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Genetic recombination: recA protein promotes homologous pairing between duplex DNA molecules without strand unwinding

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ABSTRACT

The recA protein of Escherichia coli promotes pairing in vitro between covalent circular duplex DNA and homologous circular duplex DNA containing a single stranded region. We have used a filter binding assay to investigate the frequency of homologous pairing between gapped and intact duplex DNA when unwinding of the free 3′ and 5′ ends of the gapped molecules was blocked. In order to obtain DNA without free ends, the gapped DNA was treated with trimethylpsoralen and 360 nm light so as to introduce about 6 crosslinks per DNA molecule and the double stranded regions on either side of the gaps were then digested up to the first crosslinks with exonuclease III and λ exonuclease. This treatment did not diminish the frequency of homologous pairing, an observation which is difficult to reconcile with models for recombination requiring strand unwinding before pairing.

INTRODUCTION

The recA protein of E. coli promotes homologous pairing between suitably structured DNA molecules in vitro and two forms of pairing have been investigated. First, single strand DNA fragments can be paired with intact covalent circular duplexes to produce D-loops (1, 2). Second, duplexes containing single stranded gaps (gapped duplexes) can be paired with intact covalent circular duplexes to form joint molecules (3, 4). This reaction is interesting because it resembles the homologous pairing between two duplexes in genetic recombination or postreplication repair in intact cells. Moreover, since postreplication gaps cause exchanges with an efficiency close to unity (5), it appears that gapped duplexes may be a natural substrate for recA protein (6).

According to several published models for genetic recombination (7, 8), synapsis is the result of the unwinding and displacement of a single-strand end from a nicked or gapped duplex, following which the unwound end pairs with the duplex homolog. To investigate the role of unwinding in homologous pairing between duplexes mediated by recA protein, we crosslinked gapped DNA

to prevent extensive unwinding and in preliminary observations failed to find an effect on the yield of joint molecules (3). In the present paper, we further investigate the frequency of homologous pairing between intact and gapped duplex DNA when unwinding of the free 3′ and 5′ ends of the gapped molecules had been blocked more completely by introducing interstrand crosslinks and digesting the free ends with exonucleases. The yield of joint molecules was not affected by these procedures, suggesting that strand unwinding is not required for homologous pairing.

MATERIALS AND METHODS

Enzymes and Proteins

The purification of recA protein has been described (6). DNAse I (RNAse-free, 2000 units /mg.) was purchased from Sigma. Exonuclease III was obtained from BRL. Lambda exonuclease was a generous gift from C. DasGupta.

Preparation of DNA

The preparation of ^{3}H- and ^{32}P-labeled pBR322 DNA and øX174 and fd duplex circular (RF) DNA has been described previously (3). Plasmid pBR322 DNA preparations normally contained approximately 60%, while øX and fd RF preparations contained more than 90% of covalent circular molecules (form I).

DNA with one nick per molecule

The reaction mixtures contained 50 µg/ml of ^{3}H-pBR322 DNA or fd RF DNA, 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 3mM 2-mercaptoethanol, 150 µg/ml ethidium bromide and 60 ng/ml DNAse I. Incubation was for 30 min at 20 C. After heating at 65 C for 5 min, the ethidium bromide was removed by extraction with isobutanol and the DNA was dialyzed against 50 mM Tris-HCl, pH 8.0.

Electrophoresis of an aliquot on a 1% agarose gel indicated that all the form I DNA was converted to form II DNA and no linear forms were produced. To estimate the number of nicks per molecule of DNA, an aliquot of the nicked DNA was digested with exonuclease III until 50% of the input radioactivity was rendered acid soluble. The acid insoluble material sedimented in alkaline sucrose gradient at the same position as full length strands of pBR322 or fd DNA, showing that the form II DNA bore one nick per molecule after treatment with DNAse I.

DNA with one gap per molecule

Reaction mixtures contained 50 µg/ml of nicked DNA, 50 mM Tris-HCl pH 8.0, 5mM $MgCl_2$, 10mM 2-mecaptoethanol and a saturating amount of exonuclease III, previously determined for each DNA preparation. Incubation was at 37 C.

for 5 min and produced 10% acid soluble material. In a control experiment, pBR322 form I DNA subjected to the same treatment with exonuclease III remained in covalent circular form, showing that exonuclease III did not introduce additional nicks in the DNA. Therefore, the release of 10% acid soluble material from nicked DNA corresponded to one gap per molecule averaging 800 nucleotides for pBR322, 1200 for fd. The enzymes were removed by phenol extraction and dialysis.

Crosslinking of Gapped DNA

The DNA solutions were supplemented with 1/100 volume of a saturated solution of 4,5',8 trimethylpsoralen (Paul B. Elder Co., Bryan Ohio) in ethanol and irradiated with 360 nm light as described (9).

In previously published experiments (10) we had found that a dose of 0.85 kJ/m^2 of 360 nm light in the presence of trimethylpsoralen introduced on the average one crosslink in 5.10^3 base pairs. Under identical conditions, assuming that the production of crosslinks is a linear function of dose, 5 kJ/m^2 should produce approximately 6 crosslinks per pBR322 DNA molecule (or 1 crosslink in 500 base pairs) and also 100 monoadducts (or 1 monoadduct in 75 nucleotides) (9). In order to obtain DNA without free ends, the double stranded regions on each side of the gap had to be digested to the first crosslinks with exonuclease III and λ exonuclease; it was therefore necessary to determine the activity of those two enzymes on DNA containing monoadducts.

Digestion of DNA containing monoadducts with exonuclease III and λ exonuclease

For treatment with exonuclease III, reaction mixtures contained 50 μg/ml of H^3-lambda DNA, 50mM Tris-HCl, pH 8.0, 5mM $MgCl_2$, 10 mM 2-mercaptoethanol and a saturating amount of enzyme. For treatment with lambda exonuclease, reaction mixtures contained 50 μg/ml lambda DNA, 10 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 10% DMSO, 4 μg/ml tRNA and a saturating amount of enzyme.

As previously reported (9), lambda DNA inactivated in the presence of trimethylpsoralen with 112 J/m^2 of 360 nm light contains an average of 1 crosslink and 15 to 20 monoadducts per molecule. We irradiated lambda DNA with 80 J/m^2 of 360 nm light, thus introducing approximately 0.6 crosslink and 12 monoadducts per DNA molecule. We found that saturating amounts of exonuclease III digested intact lambda DNA to 50% in 30 min and lambda DNA with monoadducts to the same extent in 100 min. Similarly, digestion to 50% of lambda DNA with monoadducts by λ exonuclease required 150 min incubation at 37 C, as opposed to 30 min for intact lambda DNA.

Digestion of crosslinked gapped pBR322 DNA with exonuclease III and λ exonuclease

^{3}H-pBR322 DNA with one gap and 6 crosslinks per molecule was treated with exonuclease III as described above. Incubation was for 100 min at 37 C and produced 5% of acid soluble radioactivity. The treatment was repeated and no additional release of acid soluble material was observed. The mixture was treated for 5 min at 65 C, dialyzed vs. 10 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, and subjected to 2 consecutive treatments with λ exonuclease. Each incubation was for 150 min at 37 C. The first incubation released 3% acid soluble material. No additional release was observed after the second incubation.

Nicking activity of exonuclease III and λ exonuclease

In order to insure that extensive treatments of the gapped DNA with exonuclease III and λ exonuclease did not introduce additional nicks (and subsequently gaps) in the double stranded regions, ^{3}H-pBR322 DNA containing 60% form I was subjected to 2 consecutive treatments with each enzyme as described above. As determined by centrifugation in alkaline sucrose gradients, the percentage of form I DNA was not affected by the enzymatic treatments. On a 1% agarose gel the gapped crosslinked DNA migrated in the same position as form II pBR322 DNA, before and after enzymatic treatments, indicating that no linear molecules had been produced.

Formation and detection of joint molecules

0.025 to 1.25 μg of ^{32}P-form I DNA and 0.025 to 1.25 μg of gapped ^{3}H-DNA were incubated with 0.1 to 0.8 mg/ml of recA protein in 50 μl of 33 mM Tris. HCl, pH 7.5, 25 mM $MgCl_2$, 2mM dithiothreithol, 100 μg/ml bovine serum albumin (BRL) and 1.2 mM ATP. The reaction was stopped by addition of EDTA to a final concentration of 50 mM, diluted 10 fold with 1.5 M NaCl, 0.15 M Na citrate (10x SSC), held 10 min at room temperature and applied to a nitrocellulose filter (Schleicher and Schuel, 0.45 micron pore size, 24mm diameter) (1, 2). The filter was rinsed with 2 x 3 ml of 10x SSC, dried and counted in 5 ml of scintillation fluid (Formula 963, NEN).

Heat stability of joint molecules

Reaction mixtures (0.5 ml) containing 5μg/ml of ^{32}P form I DNA and 5 μg/ml of either ^{3}H gapped homologous DNA or ^{3}H gapped homologous DNA crosslinked and treated with exonuclease III and λ exonuclease were incubated as above with 100 μg/ml of recA protein. The reaction was stopped as described above, except that Sarkosyl was added to a final concentration of 1%. Aliquots of 0.05 ml were withdrawn, diluted into 10x SSC and heated for 5 min at 40, 50, 60, 65 and 70 C prior to filtration.

Centrifugation

All centrifugations were performed in 5-20% neutral or alkaline sucrose gradients at 20 C and 45,000 rpm for 90 to 150 min in an SW50.1 Beckman rotor. Gradients were collected from the bottom.

Gel electrophoresis

DNA samples were analyzed on 1% agarose horizontal gels in 40 mM Tris-HCl, pH 7.9, 5 mM sodium acetate, 1 mM EDTA.

RESULTS

The following experiments were designed with the goal of comparing the yield of joint molecules obtained with covalently closed duplex DNA and duplex DNA containing single-stranded regions in the presence and in the absence of strand ends. Those experiments involving a filter binding assay were carried out in 10x SSC, sufficient to release recA protein binding from DNA (1). Moreover, since the results were not affected by adding 1% SDS, it is unlikely that the joint molecules were held together by protein.

Joint molecules of circular duplex DNA and homologous gapped DNA

In this experiment, we measured the yield of joint molecules for increasing amounts of DNA, keeping the concentration of recA protein at either 100 μg/ml or 800 μg/ml, and the ratio of gapped DNA to intact DNA at 1.0. The results shown in Fig I are for fd form I DNA reacted with homologous gapped DNA. Essentially identical results were obtained with DNA prepared from pBR322 (data not shown). At the low concentration of recA protein (100 μg/ml), we found that the yield of joint molecules varied with DNA concentration and was maximal at 5 μg/ml. The amount of enzyme was sufficient for saturation of the single-stranded regions for all the DNA concentrations examined (Fig. 1a), but not to cover all the double stranded regions for DNA concentrations higher than 5 μg/ml. We have previously suggested that recA protein binds to single stranded DNA and spreads onto the adjoining duplex in a gapped molecule (6).

At high concentration of recA protein (800 μg/ml) (Fig. 1b) the yield of joint molecules became largely independent of the DNA concentration. When fd (or pBR322) form I was replaced by non-homogolous øX form I DNA, a significant amount of homology independent pairing was observed below 2 μg/ml of DNA and at both protein concentrations.

Joint molecules of circular duplex DNA and crosslinked gapped DNA

Gapped ^{3}H-labeled pBR322 was irradiated with 360 nm light in the presence of trimethlylpsoralen and subjected to treatment with exonuclease III

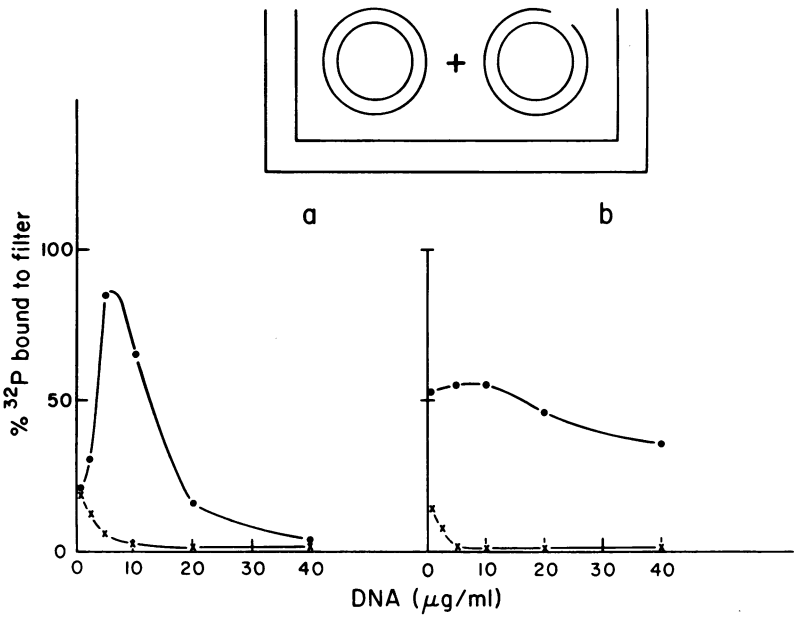

Figure 1: Formation of joint molecules of form I DNA and gapped circular duplex DNA

Equal amounts of ^{32}P-form I DNA and ^{3}H-gapped duplex fd DNA were incubated for 60 min at 37 C with recA protein as described in Methods. The yield of joint molecules was estimated by filter binding assay.
a) 100 μg/ml recA protein; b) 800 μg/ml recA protein.
●——● ^{32}P-fd RFI DNA and ^{3}H-gapped duplex fd DNA.
x——x ^{32}P-φX RFI DNA and ^{3}H-gapped duplex fd DNA.

and λ exonuclease as described in Methods. The resulting DNA was presumably gapped with crosslinks at either end. It was incubated with ^{32}P-labeled form I pBR322 in the presence of 100 μg/ml (Fig. 2a) or 800 μg/ml (Fig. 2b) of recA protein. The results are shown in Fig 2. At both concentrations, the degree of homologous pairing as a function of DNA concentration was similar to that observed with non-crosslinked gapped DNA. The reaction was dependent on homology, although homology independent pairing was significant below 5 μg/ml of DNA.

Effect of crosslinking on the heat stability of joint molecules.

Mixtures containing ^{32}P form I DNA and ^{3}H gapped homologous DNA with or without crosslinks (final DNA concentration: 10 μg/ml) were incubated with 100 μg/ml of recA protein. The conditions for incubation, termination of the

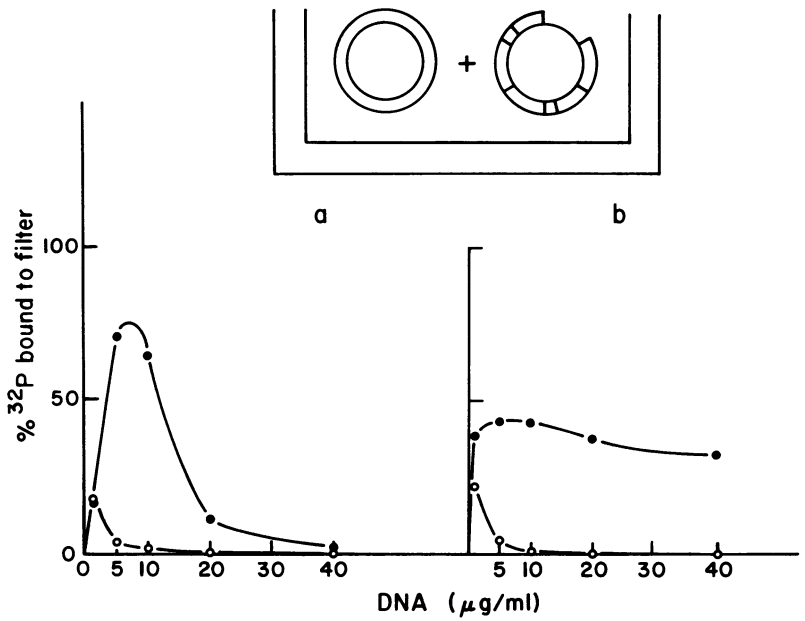

Figure 2: Formation of joint molecules of form I DNA and crosslinked gapped duplex circular DNA treated with exonucleases

Equal amounts of ^{32}P-form I pBR322 DNA and ^{3}H-gapped duplex pBR322 DNA were incubated with recA protein. Conditions for incubation and filtration were as in Fig. 1. a) 100 μg/ml recA protein; b) 800 μg/ml recA protein.
●——● ^{32}P pBR322 form I DNA and ^{3}H gapped crosslinked pBR322 DNA;
o——o ^{32}P øX form I DNA and ^{3}H gapped crosslinked pBR322 DNA.

reaction and heating are described in Methods, and the results are shown in Fig. 3. Joint molecules formed between form I DNA and gapped DNA displayed a similar heat stability whether the ends of the gap had been crosslinked or not.

DISCUSSION

In this paper we have shown that the formation of joint molecules between intact and gapped homologous duplex DNA proceeds efficiently when the ends of the gap are crosslinked. These results, together with the fact that recA protein lacks helicase activity and can promote homologous pairing without strand separation (11), are difficult to reconcile with models in which synapsis is achieved by pairing of the free end of a single-strand with one

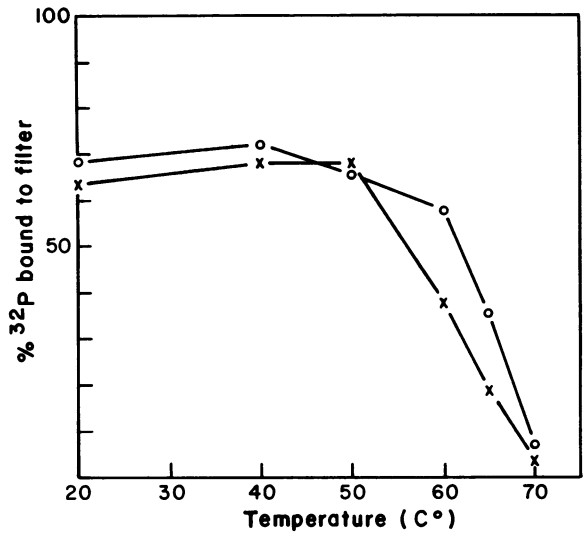

Figure 3: Heat stability of joint molecules
Conditions for incubation and heating are described in Methods.
o——o ^{32}P pBR322 form I DNA and ^{3}H pBR322 gapped DNA;
x——x ^{32}P pBR322 form I DNA and ^{3}H pBR322 gapped crosslinked DNA digested with exonucleases.

strand of duplex DNA (7, 8). We cannot exclude the possibility that the extensive treatments with exonuclease III and λ exonuclease failed to digest the ends of the gap exactly to the site of the first crosslink on each side. We feel, however that the drastic shortening of the putative invading single-strand would be reflected in the yield of joint molecules, were the displacement of a single strand required for pairing. Such unwinding may, of course, be needed for subsequent reactions in genetic recombination.

If synapsis does not take place by the displacement of a single strand to make a heteroduplex, what alternatives are to be considered? Homologous pairing may follow the loading of recA protein, which binds to the single stranded region and spreads onto the adjoining duplex (6). The loaded duplex DNA can now bind non-specifically to duplex DNA and initiate a search for homology in which the molecules are moved in relation to each other at the expense of ATP hydrolysis, leading to homologous contacts when the molecules come into register. Models of four stranded helices have been built in which specific interduplex hydrogen bonds are formed between two intact duplexes by contacts in their wide groove (12, 13). Four strand helices may be formed as

an intermediate, but have not been detected as stable entities. The three strand helix poly(dT-C).(dG-A).(U-C) is as stable in high salt as duplex DNA and appears to exist with the poly(U-C) strand hydrogen bonded in the wide grove of the duplex (14). Since this degree of stability requires all purines to be in one strand, a random sequence triple helix may be less stable, especially if all three chains are deoxyribo polymers. In the present experiments the melting temperature of the joint molecules is about 25 C below that of duplex DNA, and is slightly lower than that reported for gapped and intact duplex molecules (4). A D-loop melting temperature equal to that of duplex DNA is obtained only with long single strand fragments annealed to superhelical DNA under conditions in which extensive Watson-Crick pairing can occur (15). The lower melting temperature of joint molecules in the present experiments may reflect wide groove pairing as well as topological constraints at the ends of the gaps.

Under certain conditions (below 5 μg/ml of DNA) pairing can be detected between non-homologous molecules (Figs. 1 and 2). These non-homologous contacts may be viewed as part of the search for homology that must precede the establishment of stable, homologous pairing.

In summary, we conclude that in recombination mediated by recA protein, synapsis between gapped and intact duplex homologs may take place without strand unwinding. On the other hand, free strand ends that can unwind are no doubt needed for strand exchanges (16) and subsequent steps in recombination.

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REFERENCES

1. Shibata, T., Das Gupta, C., Cunningham, R.P. and Radding, C.M. (1979). Proc. Nat. Acad. Sci. USA 76, 1638-1642.
2. McEntee, K., Weinstock, G.M. and Lehman, I.R. (1979). Proc. Nat. Acad. Sci. USA 76, 2615-2619.
3. Cassuto, E., West, S.C., Mursalim, J., Conlon, S. and Howard-Flanders, P. (1980). Proc. Nat. Acad. Sci. USA 77, 3962-3966.
4. Cunningham, R.P., Das Gupta, C., Shibata, T. and Radding, C.M. (1980) Cell, 20, 223-235.
5. Rupp, W.D., Wilde, C.E., Reno, D. and Howard-Flanders, P. (1971). J. Mol. Biol. 61, 25-44.
6. West, S.C., Cassuto, E., Mursalim, J. and Howard-Flanders, P. (1980). Proc. Nat. Acad. Sci. 77, 2569-2573.
7. Holliday, R. (1964). Genetical Res. 5, 282-304.
8. Meselson, M.S. and Radding, C.M. (1975). Proc. Nat. Acad. Sci. USA 75, 358-361.

9. Cassuto, E., Gross, N., Bardwell, E. and Howard-Flanders, P. (1977). Biochem. Biophys. Acta 475, 589-600.
10. Cassuto, E., Mursalim, J. and Howard-Flanders, P. (1978). Proc. Natl. Acad. Sci. U.S.A. 75, 620-624.
11. West, S.C., Cassuto, E. and Howard-Flanders, P. (1981). Nature, 290, 29-33.
12. McGavin, S. (1971). J. Mol. Biol. 55, 293-298.
13. Wilson, J.H. (1979). Proc. Natl. Acad. Sci. U.S.A. 76, 3641-3645.
14. Morgan, A.R. and Wells, R.D. (1968). J. Mol. Biol. 37, 63-80.
15. Shibata, T., DasGupta, C., Cunningham, R.P., Williams, J.G.K., Osber, L. and Radding, C.M. (1981). J. Biol. Chem. In press.
16. West, S.C., Cassuto, E. and Howard-Flanders, P. (1981). Proc. Natl. Acad. Sci. U.S.A. 78, 2100-2104.