An intermediate in the phage λ site-specific recombination reaction is revealed by phosphorothioate substitution in DNA

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ABSTRACT

It has been proposed that phage lambda site-specific recombination proceeds *via* two independent strand exchanges: the first exchange forming a Holliday-structure which is then converted into complete recombinant products by the second strand exchange. If this hypothesis is correct, one should be able to trap the putative Holliday intermediate by preventing the second strand exchange. In this paper, we show that substitution of phosphorothioate for phosphate in one strand of a recombination site is an effective way to block recombination while permitting the accumulation of a novel structure. This effect is seen only when phosphorothioate is positioned at a point of potential cleavage by Int recombinase, demonstrating that the inhibition of strand exchange is highly specific. Analysis of the novel structure that accumulates in these reactions proves that it contains a Holliday joint. Holliday-structures can also be detected in unblocked recombinations but are present at very low levels. The characteristics of Holliday-structure formation that we describe substantiate the proposed recombination pathway.

INTRODUCTION

Bacteriophage λ encodes a site-specific recombination system which functions to integrate the viral genome into the chromosome of its *E. coli* host. This recombination system has been well characterized at both the genetic and biochemical levels (for reviews see refs. 1, 2). Recombination occurs between specific loci on the viral and *E. coli* chromosomes known as the phage and bacterial attachment sites, *attP* and *attB* respectively. Both attachment sites contain a 15 bp core sequence (designated as O) within which recombination occurs. The arms that flank the core are unique to each attachment site; *attP*, POP', is large (240 bp) and the P and P' arms contain multiple binding sites for the recombination proteins, whereas *attB*, BOB', is much smaller (24 bp). After recombination, the integrated viral genome is flanked by the two prophage attachment sites: *attL*, BOP', and *attR*, POB'. Integration requires two proteins: the recombinase Int, encoded by the phage, and an accessory protein known as Integration Host Factor (IHF), encoded by *E. coli*. The reverse recombination reaction, excision of the integrated prophage, requires a second viral protein, Xis, in addition to Int and IHF. Int binds to the core-arm junctions of *attP* and *attB* (3) and can make transient cleavages at specific locations within the cores (4) (Fig. 1). These cleavages are made using the topoisomerase activity of Int (5), the protein becoming covalently attached to one end of the DNA (4) thereby conserving the energy of the phosphodiester bond. Int mediates recombination by cleaving each DNA strand at the same position within the cores of *attP* and *attB*, and then rejoining the end of each *attB* strand to the corresponding ends from *attP*.

Int has been observed to make low levels of both single-strand nicks and double-strand breaks when incubated with attachment site DNA (4, 6). It has therefore been difficult to decide whether recombination involves a double-strand break mechanism or whether recombination proceeds *via* two independent strand exchanges which form and then resolve a Holliday-structure (7). There was indirect evidence suggesting that Holliday-structures might play a role in this recombination system (8, 9, 10), but initial work with *in vitro* recombination failed to detect the presumptive intermediate. In these early attempts to trap the intermediate, perturbations of the *in vitro* system which reduced recombination, such as omission of IHF, removal of supercoiling or use of attachment sites with a mutant core region, did not lead to the accumulation of novel structures but appeared to block the process at a very early step (11, 12, 13). We thought that recombination sites with substitutions of phosphorothioate for phosphate in the DNA backbone might provide a more subtle block to recombination. A phosphorothioate differs from normal (oxo) phosphate in that one of the oxygen atoms is replaced by an atom of sulphur. In phosphorothioate DNA the deoxyribose moieties continue to be linked to the phosphorus atom *via* oxygen atoms, as in natural DNA, but a bulky sulphur takes the place of a non-bridging oxygen atom (14). Our first results (6) showed that phosphorothioate substitutions could indeed inhibit the formation of completed recombinants and indicated that such inhibition might depend on the presence of a phosphorothioate residue at the site of Int attack. In this paper, we demonstrate that phosphorothioate substitution at an Int cleavage site in *attB* is both necessary and sufficient to cause inhibition of recombination. We confirm that substitutions at other locations are not inhibitory. We have also tested the expectation that, although production of completed recombinants is blocked by the presence of phosphorothioate in the Int cleavage site, the early steps in recombination proceed normally. We find that when phosphorothioate is present at the Int cleavage site in the bottom strand of *attB*, a novel species accumulates in reaction mixtures that contain Int, IHF and *attP*. This species consists of *attP* and *attB* linked by a Holliday-junction. In other work we have used this experimental strategem to explore the dependence of Holliday-structure formation on homology (15). Landy and colleagues (16) have used a related approach, blocking strand exchange by positioning specific nicks in DNA, to independently demonstrate the accumulation of Holliday structures during lambda site-specific recombination. Together, these studies establish that Holliday structures are intermediates in this recombination reaction (17).

MATERIALS AND METHODS

Materials and General Methods

The M13 sequencing primer (dTCCCAGTCACGACGT) was obtained from New England Biolabs. The B1 (dGAATTCCCTTCCCGTTTCGCTCAAGTTA) and B2 (dGAATTCCCTTCCCGTTTCGCTCAAGTTAG) primers were gifts from Gerald Zon. ³²P γ -ATP, ³²P α -dNTPs, and unlabeled α -phosphorothioate dNTPs were purchased from New England Nuclear. IHF and Int were purified as described (11, 18). The plasmids pMM290 (19) carrying *attB*, and pPH54 (20) carrying *attP* have been described previously. M13 phage were grown and transfected according to standard procedures (21) using as the host strain KK2186, a derivative of JM103 that lacks the P1 prophage (R. Zagursky, personal communication).

Recloning of Attachment Site Fragments

Plasmid pMM290, carrying a 44 bp segment of *attB* DNA derivatized with a BamHI linker at position -17 and an EcoRI linker at position +26 (19), was cut with HincII and the 780 bp fragment carrying *attB* was inserted into the HincII site of M13mp8 (21). Two clones (R568 and R570) containing the desired insert in opposite orientations were identified by their restriction patterns. A second set of M13 derivatives containing *attB* sequences were made as follows. Plasmid pMM290 was cut with EcoRI, the ends were made blunt by filling in, and BamHI linkers (dCCCGGATCCGGG; Collaborative Research) were ligated to the ends. The ligation products were restricted with BamHI and the 60 bp *attB* fragment was inserted between the BamHI sites of M13mp7 (21). Two clones (R559 and R574) containing the *attB* fragment in opposite orientations were identified by dideoxy sequencing of the viral DNA.

Synthesis of *attB* Fragments Containing Phosphorothioate

Phosphorothioates were incorporated into *attB* using a primer-extension protocol. The template was a 60 base segment carrying *attB* sequences which was cut out of the single-stranded R559 viral DNA using the method described by Been and Champoux (22); the 18 bp inverted repeats that flank the *attB* sequences in this clone were annealed to form a stem which was cleaved with BamHI endonuclease. Following extraction with a phenol:chloroform mixture and by ethanol precipitation (23), the DNA (250 µg) treated with 4 units of calf intestinal alkaline phosphatase (Boehringer Mannheim) for 30 min. at 37°C. The phosphatase was inactivated by addition of Na₂EGTA followed by incubation at 65°C for 45 min. The 60 base *attB* template was separated from the large vector segment by HPLC purification over a 125 mm x 6 mm Nucleogen 4000-10 column (Rainin) as follows; the mixture was adjusted to 30 mM sodium phosphate pH 6.5, 6 M urea, 375 mM NaCl, incubated at 37°C for 10 min., passed through a 0.45 µm Millex-HV₄ filter (Millipore) and loaded onto the column. The DNA was eluted using 30 mM sodium phosphate pH 6.5, 6 M urea with a gradient of NaCl from 375 mM to 900 mM NaCl at 15 mM/min. and a flow rate of 1 ml/min. The UV absorbance (254 nm) of the eluate was monitored and the first peak, containing the 60 mer, was collected in silanized glass tubes containing a volume of 0.5 M Na₂EDTA sufficient to give a final concentration of 5 mM. The DNA in this peak fraction was desalted and concentrated over a Nensorb-20 column (Dupont) according to the manufacturers instructions. The viral DNA of M13 clone R559 yields a template for synthesis of the bottom strand of *attB*.

This template was annealed to the primer as follows. The *attB* template strand and oligonucleotide primers were first labeled at the 5' ends using ³²P γ-ATP and T4 polynucleotide kinase (Pharmacia) and unincorporated label was removed over a Nensorb-20 column. The labeled oligonucleotides were resuspended in 1 mM Tris-HCl pH 7.6, 0.1 mM Na₂EDTA and the concentration of fragment adjusted to 0.25 µM for templates or 1.25 µM for primers. A primer-template complex was prepared by mixing 10 pmole of B1 or B2 primer and 2 pmole of template, either one or both of which were labeled, in 20 µl of 8 mM Tris-HCl pH 7.5, 50 mM NaCl, 7 mM MgCl₂, incubating at 90°C for 5 min. and allowing the mixture to cool slowly (over 30 to 60 min.) to 25°C. The annealing mixture was placed on ice and the following additions made: 3 µl of polymerase buffer (70 mM Tris-HCl pH 7.5, 70 mM MgCl₂, 50 mM NaCl, 75 mM DTT), 5 µl each of 0.5 mM solutions of dATP, dTTP, dGTP, and dCTP in 10 mM Tris-HCl pH 7.5, 1 mM Na₂EDTA, and H₂O to make a final volume of 50 µl. To make *attB* fragments containing

phosphorothioate, one dNTP was replaced by 5 μ l of a 1.0 mM solution of the corresponding α -phosphorothioate derivative (dNTP α S). The extension reaction was started by addition of 3 units of the Klenow-fragment of DNA polymerase I (Bethesda Research Laboratories), the mixture was incubated at 37°C for 30 min. and the reaction terminated by adding 5 μ l of 0.5 M Na₂EDTA and heating to 70°C for 10 min. Alternatively, reverse transcriptase was employed for the extension reaction; in this case the additions to 20 μ l of primer-template complex were 5 μ l of reverse transcriptase buffer (430 mM Tris-HCl pH 7.5, 20 mM MgCl₂, 550 mM NaCl, 100 mM DTT, 1 mg/ml BSA), 5 μ l each of 5 mM solutions of dATP, dTTP, dGTP, and dCTP or the corresponding dNTP α S derivative in 10 mM Tris-HCl pH 7.5, 200 units of murine-MLV reverse transcriptase (Bethesda Research Laboratories) and H₂O to make a final volume of 50 μ l. This mixture was incubated at 37°C for 30 min. and the reaction terminated as described for DNA polymerase reactions.

The 60 bp *attB* fragments were separated from excess primer and unextended primer-template complex by HPLC purification as described for the template except that the gradient was from 500 mM to 900 mM NaCl at 20 mM/min. Cerenkov radiation in the eluate was monitored using a Ramona radioactivity detector with a 1.2 ml liquid scintillation cell (Raytest) and the last peak, containing the double-stranded *attB* fragment, was collected. In order to limit oxidation of the incorporated sulfur, fractions were collected in silanized glass tubes containing a volume of 0.5 M Na₂EDTA sufficient to give a final concentration of 5 mM and the DNA was promptly desalted over a Nensorb-20 column. After resuspension in 50 mM Tris-HCl pH 8.0, 1 mM Na₂EDTA, the fragment concentration, based on the total Cerenkov counts and the theoretical specific activity of the *attB* fragment, was adjusted to 2.5 nM by ethidium bromide fluorescence (23). The *attB* fragments were stored at -20°C. An aliquot of each labeled *attB* was mixed with a cold *attB* x cold *attP* recombination reaction in order to check for the presence of contaminants that would inhibit recombination.

Preparation of *attB* Fragments for Analysis of the Strand Composition of α -Structures

attB fragments which were labeled at both 5' or both 3' ends were made as follows. Single-strand viral DNA from M13 clone R570 was prepared by scaling up a previously described protocol (24). A primer-template complex was prepared by annealing 20 μ g (8 pmole) of R570 single-strand DNA with 40 pmole of unlabeled B1 primer as described above. Synthesis of the bottom strand was completed using Klenow fragment and either all four dNTPs or dGTP α S plus dTTP, dCTP, and dATP essentially as described above. Double-stranded *attB* fragments (52 bp) were cleaved out of the M13 vector DNA by restriction with BamHI and EcoRI; and the DNA was either 5' or 3' end labeled. The labeled DNAs were run on a 12% polyacrylamide gel; the *attB* bands were located by autoradiography and excised from the gel. The DNA was eluted from the gel slices using the electroelution apparatus described by Hunkapiller *et al* (25) and concentrated by ethanol precipitation. The concentration and purity of these *attB* fragments were checked as described above.

Synthesis of an *attB* Fragment Containing a Single Phosphorothioate at the Int Cleavage Site

A primer-M13 template complex, prepared as described in the previous section, was first incubated with Klenow enzyme and 2 μ M dGTP α S plus 30 μ M dATP for 30 min. at 37°C. The dGTP α S was then diluted out with a 100-fold excess of dGTP. dCTP and dTTP were added to

a final concentration of 30 μ M and the mixture was incubated at 37°C for another 30 min. The extension products were restricted, end labeled and the double-stranded *attB* purified as described above. The first step adds a single phosphorothioate to the end of the primer; the dATP prevents the 3' to 5' exonuclease activity of the Klenow enzyme from nibbling back the primer. The second step completes synthesis of the bottom strand without incorporating any more phosphorothioates into the DNA.

Recombination Assays

Integrative recombination reactions (20 μ l) contained 50 mM Tris-HCl pH 8.0, 70 mM KCl, 5 mM spermidine, 1 mM Na₂EDTA, 1 mg/ml BSA, 4.5 nM of a supercoiled plasmid (pPH54) carrying *attP*, 0.25 nM *attB* fragment, ~ 2 units purified IHF and ~ 2.5 units purified Int-wt or purified Int-h. Typically, reactions were incubated at 25°C for 2 hr reactions analysed by agarose gel electrophoresis as described (15, 18).

Recombination reactions which were to be restricted were phenol extracted and ethanol precipitated. Since vacuum drying was found to induce changes in the electrophoretic mobility of Holliday structures, ethanol was permitted to evaporate at room temperature while the centrifuge tube rested horizontally. After restriction, to avoid oxidation of the sulfur residues, the samples were electrophoresed on a 5% (w/v) polyacrylamide gel (29:1, acrylamide:bisacrylamide) in 1 x TBE that had been prerun in 1 x TBE containing 0.05% (v/v) thioglycolic acid at 100 V for 1 hr and in 1 x TBE containing 5 mM β -mercaptoethanol at 100 V for 30 min..

Denaturation Analysis of α -Structures

attB fragments labeled at either both 5' or both 3' ends were used as substrates in a 5-fold scale-up of the standard recombination. The products were restricted with HindIII plus BamHI and electrophoresed on a 5% polyacrylamide gel as described above. The X-form band generated by Int-h promoted recombination of the phosphorothioate-containing *attBs* was excised from the gel as was a portion of the gel containing both the *attL* and *attR* bands generated by Int-wt promoted recombination of the non-phosphorothioate-containing *attBs*. The DNA was eluted from these gel slices using the crush-and soak method (26) with β -mercaptoethanol (5 mM) in the elution buffer and electrophoresed on an 8% (w/v) polyacrylamide gel containing 1 x TBE and 7 M urea which had been prerun in 1 x TBE containing 0.05% (v/v) thioglycolic acid at 1200 V for 60 min. and in 1 x TBE containing 5 mM β -mercaptoethanol at 1200 V for 30 min.

Time Course of Recombination

A labeled fragment, containing no sites for Hinf I, for use as an internal standard was prepared by digesting pBR322 with HindIII plus TaqI and filling in the ends with ³²P- α -dATP and cold dGTP, dTTP and dCTP. The digest was run on a 5% polyacrylamide gel, the 310 bp HindIII-TaqI fragment was excised, electroeluted and concentrated by ethanol precipitation.

A pair of *attB* fragments labeled to the same specific activity were made by annealing 5' end labeled B1 primer to a 60 base *attB* template strand as described above. This primer-template complex was divided into two aliquots; one aliquot was extended in the presence of dGTP α S and the other extended in the absence of phosphorothioate nucleotide derivatives. 20 x standard recombination reactions were prepared in parallel using 100 pmole of each of the resulting *attBs*. Equal aliquots of the standard fragment were added to each recombination mix and recombination initiated by addition of *attP*, IHF and Int-h. At various times 20 μ l aliquots were removed and

recombination terminated by extraction with phenol. After restriction with Hinf I, the recombination products were separated on 5% polyacrylamide gels which were then dried and autoradiographed. The *attR* bands (151 bp), X-form bands, internal standard bands and four blank sections were cut out of the dried gel and counted in a dry scintillation vial using Cerenkov radiation. The average value of the blanks was subtracted from the experimental values and the ratios of counts in *attR*/counts in internal standard and counts in X-forms/counts in internal standard were calculated for each sample.

In a similar experiment, 150 pmole of a non-phosphorothioate-containing *attB*, labeled at both 5' ends, was mixed with an aliquot of the labeled standard fragment and recombined with *attP* in a 30 x standard recombination reaction using Int-h. At various times three 20 μ l aliquots were removed and treated as described above. Six blanks, the combined *attR* (151 bp) and *attL* (226 bp) bands, X-form bands, and internal standard bands were cut out of the dried gels and counted. The average value of the blanks was subtracted from the experimental values and the ratios of counts in *attR* + *attL*/counts in internal standard and counts in X-forms/counts in internal standard were calculated for each sample. The mean [$m = \sum x_i / 3$] and standard error of the mean [$(s.e.)^2 = \sum (x_i - m)^2 / 6$] were calculated for each triplicate.

RESULTS

Recombination of Attachment Sites Containing Phosphorothioate

We have previously described the synthesis of linear *attB* fragments containing phosphorothioate substitutions for use as substrates in recombination assays (6). The substituted *attB* fragments were synthesized by annealing a template strand containing the *attB* sequence with an oligonucleotide primer and incorporating α -phosphorothioate derivatives of the deoxynucleotide triphosphates (α -thio-dNTPs) into the DNA during enzymatic synthesis of the complementary strand. Because of the polarity with which DNA polymerase incorporates nucleotides into DNA, an *attB* will contain a phosphorothioate at the Int cleavage site only when the bottom strand is synthesized using α -thio-dGTP or when the top strand is synthesized using α -thio-dTTP (see *attB* sequence in Fig. 1). We previously found that *attBs* synthesized with α -thio-dGTP throughout the bottom strand or with α -thio-dTTP throughout the top strand are very poor substrates for recombination, but that an *attB* synthesized with α -thio-dATP throughout the top strand recombines with normal efficiency (6). In the latter substrate none of the phosphorothioates are located at the Int cleavage site and on this basis we concluded that it was the presence of a phosphorothioate at the Int cleavage site that caused recombination to be inhibited in the first two cases. Here we test this conclusion by examining recombination of *attB* fragments containing phosphorothioate substitutions at many different locations in the bottom strand. Incorporating phosphorothioates into the bottom strand using α -thio-dATP, dCTP or dTTP does not inhibit recombination and in fact a small stimulation is observed (Fig. 2, compare lanes 2, 3 and 4 with lane 1). These results confirm that phosphorothioates at locations other than the Int cleavage site are not inhibitory; even *attBs* with substitutions in the consensus sequence for Int binding are good substrates for recombination. When α -thio-dGTP is incorporated into the bottom strand of *attB* using a primer which ends at +5, the resulting fragment is a very poor

Figure 1. *arrB* sequence. The attachment site core sequence is shown in capital letters and the sequence of the B and B' arms in lower case. Coordinates are given with reference to the central T-A base pair of the core which is defined as zero (1). The two core-arm junction Int binding sites (3) are indicated by shaded bars and the Int cleavage sites (4) are marked with an arrow. By convention, top and bottom strands refer to the attachment site as drawn.

substrate for recombination (Fig. 2, compare lane 5 with lane 1), however, an *attB* in which α -thio-dGTP has been incorporated using a primer which ends at +4 recombines efficiently (Fig. 2, compare lane 7 with lane 6). The only difference between these two substrates lies at the Int cleavage site; the first has a phosphorothioate internucleotide linkage between positions +4 and +5 whereas the latter has a phosphate linkage. This result demonstrates conclusively that the phosphorothioate at the Int cleavage site is necessary for inhibition. By carrying out the extension step in two rounds, we synthesized an *attB* in which a single α -thio-dGTP had been incorporated into the bottom strand at the Int cleavage site (see Materials and Methods). This *attB* is a poor substrate for recombination (data not shown). The presence of phosphorothioate at the site of Int cleavage is therefore both necessary and sufficient to inhibit recombination.

Figure 2. Recombination of *attB*s containing phosphorothioate. Autoradiograph of an agarose gel showing the products of recombination between a supercoiled plasmid (4.4 kb) carrying *attP* and various labeled DNA fragments (60 bp) carrying *attB*. Recombination was mediated by Int-wt for 30 min. The *attB* substrates in lanes 1 to 5 were made using an oligonucleotide (B1) ending at the +5 position to prime synthesis of the bottom strand. The *attB* substrates in lanes 6 and 7 were made using a primer (B2) ending at the +4 position. Synthesis of the bottom strand was completed using DNA polymerase and all four dNTPs (lanes 1 and 6) or one α -phosphorothioate dNTP derivative plus the remaining three dNTPs as follows: α -thio-dATP (lane 2), α -thio-dCTP (lane 3), α -thio-dTTP (lane 4) and α -thio-dGTP (lanes 5 and 7). Autoradiography was for 6 hr with an intensifying screen. NC, L, and SC mark the positions of the nicked circular, linear and supercoiled forms of the *attP* substrate. The recombinant products comigrate with the linear form of the *attP* substrate; the *attB* substrate has run off the bottom of the gel.

A little completed product, comigrating with the linear substrate, is visible even in recombinations inhibited by phosphorothioate (Fig. 2, lane 5) and is more obvious in a longer exposure (Fig. 4, lane 5). We considered that some of this product may have come from a small fraction of the substrate which did not have phosphorothioate at the critical position. Such a substrate could have arisen due to contamination of α -thio-dGTP with oxo-dGTP, misincorporation of one of the other three dNTPs at the critical position, or by oxidation of the phosphorothioate subsequent to incorporation into the DNA (27). Not all the residual product can be explained in this way since *attB* substrates in which the only label is ^{35}S -phosphorothioate at the Int cleavage site still produce a trace of labeled recombinant product (Kitts and Nash, in preparation). We conclude that cleavage of DNA by Int, as for many other enzymes (14), is inhibited substantially but not completely by the presence of a phosphorothioate linkage.

The phosphorothioate in an α -thio-dNTP introduces an additional chiral center into the molecule, these compounds therefore exist as two diastereoisomers, R_p and S_p . DNA polymerases are stereoselective, therefore, the phosphorothioates in the substrates described above will all be the R_p diastereoisomer (14). In order to make a preliminary determination of the effect of S_p phosphorothioate on recombination, an *attB* fragment was constructed by enzymatic extension of a primer which was chemically synthesized so as to contain a racemic mixture of phosphorothioates at the bottom strand Int cleavage site (a generous gift of Gerald Zon). This substrate recombines with an efficiency which is intermediate between that of a similar *attB* containing no phosphorothioate and one that contains a single R_p phosphorothioate at the bottom strand Int cleavage site (data not shown). We infer that only the R_p phosphorothioate inhibits recombination to a significant extent. We note, however, that we have not directly measured the chemical composition of the *attB* substrate; confirmation that Int is stereoselective must await either the synthesis of an *attB* containing pure S_p phosphorothioate at the critical position or an analysis of the stereochemistry of the phosphorothioate in the product.

Formation of Holliday-Structures During *in vitro* Recombination

Although formation of completed recombinants is blocked by a phosphorothioate at the Int cleavage site, one might expect that the early steps in recombination could still proceed normally. A single strand exchange would generate a Holliday-junction (7) and in our assay this would form part of an α -structure (Fig. 3). Although in the α -structure one strand of *attP* is not covalently closed, base pairing in the arms will constrain the circular portion of the molecule and the α -structures should therefore have a mobility similar to that of the covalently closed *attP* substrate. Indeed, when the sensitivity of our assay is increased, a product with this mobility can be detected in a recombination reaction which is inhibited by the presence of a phosphorothioate at the Int cleavage site in the bottom strand of *attB* (Fig. 4, lane 5). Figure 4, lane 6 shows that a higher level of this presumptive α -band is generated when recombination of the phosphorothioate *attB* is mediated by the variant Int protein known as Int-h. [Int-h was isolated as a variant of Int able to carry out some residual recombination in an IHF mutant strain (28). Recombination mediated by Int-h is, however, strongly stimulated by active IHF (28, 30)]. Int-h recombinations do not yield higher levels of the presumptive α -band simply because Int-h is able to overcome the effects of the phosphorothioate substitution since the amount of completed recombinant made by Int-h is similar to that produced by Int-wt (Fig. 4, compare lanes 5 and 6).

Figure 3. Diagram of the *in vitro* recombination assay. The substrates for recombination (left) are a supercoiled plasmid (4.4 kb) carrying *attP* (POP') and a small (52-60 bp) linear labeled DNA fragment which carries *attB* (BOB'). Recombination of one strand of *attP* with the corresponding strand of *attB* makes an α -structure (center) containing a Holliday-junction at the attachment sites. Recombination of the second pair of strands generates the completed recombinant product (right) containing *attL* (BOP') and *attR* (POB'). The *attB* fragment makes a negligible contribution to the size of the products, therefore the completed recombinant product will comigrate with the linear form of the *attP* plasmid and α -structures will comigrate with the covalently closed *attP* plasmid. Sites for the endonucleases Hind III (P) and BamHI (B) are marked. *attP* and *attB* are not drawn to scale.

In addition to α -structures, other forms of association between *attP* and *attB* (such as non-covalent protein-DNA complexes, D-loops, or hemicatenanes) might be expected to comigrate with the covalently closed *attP* substrate. We have examined the structure of the material in the presumptive α -band by restriction analysis. If *attB* and *attP* were linked in an α -structure, restriction with enzymes that cut on either side of the *attP* site should liberate an X-form containing the Holliday-junction (Fig. 3). When the products of a recombination reaction similar to that shown Fig. 4, lane 6 are restricted and separated on a polyacrylamide gel, a band with the slow mobility expected for X-forms can be seen (Fig. 5A, lane 1). The presence of this band indicates that the association between *attB* and the *attP* fragment can survive phenol extraction, therefore, the two attachment sites are not held together in a purely non-covalent protein:DNA complex. Since the novel species partitions into the aqueous phase on extraction with phenol, neither does it have protein covalently attached to the DNA. In order to determine whether the *attB* DNA strands were covalently linked to *attP* in this species, we looked at the sizes of the single-strands which were released when the DNA in the putative X-form band was denatured. The presence, in this material, of labeled single-strands which comigrate with the labeled strands from *attL* and *attR* (Fig. 5B, lanes 2 and 3) is evidence that recombination has joined *attP* and *attB* DNA. Furthermore, the pattern of labeled strands that we observe is diagnostic for X-forms made by top strand exchange. If the *attB* substrate is labeled at both 3' ends, denaturation of an X-form which had been formed by recombination between the top strands of *attP* and *attB* would yield one labeled single-strand containing *attB* sequences and another carrying *attR* sequences (Fig. 5C). On the other hand, if the *attB* was labeled at both 5' ends, denaturation of the same X-form would again yield a labeled *attB* strand but the second labeled strand would carry *attL* sequences (Fig. 5C). Upon denaturation, the X-forms generated in our recombination reactions give labeled products of the sizes predicted for this structure (Fig. 5B, lanes 2 and 3). (Although the region of the gel containing the *attB* strands is not shown, both 3' and 5' labeled X-forms release a labeled strand which comigrates with strands from the denatured *attB* substrate.) An X-form made by exchange of the bottom strands would give the opposite pattern

Figure 4. The formation of α -structures. Autoradiograph of an agarose-EtBr gel showing the products of 2 hr. recombination reactions. Recombination mixtures contained IHF, Int-wt or Int-h as indicated. The *attB* substrate used in lanes 1 to 4 contained only oxophosphates. The *attB* substrate used in lanes 5 to 8 was made by incorporating α -thio-dGTP into the bottom strand at all positions 3' to +5 and therefore contained a phosphorothioate at the Int cleavage site. NC, L, and CC mark the positions of the nicked circular, linear and covalently closed forms of the *attP* substrate. The positions of completed recombinant products (R) and α -structures (α) are indicated. The *attB* substrate has run off the bottom of the gel. The higher molecular weight band seen in some lanes corresponds to products containing more than one copy of the *attP* plasmid. Autoradiography was for 72 hr. with an intensifying screen.

(*attL* from 3' labeled *attB* and *attR* from 5' labeled *attB*), however, no products of these sizes can be detected (Fig. 5B, lanes 2 and 3). Other structures, including D-loops and hemicatenanes, would not release single-strands with the same length as *attL* or *attR*. We conclude that the novel species is an X-form containing a Holliday-junction made by top strand exchange; the X-forms, and by inference the α -structures, have therefore been made by recombining the non-phosphorothioate-containing top strand of *attB* with the top strand of *attP*. (The consequences of blockage of top strand exchange by phosphorothioates will be presented elsewhere.) On a gel which would clearly resolve strands which differed by 2 or 3 nucleotides, the length of the recombinant strands in the X-forms is indistinguishable from the lengths of the strands in the completed recombinants (data not shown). This result implies that the strand exchange that generates these Holliday-structures maintains the fidelity of the complete recombination process, e.g., no Holliday structures are observed that result from the anti-parallel alignment of attachment sites (i.e., top strand of one site exchanged with the bottom strand of another).

The losses entailed in eluting DNA species from the gel, in conjunction with the low yield of α -structures from Int-wt recombinations, prevented us from analyzing the structure of these products using the method described above. We therefore developed an alternative method in which the restricted products of recombination are separated in one direction under native

Figure 5. Restriction and denaturation analysis of α -structures. (A) Autoradiograph of a native polyacrylamide gel showing the products of 2 hr., Int-h mediated recombinations after phenol extraction, ethanol precipitation and restriction with Hind III and BamHI. Lane 1, the *attB* substrate contained phosphorothioate at the Int cleavage site in the bottom strand; lane 2, the *attB* contained no phosphorothioate; lane 3, HindIII-BamHI *attP* fragment; lane 4, size marker. X denotes the position of X-forms. Sizes of the various fragments are indicated on the right-hand side. Autoradiography was for 96 hr with an intensifying screen. (B) Autoradiograph of a denaturing polyacrylamide gel showing the labeled single-strands contained within the recombinant products. The products generated by Int-h mediated recombination of an *attB* containing phosphorothioate in the bottom strand, and those generated by Int-wt mediated recombination of a non-phosphorothioate-containing *attB* were restricted with Hind III and BamHI, separated on a native acrylamide gel as described in (A). DNA from the X-form band of the Int-h recombination reaction was eluted and rerun on a denaturing gel (lanes 2 and 3). Similarly, the *attL* and *attR* fragments from the Int-wt recombination were rerun on the denaturing gel to provide markers (lanes 1 and 4). The *attB* substrates used to generate the products rerun in lanes 1 and 2 were labeled at both 3' ends; the products rerun in lanes 3 and 4 were made from *attB* substrates labeled at both 5' ends. Autoradiography was for 2 weeks with an intensifying screen. (C) Diagrams of X-forms containing Holliday-junctions made by recombination of the top strand of *attP* with the top strand of an *attB* that is either labeled at both 3' ends or labeled both 5' ends. Arrowheads indicate the 3' end of each DNA strand; ●, radioactive label. The lengths of the various arms are not to scale.

conditions and then under denaturing conditions in a perpendicular direction, thereby keeping sample losses to a minimum. Using this 2D-gel method to analyze the strand composition of X-forms made in Int-wt recombinations blocked by phosphorothioate in the bottom strand of *attB*, we confirmed that Int-wt makes Holliday-junctions that have the same structure as those made by Int-h (data not shown).

In addition to giving higher yields of α -structures in recombination reactions inhibited by phosphorothioate, Int-h also boosts the production of α -structures from an *attB* which contains no phosphorothioate so that an α -band is readily detectable (Fig. 4, lane 2). We have confirmed that this band also contains a Holliday-junction using the 2D-gel method (Kitts and Nash, in preparation). In fact, on longer exposures a faint α -band can also be detected following Int-wt mediated recombination of the non-phosphorothioate *attB* (data not shown). These results indicate that production of α -structures is not only a consequence of phosphorothioate substitution but occurs even in unblocked recombinations.

On agarose-EtBr gels most of the α -structures migrate with the covalently closed *attP* plasmid with only a small fraction migrating in the nicked circle position (Fig. 4, lanes 2, 5 and 6). Agarose gels run without EtBr show that the α -structures are not significantly more relaxed than the unrecombined *attP* plasmids (data not shown). The two halves of a strand exchange must, therefore, be closely coupled; when cleavage of the bottom strand of *attB* is blocked by phosphorothioate, the bottom strand of *attP* is not nicked, or if it is nicked it is not released.

The Relationship Between Holliday-Structure Formation and Recombination

The formation of both α -structures and complete recombinants is dependent upon Int and IHF (Fig. 4, lanes 3, 4, 7 and 8). Likewise, in Int-h mediated recombinations inhibited by phosphorothioate in *attB*, α formation and production of completed recombinants both require a supercoiled *attP* partner: nicked circular *attP*, or supercoiled plasmids carrying *attB*, *attL*, *attR* or an *attP* with a defective H1 IHF binding site (29), cannot efficiently replace supercoiled *attP* (data not shown). These results imply that Holliday-structures are products of the normal recombination process.

In Fig. 6A we compare the time course for production of Holliday-junctions, assayed as X-forms, from recombination with a phosphorothioate substituted *attB* and the time course for production of completed recombinants from a parallel unblocked recombination reaction; we find that these two products are formed with comparable rates. At least in this most favorable case, when Int-h is acting on a phosphorothioate substituted *attB*, the rate of Holliday-structure formation is sufficiently rapid to account for the rate of recombination observed with unsubstituted *attBs*. Figure 6B compares the time course for production of X-forms and completed recombinants in a single unblocked recombination reaction. We find, as would be expected if Holliday-structures are precursors to recombinants, that X-forms reach a maximum before the completed recombinants plateau. The observed time courses are consistent with the view that Holliday-structures are intermediates on the normal recombination pathway. It remains to be seen whether our data can be fitted into a quantitative kinetic scheme that provides a simple way of accounting for the different levels of Holliday-structures seen with Int-h versus Int-wt and in blocked versus unblocked recombination reactions.

Figure 6. Time courses comparing Holliday-structure formation and production of completed recombinants. (A) The 5' end of the B' arms of *attB* fragments containing either no phosphorothioate or phosphorothioate at the Int cleavage site in the bottom strand were labeled to the same specific activity. An equal number of counts of each *attB* were separately mixed with the same amount of a labeled fragment which carried no attachment site sequences that was used as an internal standard. These substrates were incubated with Int-h, IHF and *attP* in parallel reactions. Aliquots were removed at various times, restricted with *Hinf* I and run on acrylamide gels like that shown in Fig. 5A. The product and internal standard bands were excised from the dry gel, and counted by Cerenkov radiation. The counts in the product bands were normalized to the internal standard to give a relative amount of product. ●—● *attR* fragments made by recombination of the non-phosphorothioate-containing *attB*. O---O X-forms made by recombination of the phosphorothioate *attB*. (B) An *attB* fragment containing no phosphorothioate and which was labeled at both 5' ends was mixed with an internal standard labeled fragment and incubated with Int-h, IHF and *attP*. At each timepoint, three aliquots were removed and the products assayed as described in (A). ●—● (left-hand scale) *attL* plus *attR* fragments; O---O (right-hand scale) X-forms. The data are plotted as mean $\pm$ standard error.

DISCUSSION

The use of attachment sites containing phosphorothioate substitutions has enabled us to study a partial reaction mediated by the phage λ site-specific recombination system. The primer-extension protocol that we employ for synthesizing the substituted DNA allows any nucleotide derivative that is a substrate for DNA polymerase or that can be chemically incorporated into an oligonucleotide to be introduced into the recombination site. By varying the template strand, the length of the primer and the choice of substituted or unsubstituted dNTPs, nucleotide analogues can be placed at predetermined positions in either strand of the DNA fragment. A difficulty arises when runs of the same nucleotide exist in the template; in this case, multiple phosphorothioate substitutions are hard to avoid. Fortunately, oligonucleotide-directed mutagenesis of the naturally occurring site can often be used to create a functional site in which the run is disrupted, a strategy we have exploited in this work. The ability to vary specific structural features of the DNA has many potential applications. For instance, it allows one to ask what features of the DNA are important for protein:DNA interactions in more detail than the current methods of ethylation and methylation interference (31).

In this paper we show that a phosphorothioate at the Int cleavage site in the bottom strand of *attB* is both necessary and sufficient to cause a strong inhibition of the integration reaction. We

Figure 7. A hypothetical reaction scheme involving a Holliday-structure intermediate. The phage, bacterial and prophage attachment sites are represented as P, B, L and R respectively; Holliday-structures are represented as α . An asterisk indicates that the DNA molecule(s) are part of a complex that also involves recombination proteins. The rate constants for the forward reactions are designated k_1 to k_5 , and those for the reverse reactions as k_{-1} to k_{-3} . For a discussion of the various steps see the text.

also demonstrate that phosphorothioates at other locations have only a small effect on recombination. These findings lead us to infer that phosphorothioate inhibits recombination by making the DNA resistant to cleavage by Int, possibly because the sulfur atom prevents intimate contact between the Int active site and the DNA backbone. Certain phosphorothioate substitutions result in a small stimulation of recombination; to our knowledge, this is the first report of a stimulatory effect resulting from phosphorothioate substitution. We imagine that the substituted attBs may be better substrates for recombination because the binding of Int to phosphorothioate DNA might be enhanced or because phosphorothioates might induce an altered DNA structure (32, 33) that favors recombination.

An early model for phage λ site-specific recombination proposed that the mechanism involved the formation of double-strand breaks at the attachment sites which were then rejoined to give the recombinant products (34, 35, 36). An alternative proposal was that recombination proceeds *via* a Holliday-structure intermediate; recombination between one pair of strands would form a Holliday-junction between the attachment sites, and a second strand exchange would convert this structure into the completed recombinant products. In this paper we have shown that Holliday-structures accumulate to significant levels *in vitro* when recombination is blocked by a phosphorothioate substitution in the bottom strand of attB. We also demonstrate that a low level of Holliday-structures is formed even in unblocked recombination reactions. Furthermore, we show that Holliday-structure formation depends on many of the same factors required for the complete recombination reaction and that the time course for the formation of Holliday-structures is compatible with their role as intermediates on the normal recombination pathway. We find that the variant Int protein known as Int-h generates higher levels of Holliday-structures than Int-wt; consequently, our strongest evidence that Holliday-structures are intermediates in phage λ recombination comes from data obtained using Int-h. Wherever possible, however, we have carried out parallel experiments with Int-wt and have always observed qualitatively similar results. We believe, therefore, that both Int-wt and Int-h, working in the presence of IHF, use a recombination mechanism involving Holliday-structure intermediates. Recently, Landy and coworkers have found that attBs containing a nick at one Int cleavage site also give high levels of Holliday-structures when recombined *in vitro* (16). Nash *et al.* (37) have reported experiments using a very different approach that also argue against the double-strand break mechanism for recombination. Although there can now be little doubt that phage λ site-specific recombination

proceeds *via* two independent strand exchanges which make and then resolve a Holliday-structure intermediate, in normal recombination reactions such intermediates must be short lived since under these conditions they are present at very low levels.

In Fig. 7, we present a plausible scheme for a recombination pathway involving a Holliday-structure intermediate. In this scheme, the first step is formation of a synaptic structure within which the cores of *attP* and *attB* are juxtaposed. We imagine that this structure is built upon a scaffold of Int and IHF protomers bound to the arms of *attP* (38, 39, 40); hence the first strand exchange will generate a Holliday-structure which is also part of a protein:DNA complex (step 2). Subsequent exchange of the unrecombined strands will yield a protein:DNA complex containing *attL* and *attR* (step 3); we imagine that, in the absence of Xis, dissociation of this complex (step 4) is irreversible. We believe that the complex containing the Holliday-structure is a very reactive intermediate and does not normally accumulate because both the forward rate (step 3) and backward rate (step -2) are fast; even when the second strand exchange (step 3) is inhibited, Holliday-structures do not accumulate to high levels because they are efficiently resolved back to substrates (step -2). The high level of Holliday-structures that we observe in our best case appears to result from a fortunate conjunction between the effects of Int-h and of phosphorothioate substitution.

The rationale for the accumulation of Holliday structures with substrates that contain phosphorothioates is straight-forward: the sulfur substituent simply interferes with k_3 . Similar effects can be seen when second strand exchange is blocked by non-homology (15) or by an artificially introduced nick (16). In the latter case, the resulting nicked Holliday structure accumulates to very high levels, suggesting that the k_2 process is also blocked. Why should Int-h yield higher levels of the intermediate? Int-h generates higher levels of Holliday-structures than Int-wt in both blocked and unblocked recombination reactions, therefore the alteration in Int-h must affect at least one step that precedes the second strand exchange. A plausible explanation of our results is that Int-h makes higher levels of the intermediate because the complex between Holliday-structure complex involving Int-h dissociates more readily than does the Int-wt complex (step 5 in Fig. 7) and that free Holliday-structures are more stable than the protein bound forms. A mechanism similar to this has been proposed to account for the observation that three variants of the Cre recombinase generate elevated levels of Holliday-structures during *in vitro* recombination reactions (41). However, we do not favour this explanation for our results because the yield of Holliday-structures is very sensitive to the method used to stop the recombination reaction; the maximum yield of Holliday-structures is only obtained when recombination is terminated rapidly using harsh protein denaturants (P. Kitts, unpublished observations). This suggests that in the recombination mixture the Holliday-structures are part of a protein:DNA complex rather than being free in solution. An alternative explanation, and one which might also explain why Int-h does better than Int-wt under various suboptimal recombination conditions (28, 30), is that Int-h makes stronger protein:protein or protein:DNA interactions than Int-wt, i.e., protein:DNA complexes involving Int-h are more stable than those involving Int-wt. (In the scheme presented in Fig. 7, this would mean that the rate constants k_1 and k_4 for Int-h would be smaller than the corresponding rate constants for Int-wt). Recombination mediated by Int-h would therefore accumulate higher levels of all the protein:DNA complexes, including Holliday-structure intermediates. This scheme predicts little effect of Int-h on the overall recombination reaction rate

in agreement with previous observations (30), because our assays do not distinguish between *attL* and *attR* that are complexed with protein (LR^{*}) and free recombinant products (L+R). It is known that Int-h differs from Int-wt by a single amino-acid substitution which alters the charge on the protein (42) and this might well affect the interactions of Int with other proteins or with DNA. If Int-h does indeed make tighter protein:DNA complexes, then this might be observed using the Intasome assay developed by Richet *et al.* (39). A final explanation for the effect of Int-h on Holliday structures invokes the possibility that Int-h affects branch migration. We have suggested before (15) that the Holliday structure intermediate is prone to reversal and escapes this fate only if branch migration moves the joint away from the Int protomers that formed it. Int-h might reduce k_2 by promoting such branch migration. To be consistent with the lack of a dramatic effect of Int-h on the overall speed of recombination (30), one might postulate that branch migration promoted by Int-h slows down after moving a few nucleotides, so that k_3 is also reduced. Measuring the effect of Int-h on the kinetics of resolution of artificially formed Holliday structures should provide a test of this explanation.

The site-specific recombination systems involving the Cre and FLP proteins appear to be distantly related to the phage λ system (43) and more closely related to one another (44, 45). There is evidence that recombination mediated by the Cre protein, like that promoted by Int, proceeds *via* a Holliday-structure intermediate (41, 46) and it seems likely that FLP mediated recombination also involves single-strand exchanges. On the other hand, the family of site-specific DNA resolvases and DNA inverting enzymes may use double-strand breakage for strand exchange. In these systems, the change in linking number that accompanies recombination (47, 48, 49) is consistent with double-strand breaks, as is the accumulation of minor species from iterative rounds of recombination (49, 50). Phosphorothioate-substituted derivatives of the substrates for these recombination systems could be made using our methods and might prove to be valuable tools for investigating the mechanism of recombination in these systems.

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REFERENCES

1. Weisberg, R.A. and Landy, A. (1983) In Hendrix, R.W., Roberts, J.W., Stahl, F.W. and Weisberg, R.A. (eds.), *Lambda II*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, pp. 211-250.
2. Sadowski, P. (1986) *J. Bacteriol.* **165**, 341-347.
3. Ross, W. and Landy, A. (1983) *Cell* **33**, 261-272.
4. Craig, N.L. and Nash, H.A. (1983) *Cell* **35**, 795-803.
5. Kikuchi, Y. and Nash, H.A. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 3760-3764.

6. Kitts, P., Richet, E. and Nash, H.A. (1984) Cold Spring Harbor Symp. Quant. Biol. **49**, 735-744.
7. Holliday, R. (1964) Genet. Res. **5**, 282-304.
8. Echols, H. and Green, L. (1979) Genetics **93**, 297-307.
9. Enquist, L.W., Nash, H. and Weisberg, R.A. (1979) Proc. Natl. Acad. Sci. USA **76**, 1363-1367.
10. Hsu, P.-L. and Landy, A. (1984) Nature **311**, 721-726.
11. Nash, H.A. and Robertson, C.A. (1981) J. Biol. Chem. **256**, 9246-9253.
12. Pollock, T.J., and Nash, H.A. (1983) In Chater, K.F., Cullis, C.A., Hopwood, D.A., Johnston, A.A.W.B. and Woolhouse, H.W. (eds.), Genetic Rearrangement, Croom Helm, London, pp. 41-57.
13. Nash, H.A. and Pollock, T.J. (1983) J. Mol. Biol. **170**, 19-38.
14. Eckstein, F. (1985) Ann. Rev. Biochem. **54**, 367-402.
15. Kitts, P.A. and Nash, H.A. (1987) Nature **329**, 346-348.
16. Nunes-Düby, S.E., Matsumoto, L. and Landy, A. (1987) Cell **50**, 779-788.
17. Leach, D. (1987) Nature **329**, 290-291.
18. Nash, H.A. (1983) Methods Enzymol. **100**, 210-216.
19. Mizuuchi, M. and Mizuuchi, K. (1985) Nucleic Acids Res. **13**, 1193-1208.
20. Hsu, P.-L., Ross, W. and Landy, A. (1980) Nature **285**, 85-91.
21. Messing, J. (1983) Methods Enzymol. **101**, 20-78.
22. Been, M.D. and Champoux, J.J. (1983) Methods Enzymol. **101**, 90-98.
23. Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning, a Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
24. Zinder, N.D. and Boeke, J.D. (1982) Gene **19**, 1-10.
25. Hunkapiller, M.W., Lujan, E., Ostrander, F. and Hood, L.E. (1983) Methods Enzymol. **91**, 227-236.
26. Maxam, A.M. and Gilbert, W. (1980) Methods Enzymol. **65**, 499-560.
27. Burgers, P.M.J. and Eckstein, F. (1978) Proc. Natl. Acad. Sci. USA **75**, 4798-4800.
28. Miller, H.I., Mozola, M.A. and Friedman, D.I. (1980) Cell **20**, 721-729.
29. Gardner, J.F. and Nash, H.A. (1986) J. Mol. Biol. **191**, 181-189.
30. Lange-Gustafson, B.J. and Nash, H.A. (1984) J. Biol. Chem. **259**, 12724-12732.
31. Siebenlist, U. and Gilbert, W. (1980) **77**, 122-126.
32. Jovin, T.M., van de Sande, J.H., Zarling, D.A., Arndt-Jovin, D.J., Eckstein, F., Földner, H.H., Greider, C., Grieger, I., Hamori, E., Kalisch, B., McIntosh, L.P. and Robert-Nicoud, M. (1983) Cold Spring Harbor Symp. Quant. Biol. **47**, 143-154.
33. Cosstick, R. and Eckstein, F. (1985) Biochemistry **24**, 3630-3638.
34. Signer, E.R. and Weil, J. (1968) Cold Spring Harbor Symp. Quant. Biol. **33**, 715-719.
35. Kaiser, A.D. and Wu, R. (1968) Cold Spring Harbor Symp. Quant. Biol. **33**, 729-734.
36. Shulman, M. and Gottesman, M. (1973) J. Mol. Biol. **81**, 461-482.
37. Nash, H.A., Bauer, C.E. and Gardner, J.F. (1987) Proc. Natl. Acad. Sci. USA **84**, 4049-4053.
38. Better, M., Lu, C., Williams, R.C. and Echols, H. (1982) Proc. Natl. Acad. Sci. USA **79**, 5837-5841.
39. Richet, E., Abcarian, P. and Nash, H.A. (1986) Cell **46**, 1011-1021.
40. Richet, E., Abcarian, P. and Nash, H.A. (1988) Cell **52**, 9-17.
41. Hoess, R., Wierzbicki, A. and Abremski, K. (1987) Proc. Natl. Acad. Sci. USA **84**, 6840-6844.
42. Bear, S.E., Clemens, J.B., Enquist, L.W. and Zagursky, R.J. (1987) J. Bacteriol. **169**, 5880-5883.
43. Argos, P., Landy, A., Abremski, K., Egan, J.B., Haggard-Ljungquist, E., Hoess, R.H., Kahn, M.L., Kalionis, B., Narayana, S.V.L., Pierson, L.S., III, Sternberg, N. and Leong, J.M. (1986) EMBO J. **5**, 433-440.
44. Hoess, R.H. and Abremski, K. (1984) Proc. Natl. Acad. Sci. USA **81**, 1026-1029.
45. Meyer-Leon, L., Senecoff, J.F., Bruckner, R.C. and Cox, M.M. (1984) Cold Spring Harbor Symp. Quant. Biol. **49**, 797-804.
46. Sternberg, N. (1981) J. Mol. Biol. **150**, 603-608.

47. Boocook, M.R., Brown, J.L. and Sherratt, D.J. (1987) In McMacken, R. and Kelly, T.J. (eds.), DNA replication and recombination, Alan R. Liss, Inc., New York, pp 703-718.
48. Kahmann, R., Mertens, G., Klippel, A., Bräuer, B., Rudt, F. and Koch, C. (1987) In McMacken, R. and Kelly, T.J. (eds.), DNA replication and recombination, Alan R. Liss, Inc., New York, pp 681-690.
49. Kanaar, R., van de Putte, P. and Cozzarelli, N.R. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 752-756.
50. Wasserman, S.A., Dungan, J.M. and Cozzarelli, N.R. (1985) *Science* **229**, 171-174.