclease because the substrate DNA was isolated from a modification plus host. There was a requirement for Mg\(^{2+}\) and a nucleoside 5'-triphosphate (Table 2). The nucleoside 5'-triphosphate requirement was satisfied by rATP and could be partially satisfied (80%) by a mixture of the four dNTPs. Spermidine (2.5 mM) stimulated the reaction, and KCl inhibited it.

Requirement for DNA Gyrase. Novobiocin and oxolinic acid, specific inhibitors of DNA gyrase (35), inhibited the reaction by about 80% (Table 2). Variable results were obtained with novobiocin but at least 50% inhibition was always observed. Tetramer DNA relaxed by E. coli \(\omega\) protein (36) was
as good a substrate as was negatively supercoiled plasmid DNA (data not shown). Novobiocin and oxolinic acid inhibited the reaction with relaxed substrate DNA to the same extent (80%) as with native supercoiled substrate DNA. In all experiments, the substrate DNA remained covalently closed (>70–80%) as judged by electrophoresis on agarose gels. The possibility that circular DNA containing single-strand breaks would function as a substrate was not investigated.

Requirement for the recA Protein. Extracts prepared from recA strains were less active (<15%) than wild-type extracts (Table 3). Activity in a recA extract was restored by the addition of a recB recC recF extract (recA protein donor). Addition of homogeneous recA protein also restored activity in a recA extract. Thus, the recA protein appears to be required.

Requirement for Either the recBC Nuclease or the recF Protein. Extracts from recB recC recF strains were less active than extracts of wild-type strains, recB recC strains, or recF strains (Tables 3 and 4). Activity was restored to the recB recC recF extract by adding either exonuclease V (recBC nuclease) or a recA recB recC extract (recF protein donor). Therefore, the reaction also requires either the recBC protein (exonuclease V) or the recF protein. Additional components are probably required because the recA protein and exonuclease V together were insufficient to carry out the reaction (Table 4).

**DISCUSSION**

The results presented here demonstrate that extracts of E. coli catalyze conversion of a tetramer plasmid DNA molecule into smaller circular product molecules. This reaction is similar to that observed in vivo (28, 29, 33) (Fig. 1). The formation of molecules resembling figure 8 recombination intermediates suggests that the reaction occurs by the pathway suggested for the recombination of bacteriophage φX174, SI3 and G4 replicative form DNAs, and PMB9 plasmid DNA (10, 27, 32, 33). Catenanes could be intermediates in, or byproducts of, the processes by which figure of 8 molecules are resolved to form progeny. Catenanes are formed during bacteriophage λ integrative recombination in vitro (37) and catenanes are recombinant in vivo (38, 39). Furthermore, enzymes that resolve catenanes into free circles exist in E. coli (40). Studies of the kinetic behavior of catenanes and figure of 8 molecules will be required to demonstrate that they behave as intermediates. The major products observed (>70%) during the in vitro reaction resemble the terminal products observed in vivo, suggesting that the in vitro system can catalyze the entire recombination reaction.

The recA protein appears to be required in all of the E. coli general recombination pathways (1). However, general recombination is not completely deficient in recB recC strains (1, 2), presumably due to residual recombination by the recF pathway. General recombination is not significantly blocked.

![Figure 3](https://example.com/figure3.png)

**FIG. 3.** Additional DNA molecules observed in reaction mixtures. (A) Catenane of two tetramers. (B) Replicating tetramer.
Table 4. Requirement for either recBC protein or recF protein

<table>
<thead>
<tr>
<th>Extract*</th>
<th>Product molecules,</th>
<th>%</th>
<th>n</th>
</tr>
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<tbody>
<tr>
<td>25 µl recBC recC recF</td>
<td></td>
<td>2.4</td>
<td>1057</td>
</tr>
<tr>
<td>25 µl recB recC recF</td>
<td></td>
<td>7.1</td>
<td>533</td>
</tr>
<tr>
<td>+ 10 units exonuclease V</td>
<td>6.3</td>
<td>563</td>
<td></td>
</tr>
<tr>
<td>12.5 µl recBC recF +</td>
<td></td>
<td>5.8</td>
<td>542</td>
</tr>
<tr>
<td>12.5 µl recA recB recC</td>
<td>5.0</td>
<td>533</td>
<td></td>
</tr>
<tr>
<td>25 µl recA recB recC</td>
<td>1.9</td>
<td>516</td>
<td></td>
</tr>
<tr>
<td>25 µl recB recC</td>
<td>5.8</td>
<td>542</td>
<td></td>
</tr>
<tr>
<td>25 µl recF</td>
<td>5.0</td>
<td>533</td>
<td></td>
</tr>
<tr>
<td>1 µg recA protein +</td>
<td></td>
<td>1.2</td>
<td>550</td>
</tr>
</tbody>
</table>

* Reaction conditions and strains were as in legends to Figs. 1 and 2 and Table 3 except for the indicated strain changes and additions. The E. coli recA13 recB21 recC22 strain was JC5547.

in recF strains but is almost completely blocked in recB recC recF strains (1). The results presented here demonstrate that intramolecular recombination of the tetramer substrate in vivo and in vitro requires direct action of the recA protein and either the recBC protein (exonuclease V) or the recF protein. These proteins are required early in the reaction because their absence does not lead to production of detectable intermediates or products. Thus, the reaction is catalyzed by some of the pathways of general recombination in E. coli. Recombination by the recE pathway (1) has not yet been tested.

Although the reaction described here utilizes many of the gene products required for E. coli general genetic recombination, it is possible that the product molecules were formed by illegitimate recombination rather than by a homologous excision event. This possibility seems unlikely because measurements showed that the product molecules were homogeneous DNA species of monomer, dimer, and trimer lengths.

DNA gyrase functions in E. coli to produce circular DNA molecules with a negative superhelical density (35) and can catenate and uncatenate circular DNA molecules (40). Novobiocin and oxolinic acid, inhibitors of DNA gyrase (35), partially inhibit the in vitro recombination reaction and appear to inhibit early steps in the reaction. These results suggest that negative superhelical DNA could be the preferred substrate for the reaction. Another possibility is that DNA gyrase could do something other than keep the substrate DNA supercoiled when relaxed DNA functions as a substrate (=20%) in the presence of novobiocin and oxolinic acid. The exact role of DNA gyrase can best be resolved when it is possible to reconstitute the reaction from purified proteins.

The results presented here demonstrate that DNA molecules designed to undergo intramolecular recombination events will be useful in studying E. coli general recombination. In vitro complementation observed for the recF protein should provide an assay for the purification of the recF protein. In vitro reconstitution assays, like those used to study DNA replication, should make it possible to purify other proteins involved in recombination. Such proteins must exist because recA protein and exonuclease V (recBC protein) are insufficient to carry out recombination (Table 4).

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