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HOMOLOGOUS PAIRING IN VITRO INITIATED BY DNA SYNTHESIS

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A number of models have been proposed for the initiation of general genetic recombination. One of these, originally proposed by Meselson and Radding, imagines that the single-stranded 5' tail that results from strand displacement DNA repair synthesis can initiate homologous recombination by invading a homologous duplex. The resultant D-loop intermediate is then processed into mature products. We demonstrate here that an in vitro system composed of the bacteriophage T4 uvsX protein (a RecA-like "strand transferase") and part of the T4 DNA polymerase holoenzyme efficiently mediates pairing between nicked double-stranded circular and linear duplex DNAs, thereby demonstrating the feasibility of a key step in the Meselson-Radding model.

General genetic recombination is a complex process that proceeds in a number of steps (1). Work over the last ten years with the *Eschericia coli* recA protein (2) and similar factors isolated from other organisms has greatly enhanced our understanding of homologous strand exchange, a central phase of the recombination cycle. However, the mechanisms by which homologous pairing is intiated are less well characterized biochemically. Most models include the production of single-stranded DNA, which serves as a substrate for protein-mediated strand invasion. This has been proposed to occur by a variety of mechanisms, including recBCD protein-mediated unwinding of duplex DNA (3), exonucleolytic digestion of double-strand breaks (4) and incomplete replication of chromosomal ends (5). Yet another model, originally proposed by Meselson and Radding (6), supposes that the single-stranded 5' tails released by strand displacement DNA repair synthesis might be a substrate for homologous pairing. The resulting displacement loop (D-loop) intermediates is then presumably processed into mature recombinants (Figure 1). This proposal is attractive in that it provides a rationalization for the recombinagenic nature of nicks. However, the feasability of the model has never been tested in vitro with purified proteins. We show here that strand displacement synthesis catalyzed by part of the

Abbreviations: gp32, gene product 32, D-loop, displacement loop, EDTA, disodium salt of ethylene diamine tetraacetic acid, SDS, sodium dodecylsulfate.

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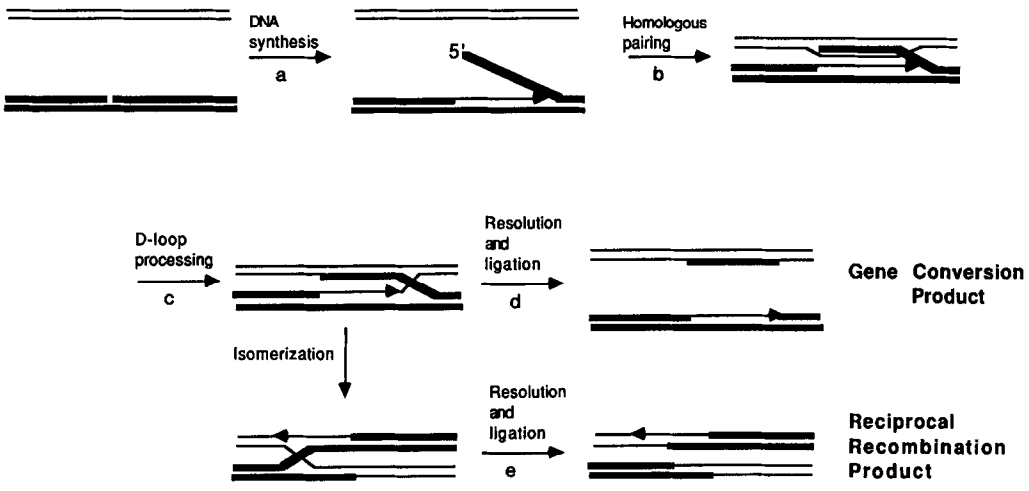


Figure 1: A simple model for replication-dependent homologous recombination originally proposed by Meselson and Radding. a) Strand displacement synthesis originating from a nick results in the production of very long 5' tails. b) These single-stranded DNA can be employed as substrates in protein-mediated D-loop formation. c) Specific nucleases and DNA ligase are then proposed to process the D-loop into complete Holliday junctions. The crossed strand intermediates are cleaved and the resulting nicks ligated to yield mature recombinants. Cleavage of the crossing strands could occur before (d) or after an isomerization (e) which transposes the crossing and non-crossing strands (17). The latter process yields products with exchange of flanking markers rather than simple "patch" recombinants.

bacteriophage T4 DNA polymerase holoenzyme can indeed initiate homologous pairing mediated by the uvsX protein, the T4 analogue of RecA, thus substantiating an important feature of the Meselson-Radding model.

Materials and Methods

Proteins and DNAs: The T4 gene 32 (7), 43 (8), 44/62 and 45 (9) products were purified by the literature procedures from overproducing strains generously provided by Dr. William Konigsberg (Yale University). The uvsX protein was purified by the method of Hinton and Nossal (10) from an overproducing strain provided by Dr. Teiichi Minagawa (Kyoto University). The purified recA protein and the phage fd geneII protein were gifts from Dr. Steven Kowalczykowski (Northwestern University Medical School) and Dr. Jack Berry (UCSF), respectively.

Supercoiled and single-stranded M13 DNAs were produced by the method of Messing (11). 3'-^{32}P-labeled linearized M13 DNA was produced by cleavage of the supercoiled molecule with HpaI restriction endonuclease (New England Biolabs) followed by the "chew back, fill-in procedure of O'Farrell (12). The specific activity of the DNA employed was 200 cpm/pmol. Gene II protein-nicked M13 DNA was produced as described previously (13).

Coupled DNA synthesis/homologous pairing reactions: Each reaction contained 100 µg/ml gene 32 protein, 4 µg/ml gene 43 protein, 25µg/ml each of the gene 45 and 44/62 products, 20 µg/ml of creatine phosphokinase, 5 µg/ml 3'-^{32}P-labeled HpaI-cut M13 DNA (as base pairs) and 5 µg/ml gpII-nicked circular M13 DNA. The concentration of the uvsX protein given in the figure captions. The buffer employed for reactions containing the uvsX protein included 20 mM Tris acetate (pH=7.4), 10 mM magnesium acetate, 1 mM DTT, 0.5 mM EDTA, 10 mM creatine phosphate, 67 mM potassium acetate and 0.15 mM each of dATP, dGTP and TTP.

Upon addition of the gene 43 product (DNA polymerase) the reaction was incubated for two minutes at 37°C in the absence of dCTP in order to allow most of the nicked molecules to acquire a polymerase holoenzyme, thereby synchronizing replication. At this time (T=0), dCTP was added to 0.15 mM. Aliquots were removed at the times indicated and made 25 mM in Na_3EDTA and 1% in SDS. The samples were electrophoresed through a 0.8% agarose gel at 8V/cm. The gels were then dried and the DNA bands visualized by autoradiography. The band intensities were quantitated by scanning densitometry using a home-built instrument.

Other homologous pairing reactions: RecA-mediated homologous pairing reactions between circular single-stranded and HpaI-linearized double-stranded M13 DNAs were conducted as described by Cox and Lehman (14).

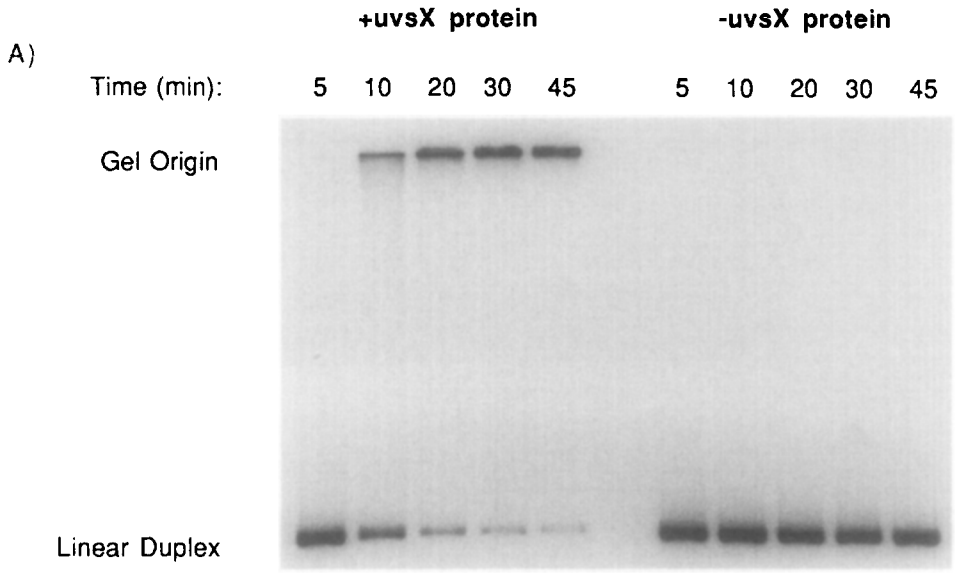
Results and Discussion

UvsX protein-mediated homologous pairing is triggered by strand displacement DNA synthesis: The products of the bacteriophage T4 genes 43 (DNA polymerase), 44/62 and 45 (accessory factors) constitute the part of the viral polymerase holoenzyme necessary to catalyze leading strand synthesis (15). This complex is capable of efficient strand displacement synthesis in the presence of the gene 32 product, a helix-destabilizing protein (16). When M13 DNA that has been nicked with the phage fd gene II protein (17) is added to a solution containing these proteins, ATP and deoxynucleotide triphosphates (dNTPs), rolling circle DNA synthesis ensues, producing a long 5' tail (13). If the Meselson-Radding scheme is correct, this tail should be a substrate for the protein-mediated invasion of a homologous duplex.

To test this postulate, we added 3'-^{32}P end-labeled linear double-stranded M13 DNA and the T4 uvsX protein to the rolling circle reaction and asked whether the labeled DNA suffers invasion by the 5' tail of the replicating molecule. The uvsX protein is the phage analogue of RecA and has been shown to catalyze synapsis between homologous single and double-stranded DNAs in the presence of the gene 32 protein and ATP (10,18,19). To monitor the reaction, aliquots were removed at various times, quenched by adding EDTA and SDS, and electrophoresed through an agarose gel. If homologous pairing does occur, the labeled DNA will become incorporated into much higher molecular weight material.

Figure 2 shows the results of such an analysis. With time, the band on the autoradiogram corresponding to the linear duplex is incorporated into material that barely enters the gel, indicating that homologous pairing between the replicating molecule and the linear duplex has occured. As expected, the reaction is absolutely dependent on the presence of the uvsX protein (Figure 2).

Electron microscopy reveals complex, aggregated products: Analysis of the products by electron microscopy confirms the presence of the expected product (Figure 3a). However, the majority of joint molecules are more complex. This was expected based upon earlier studies of UvsX-promoted pairing (20); the aggregates are the result of secondary pairing reactions. For example, the 5' tail can invade another circular molecule (Figure 3b), or itself (Figure 3c, lower left). If the 5' end of the latter intermediate is released by subsequent branch migration, it can then invade another DNA, for example a linear molecule, resulting in the product shown in Figure 3c (middle right). Many other combinations are possible and a number of these are observable in the electron microscope as well as very large aggregates consisting of many DNA molecules (data not shown).



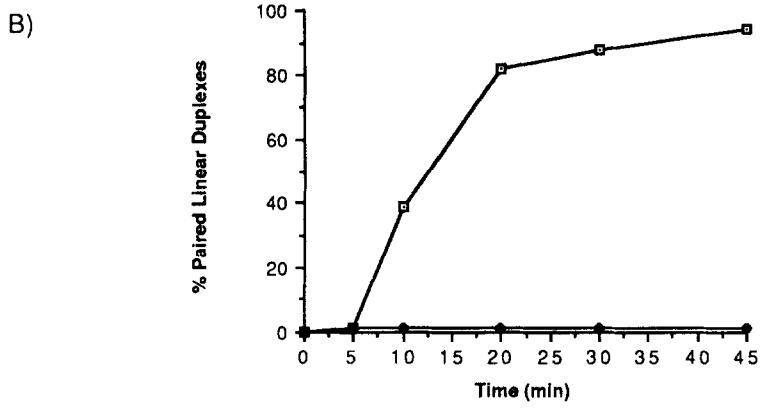


Figure 2: The T4 uvsX and gene 32 proteins catalyze homologous pairing between double-stranded linear DNA and nicked double-stranded circular DNA undergoing rolling circle DNA synthesis. A) The ^{32}P-labeled DNA is incorporated into very slowly migrating products as expected from invasion of the duplex by a long 5' single-stranded tail. The reaction is completely dependent on the presence of the uvsX gene product. In its absence, the R_f of the duplex is unchanged even though extensive DNA synthesis occurs (see ref. 13). B) Quantitation of the autoradiogram by scanning densitometry shows that almost all of the labeled molecules participate in homologous pairing (Filled squares="+uvsX protein" (2.5 μM), filled diamonds="-uvsX protein".)

Homologous pairing requires efficient DNA synthesis: Experiments in which various reaction components were omitted further define the requirements of the reaction (Table 1). Homologous pairing was not observed in the absence of the DNA polymerase or dCTP, demonstrating that DNA synthesis is strictly necessary for pairing. However, only a 3-4 fold lower level of pairing was observed when the circular DNA was not nicked with the

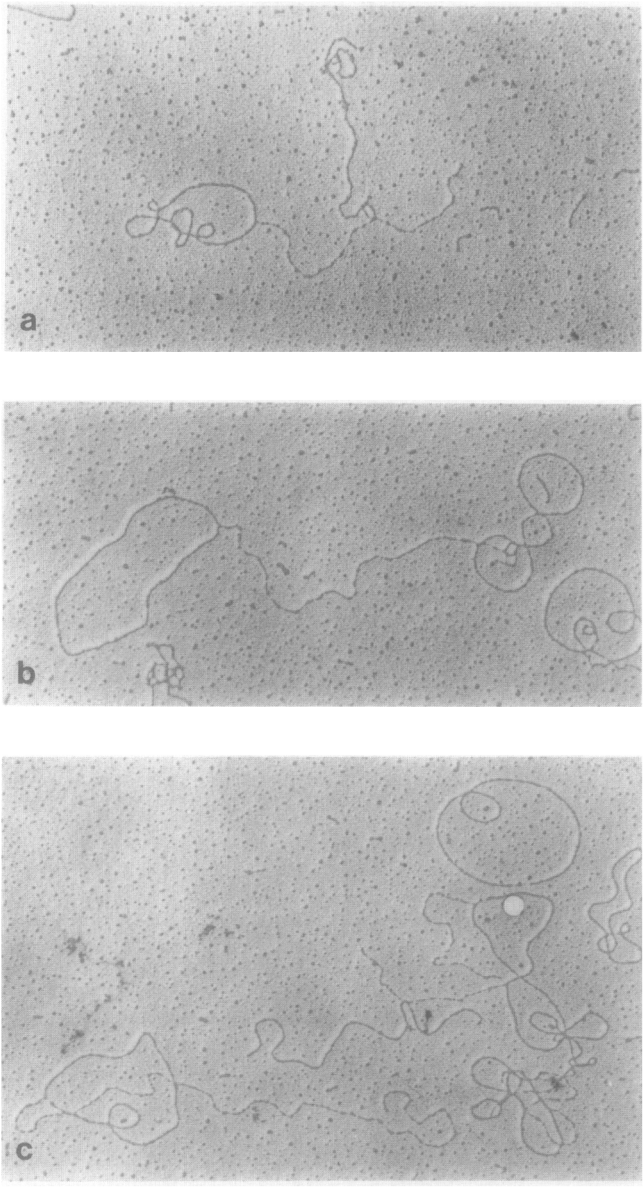


Figure 3: Electron microscopy reveals the presence of D-loops formed by uvsX protein-mediated invasion of a linear duplex DNA by the 5' tail of a molecule undergoing rolling circle DNA synthesis. See text for a description of each intermediate.

gene II protein. When the closed circular DNA was omitted altogether, a much less efficient, but readily detectable, reaction occured (Table 1). Likewise, a trace of paired product was observed when ^{32}P-labeled labeled, non-homologous linear pBR322 DNA was employed in place of linearized M13 DNA. We believe that these inefficient reactions are due to the nicked molecules present in any plasmid preparation. These spurious nicks provide primer template junctions, thereby allowing strand displacement synthesis and

Table 1: Requirements for in vitro homologous pairing primed by DNA synthesis

Reaction	Compound Omitted	% Pairing
1	None	74
2	uvsX protein	0
3	DNA polymerase	0
4	fd phage geneII protein	25
5	circular duplex DNA	7
6	mp19 linear DNA (pBR322 DNA substituted)	9
7	dCTP	0.6

Reactions of the type shown in Figure 2 were carried out with various components omitted in order to delineate the requirements for efficient homologous pairing. Each reaction was incubated for 30 minutes. The data shown above were obtained by quantitative scanning densitometry of autoradiograms of the type shown in Figure 2. "% Pairing" refers to the percentage of labeled molecules that were observed to migrate more slowly than the linear duplex starting material. In reaction six, EcoRV-restricted pBR322 DNA was substituted for M13 linear DNA.

homologous pairing to occur at low levels[1]. Of course, such spurious nicks will also be present in the preparations of linear duplex DNA. Observation of the expected products from these reactions by electron microscopy and the suppression of "spurious" pairing by the addition of DNA ligase (not shown) support this interpretation.

Protein affinity chromatography studies have indentified numerous specific protein-protein contacts between various T4 replication and recombination proteins (21,22), and some of these interactions have been shown to be functionally important. For example, Formosa and Alberts have shown that the recA protein cannot substitute for UvsX in "bubble migration" DNA synthesis catalyzed by the T4 polymerase holoenzyme (23). Therefore, it is of interest to determine whether there is any evidence for a similar direct coupling between the T4 replication and recombination machines in the Meselson-Radding reaction. Unfortunately, we found that the recA protein does not mediate efficient pairing between rolling circle intermediates and homologous duplexes under the conditions used for the Figure 2 experiment, even if the replication proteins have been removed prior to pairing (data not shown). However, the same RecA preparation does catalyze strand exchange between single-stranded circular and homologous double-stranded linear DNAs. We conclude that the tailed circles are simply poor substrates for RecA, and that the *E. coli* strand transferase cannot be used as an isofunctional substitute for UvsX in this reaction. Therefore, we cannot comment at this time about the possible importance of interactions between the T4 replication and recombination proteins in this reaction.

[1] We have previously quantified this "background" DNA synthesis (see ref. 13). For a typical preparation of double-stranded M13DNA, gpII-nicked molecules supported 4-8 fold greater levels of synthesis than untreated DNAs. The products in both cases are the result of rolling circle replication. The 5' tails can be very long (greater than 20,000 nucleotides).

The experiments reported here demonstrate that homologous pairing between a duplex DNA undergoing strand displacement DNA synthesis and a linear duplex is feasible in vitro, substantiating a key step of the Meselson-Radding model of homologous recombination. As expected from the model, formation of joint molecules requires homologous DNAs and is completely dependent on the presence of the uvsX protein, the T4 DNA polymerase holoenzyme and all four deoxynucleotide triphosphates. This interesting system, composed of six highly purified proteins, presumably provides a good model for the events that may occur in vivo during DNA repair synthesis (24) and helps to rationalize the recombinagenic nature of nicks in DNA (25).

Acknowledgments

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