Analysis of Higher Order Intermediates and Synapsis in the Bent-L Pathway of Bacteriophage λ Site-specific Recombination*

(Received for publication, April 29, 1998, and in revised form, July 8, 1998)

Anca Mara Segall‡

From the Department of Biology and Molecular Biology Institute, San Diego State University, San Diego, California 92182-4614

The integrase protein of bacteriophage λ mediates recombination via four distinct pathways. The recent in vivo reconstitution of the efficient bidirectional reaction between two attLtenP1 target sites now allows comparisons of this pathway, known as the bent-L pathway, with the inefficient bidirectional straight-L pathway and with the efficient but unidirectional pathways of integration and excision. To this end, a series of higher order intermediates of the bent-L pathway was characterized using gel mobility shift assays, two-dimensional gel analysis, and footprinting. The analysis spans the initial binding of proteins to individual DNA target sites, synapsis of two partner DNA targets, and strand exchange. This study identifies a presynaptic “checkpoint” of recombination. It shows that synapsis is a slow step in the recombination reaction, while subsequent strand exchange is comparatively fast. Synaptic complexes contain a preponderance of recombinant products, suggesting that an energetically favorable but somewhat subtle conformational change drives strand exchange. In addition, comparison of wild-type integrase with a catalytically defective mutant of integrase, IntF, showed that, in addition to the synthesis defect, this mutant has different DNA-binding properties than the wild-type protein.

Site-specific recombination is ubiquitous in nature. It is involved in faithful segregation of bacterial, plasmid, and chromosome loci in cell division, control of gene expression, and in generating genetic diversity. Many features of the mechanism and the control of recombination are still largely unknown. For example, we do not yet fully understand the determinants of recombination efficiency at the molecular level.

Bacteriophage λ site-specific recombination (SSR) has served as a paradigm for site-specific recombination reactions since its reconstitution in vitro (reviewed in Refs. 1 and 2). The recombinase integrase (Int) binds to specific loci, att sites, where it cuts and reseals DNA strands, resulting either in the joining or the separation of the phage λ and the Escherichia coli genomes. Int carries out recombination via four distinct pathways, aided by up to three accessory factors (3). Int has two specific DNA binding domains, one of which is also the catalytic domain (4, 5). The catalytic domain binds to the core sequences present in all att sites, where it performs strand exchange. An N-terminal DNA binding domain contacts the arm sequences which are present in the phage recombination locus attP and in the two loci that flank the prophage, attL and attR. Arm binding is more stable than core binding and serves to deliver the catalytic domain to the regions where strand exchange takes place. The chemistry of recombination takes place in synaptic complexes in which the two substrates are held together by Int, together with the aid of the accessory proteins (6–9). However, these complexes have been relatively elusive, and their formation and geometry are poorly understood for the λ SSR reactions. Our understanding of the overall pathway of recombination is also incomplete; in particular, the rate-limiting step of the reaction has not been identified.

Recently, two related pathways of λ site-specific recombination, named straight-L and bent-L recombination, have been reconstituted in vitro (3, 8). Both pathways occur in vivo (3, 10, 11) and nominally use the same recombination targets, two attL sites (Fig. 1). These pathways are also similar to each other in that, unlike integration or excision, they are bidirectional; the structure of the recombination products is the same as that of the substrates. However, the straight-L pathway is inhibited by IHF and is stimulated by Int binding at the core-proximal P1 arm binding site, while the bent-L pathway requires the IHF protein and, in vitro, is inhibited by binding of Int to the P1 arm site (Fig. 1). Thus, the nucleoprotein intermediates assembled in the two pathways differ significantly (3). The two pathways also differ in their efficiencies; both in vitro and in vivo, the straight-L pathway converts only 5–10% of substrates into products, while the bent-L pathway converts up to 50–60% of substrates into products. Thus, the efficiency of the bent-L pathway resembles that of excusive and integrative recombination. While straight-L recombination is arguably the simplest pathway mediated by Int, requiring only target DNA and Int, bent-L recombination appears to be the least stringent, “default” pathway; many mutants of Int that cannot perform integration or excision can still carry out bent-L recombination (3). The bent-L pathway can therefore be used to identify the features of Int required for catalysis and allows separation of these features from those required for determining directional recombination. Furthermore, comparing these two pathways will give insight into what factors affect the efficiency of recombination via more complex reactions.

In order to compare the two pathways, a detailed characterization of the protein-DNA intermediates of the bent-L pathway is necessary. This work describes the process of substrates and proteins through several higher order complexes, including a synaptic intermediate in which the paired DNA substrates are held together by Int and IHF. The synaptic
Figure 1. Comparison of the bent-L and straight-L pathways of recombination. The B and C sites bind the catalytic domain of Int, while the P1, P2, and P3 sites bind the arm domain of Int. Three base substitution mutations, known collectively as tenP1, lower the affinity of Int for the P1 site (14). These three mutations are necessary for bent-L recombination in vitro but not in vivo (3). IHF binds to the H site.

The hallmark of a synaptic complex is that its electrophoretic mobility depends on the size of two DNA substrates rather than one, since the two DNA molecules are held together noncovalently by Int (and appropriate other host factors). Indeed, the slower complexes in Fig. 2, lanes 4 and 5, fit this definition of a synaptic complex. In lane 5, this same complex contains two labeled att sites; in lane 5, this same complex forms, but an even slower complex is present and consists of unlabeled 496-bp DNA with 4 attL sites, while the latter contains both labeled sites and a 4-fold excess of unlabeled 496-bp DNA with 4 attL sites. The faster two protein-DNA intermediates, labeled IHF and UMC1, do not shift their mobility in the presence of the unlabeled att site, and thus probably contain a single DNA molecule. The same is true for the intermediate labeled UMC2 in lanes 6 and 9–11 (e.g. compare with lanes 3–12 in Fig. 3, in which only the labeled att site is present). Note that the UMC2 is more prominent at lower temperatures or in the absence of spermidine at 37°C. The fastest moving complex consists of a DNA-IHF complex (it is assembled in the presence of DNA and IHF only; e.g. Fig. 3, lane 19), while the UMC1 and UMC2 complexes require both IHF and Int.

The analysis of intermediate complexes in Phage λ Bent-L Recombination 24593

Identification of Protein-DNA Complexes, Including a Synaptic Complex, in the Bent-L Pathway—In order to understand differences between the bent-L pathway and the other pathways of λ SSR, I characterized the higher order protein-DNA complexes which serve as intermediates in the bent-L pathway. Reactions containing labeled attLtenP1 DNA and Int or Int and IHF were assembled and analyzed using electrophoretic mobility shift assays (Fig. 2). The reactions in lanes 4 and 5 differ in that the former contains only labeled 187-bp att sites, whereas the latter contains both labeled sites and a 4-fold excess of unlabeled 496-bp DNA with attLtenP1 sequences. The faster two protein-DNA intermediates, labeled IHF and UMC1, do not shift their mobility in the presence of the unlabeled att site, and thus probably contain a single DNA molecule. The same is true for the intermediate labeled UMC2 in lanes 6 and 9–11 (e.g. compare with lanes 3–12 in Fig. 3, in which only the labeled att site is present). Note that the UMC2 is more prominent at lower temperatures or in the absence of spermidine at 37 °C. The fastest moving complex consists of a DNA-IHF complex (it is assembled in the presence of DNA and IHF only; e.g. Fig. 3, lane 19), while the UMC1 and UMC2 complexes require both IHF and Int.

ExPERIMENTAL PROCEDURES

DNA Substrates—All DNA substrates were synthesized using 30 cycles of a polynucleotide chain reaction with VENT polymerase (New England Biolabs). The short (187 bp) labeled substrate spans coordinates 69 to +110 of attL sequence (12). In addition, the primers contain EagI restriction sites not present in the template sequence (8). The longer (496 bp) unlabeled fragments span coordinates −250 to +246 in the attL sequence. The templates used for polymerase chain reactions contained either the attL* site (pHNS72) (13) or the attLtenP1 site (pTN122) (14). The ten mutations change the P1 arm binding site from AGGCTCAGT to AGGGCTCAGT. The template plasmids were the generous gifts of Howard Nash and Jeffrey Gardner, respectively.

Gel Mobility Shift and in Vitro Recombination Assays—Standard reactions were performed in a volume of 20 μl and contained between 0.5 and 4 nM labeled att site, as specified in the figure legends, or 0.5–2 nM labeled att site in addition to 2 nM unlabeled att site when recombination was assayed. Reactions also contained 0.67 μM of sonicated salmon sperm DNA as a nonspecific competitor, 44 mM Tris-Cl (pH 8.0), 60 mM KCl, 0.05 mM MgCl2, 0.15 μM bovine serum albumin, 11 μM Tris borate (pH 8.9), 1 mM EDTA, and 13.6% (v/v) glycerol. Spermidine (5 mM) was present unless specified otherwise. Unless otherwise noted, Int was present at 50 nM and IHF at 35 nM. These proteins were purified and generously supplied by Carol Robertson, Shuwei Yang, and Howard Nash.

Gel mobility shift reactions were incubated for the specified length of time (generally 90 min) at 37 °C and layered without loading dyes onto 5% native polyacrylamide, 0.5× Tris/borate EDTA gels (29:1 acrylamide:bisacrylamide). Depending on gel size, electrophoresis was performed at 165 V or 245–325 V at 4–6 °C. Electrophoresis at room temperature shows fewer synaptic complexes and more “stroking” of radioactivity below the synaptic complexes toward complexes containing single DNA molecules. The streaks consist of recombinant products as well as uncombined substrate (e.g. see Fig. 6).

Recombination reactions were incubated for the specified length of time (usually 90 min) at 37 °C (unless otherwise indicated). Reactions were then stopped by the addition of 5 μl of 10% SDS with a little bromphenol blue dye, layered onto 5% polyacrylamide Tris/SDS gels, and electrophoresed in Tris/Tricine/SDS buffer (15) at 100 mA constant current for 5 h.

Both electrophoretic mobility shift and recombination assays were quantitated after drying gels and exposing them to a phosphor screen. Radioactive counts in relevant bands were measured using a Molecular Dynamics PhosphorImager.

Two-dimensional Gel Analysis—Mobility shift reactions were assembled and electrophoresed as described above. Gels were exposed wet to x-ray film for 1 h or more at room temperature or 4 °C, then appropriate lanes were cut from the gel and bathed in SDS/bromphenol blue loading dye for a few minutes. Lanes were then inserted into a large well of a Tris/SDS gel and electrophoresed at 100 mA constant current for 6 h, as above. The gel was then dried, exposed to a phosphor screen, and visualized with the Molecular Dynamics instrument.

Footprinting Analyses—Copper phenanthroline and dimethyl sulfate footprinting were done as described (3). Footprinting with 5-phenyl-1,10-copper phenanthroline (50CuOP) was carried out as described by Mazumder (16). In all cases, free DNA was separated from the various protein-bound species using gel electrophoresis; since this involves extraction from acrylamide gels, reactions were doubled and loaded two per gel lane so that each band contained DNA equivalent to four reactions, to make up for the large amount of DNA lost during the extraction process. In the case of the phenanthroline reagents, gel shift analysis was performed as usual; the wet gels were immersed in footprinting and stop solutions, then exposed to x-ray film (17). The appropriate gel slices were cut from the gel and DNA was extracted by soaking in Tris EDTA or 0.4 M ammonium acetate, 1 mM EDTA over-night at 37 °C in a shaker incubator. Dimethyl sulfate footprinting was carried out in solution, but the reaction was followed by gel electrophoresis in order to separate free and protein-bound DNA species. The DNA representing specific complexes was isolated from the gel slices and treated with NaOH for G → A cleavage (18). All footprinting reactions were electrophoresed through 6% polyacrylamide (19:1 acrylamide:bisacrylamide), 7 M urea gels. The gels were dried and exposed to film (Kodak XAR), and the resulting autoradiographs were scanned on a flatbed scanner into Adobe Photoshop. Results

Identification of Protein-DNA Complexes, Including a Synaptic Complex, in the Bent-L Pathway—In order to understand differences between the bent-L pathway and the other pathways of λ SSR, I characterized the higher order protein-DNA complexes which serve as intermediates in the bent-L pathway. Reactions containing labeled attLtenP1 DNA and Int or Int and IHF were assembled and analyzed using electrophoretic mobility shift assays (Fig. 2). The reactions in lanes 4 and 5 differ in that the former contains only labeled 187-bp att sites, whereas the latter contains both labeled sites and a 4-fold excess of unlabeled 496-bp DNA with attLtenP1 sequences. The faster two protein-DNA intermediates, labeled IHF and UMC1, do not shift their mobility in the presence of the unlabeled att site, and thus probably contain a single DNA molecule. The same is true for the intermediate labeled UMC2 in lanes 6 and 9–11 (e.g. compare with lanes 3–12 in Fig. 3, in which only the labeled att site is present). Note that the UMC2 is more prominent at lower temperatures or in the absence of spermidine at 37 °C. The fastest moving complex consists of a DNA-IHF complex (it is assembled in the presence of DNA and IHF only; e.g. Fig. 3, lane 19), while the UMC1 and UMC2 complexes require both IHF and Int.
formation of the synaptic complex was investigated. λ SSR is greatly inhibited in the absence of spermidine or at 0 °C (e.g. Fig. 2, lower panel, lanes 3, 6, and 11). Note that recombination cannot be seen in lanes 1 and 4 because the substrates and the products in these reactions are the same size. While the SL-BMC forms both in conditions which do and in conditions which do not support recombination (Fig. 2, lane 2 versus lane 3) (8), the BL-BMC is assembled to a significant extent only in recombination-permissive conditions (Fig. 2). MgCl₂ can substitute for spermidine to a significant extent both for recombination and for BL-BMC formation (Fig. 2, lanes 7 and 8). Both bent-L recombination and the BL-BMC share temperature optima at 30–37 °C (Fig. 2, lanes 5 and 9–11). In contrast, integration, straight-L, and even the more temperature-resistant excision pathways yield more products at 25 °C than at 37 °C in vitro (Ref. 3; reviewed in Landy and Weisberg (12)). The IntF protein forms BL-BMC with much lower efficiency than does the wild-type protein (see below). All of these observations suggest that efficient assembly of the BL-BMC requires recombination-permissive conditions.

The time course of forming both unimolecular and bimolecular intermediates was investigated next. At both 0 and 37 °C, the wild-type protein assembles UMC1 and UMC2 within 5 min (Fig. 3, lanes 2 and 12). In the case of wild-type Int at 37 °C, the UMC2 intermediates gradually disappear and are replaced by BL-BMC intermediates, suggesting that UMC2s are precursors of BL-BMCs. The same inverse relationship between UMC2 and BL-BMC can be seen when reactions are incubated at 0, 25, 30, and 37 °C (Fig. 2); the fewer BL-BMCs assemble, the more UMC2s accumulate. At 0 °C, the conversion of UMC2 into BL-BMC occurs rarely (Fig. 3); the small amount of BL-BMC formed within 5 min does not increase significantly with time. Since very little recombination occurs at 0 °C (<3%) and only a small amount of BL-BMCs assemble at 0 °C, it is simplest to believe that the BL-BMCs present at 37 °C after 15–30 min have undergone recombination. To test whether BL-BMCs form before or after recombination, the ability of IntF to form BL-BMCs was explored. As seen in Fig. 3, this mutant protein does not assemble BL-BMCs as efficiently as the Int⁺ protein, although the BL-BMCs do accumulate

S. D. Goodman and H. A. Nash, personal communication.
slightly with time. While BL-BMCs can form in the absence of strand exchange, the IntF data agrees with the previous observation that conditions which favor strand exchange also favor BL-BMC accumulation.

The Stability of Intermediates Formed by Int\(^+\) and IntF—The mutant protein does not accumulate any UMC2 species and releases increasing amounts of DNA-IHF complexes with time (Fig. 3). Two possibilities exist; IntF protein may either not form the UMC2 at all, or it may form less stable UMC2 than Int\(^+\). The stability of all of the complexes was tested by allowing Int to form complexes for 90 min, then adding 10-fold excess of specific competitor DNA to reactions and incubating for 10 min before loading (Fig. 4, lanes 4, 7, 10, and 13). As a control, competitor DNA was also added before adding Int (Fig. 4, lanes 3, 6, 9, and 12). The Int\(^+\) and IntF proteins were compared at both 0 °C and 37 °C. Several conclusions can be drawn. First, the BL-BMC complex, once formed, is stable to challenge by specific competitor DNA to reactions and incubating for 10 min before loading (Fig. 4, lanes 4, 7, 10, and 13). As a control, competitor DNA was also added before adding Int (Fig. 4, lanes 3, 6, 9, and 12). The Int\(^+\) and IntF proteins were compared at both 0 °C and 37 °C. Several conclusions can be drawn. First, the BL-BMC complex, once formed, is stable to challenge by specific competitor DNA to reactions and incubating for 10 min before loading (Fig. 4, lanes 4, 7, 10, and 13). As a control, competitor DNA was also added before adding Int (Fig. 4, lanes 3, 6, 9, and 12).

Are UMC1 Complexes Precursors to UMC2 Complexes?—In order to investigate further the relationship of the various complexes to each other, the effect of Int concentration on assembly of UMC1, UMC2, and BL-BMC complexes was tested (Fig. 5). At limiting Int concentrations, IHF-DNA and UMC1 complexes predominate. As the Int concentration is increased at 0 °C, more UMC2 species appear at the expense of UMC1. At 37 °C, where recombination is efficient, BL-BMCs accumulate instead of UMC2 species. The UMC2 complexes can and do accumulate at 37 °C, but only in the absence of significant recombination (Fig. 2, lane 6). The reactions with Int\(^+\) protein suggest most clearly that UMC1 precedes UMC2, which in turn precedes BL-BMC complexes. The same trend, although much less pronounced, is seen for the IntF protein. Differences between Int\(^+\) and IntF are seen in the assembly of all three complexes, especially those of UMC2 and the BL-BMC (Figs. 3–5). This suggests that the hydroxyl group of tyrosine 342 may stabilize important protein-DNA interactions in the bent-L intermediates.

Direct Demonstration That the BL-BMC Is a Recombination Intermediate—Since the BL-BMCs accumulate best in conditions which favor recombination, the relationship between recombinant products and BL-BMCs was explored directly. Two-dimensional gel electrophoresis was performed to determine whether recombinant products were associated with any of the higher order complexes described above. Reactions containing Int, IHF, and attLtenP\(^+\) sites of two different lengths were assembled and electrophoresed in the first dimension through a native polyacrylamide gel. The appropriate lane was excised from the gel, loaded on top of a SDS-containing gel, and electrophoresed in the second dimension (Fig. 6). All of the recombinant products are found at the position of the BL-BMC formed between the labeled short site and the unlabeled long site (the position marked with a double asterisk). Quantitation shows that this BL-BMC is composed mostly of recombinant products (83%) and relatively few substrate molecules (17%). In agreement with data showing that efficient formation of BL-BMC requires recombination-permissive conditions, this experi-
of BL-BMCs encompasses two steps, synopsis prior to strand exchange and strand exchange. Thus, it is important to distinguish whether strand exchange itself is fast or slow relative to synopsis. This was again tested by two-dimensional gel analysis; reactions incubated for 5, 15, 30, and 60 min were first electrophoresed using native polyacrylamide gel electrophoresis; individual lanes were then excised and electrophoresed through SDS-containing gels to measure the percentage of substrates and recombinant products associated with the respective BMCs formed. The results (Table I) indicate that the low percentage of BL-BMCs formed after 5 min already contain about 50% recombinant products, and nearly 80% after 15 min incubation. These data clearly show that the rate of strand exchange is fast relative to synopsis and suggest that synopsis prior to strand exchange may be the rate-limiting step in the bent-L reaction among the steps described in this work.

**Footprinting Analysis of Intermediates in the Bent-L Pathway and Comparison with SL-BMC Complexes**—The protein and DNA site requirements of the bent-L pathway predict that it depends both on bending by IHF at the \( H^+ \) site and on the absence of Int from the P'1 site (3). The actual configuration of individual protein-DNA intermediates was confirmed in this work by DNA footprinting analysis of specific complexes isolated by gel electrophoresis. In order to maximize the number of synaptic complexes available for analysis, they were assembled at their respective optimal temperatures, 25 °C for SL-BMCs and 37 °C for BL-BMC. “In gel” copper phenanthroline footprinting was performed as described previously (8, 16, 17) using both 1,10-phenanthroline copper (CuOP) and 50CuOP. The 50CuOP agent is reported to cut single-stranded regions of DNA more efficiently than does CuOP, as well as cutting distorted double-stranded DNA such as is found at the leading edge of transcription complexes (19). The top strand footprints of the att site with 50CuOP reveal that IHF is indeed present in the UMC1, UMC2, and BL-BMC complexes (Fig. 7A; a summary of the footprinting data is presented in Table II). The BL-BMC complex also shows enhanced cleavage at the −2 coordinate of the att core, which is the position of top strand cleavage. However, no strong Int footprints were seen on the arm binding sites. In contrast, bottom strand footprints with 50CuOP both confirm the presence of IHF at \( H^+ \) in UMC1, UMC2, and BL-BMC complexes and show strong Int footprints at the P’2 and P’3 arm sites for BL-BMC and UMC2 complexes (Fig. 7B and data not shown). As predicted, the P’1 arm site is free in all the \( attLtenP'1 \) intermediates but is occupied in the SL-BMC. The +4 position in the core, where bottom strand cleavage occurs, shows somewhat enhanced cleavage in the BL-BMC formed by Int” at 37 °C (Fig. 7C); this enhanced cleavage is not seen in the BL-BMCs formed with Int” at 0 °C, with IntF at 37 °C, or in the UMC1 or UMC2 species. It is also not seen in the SL-BMC. Other subtle differences in susceptibility to cleavage, for example at position 0 in the core, must reflect slight changes in the conformation of the protein-DNA intermediates. Footprints obtained with CuOP show the same

**Analysis of Intermidates in Phage \( \lambda \) Bent-L Recombination**

FIG. 6. Two-dimensional electrophoretic analysis of a bent-L recombination reactions. The reactions contains 2 nM labeled and 2 nm unlabeled \( attLtenP'1 \) sites. The former is 187 bp long, the latter is 496 bp long. A reaction containing Int” and incubated at 37 °C is shown. In the first dimension, reactions incubated 90 min were electrophoresed in a native polyacrylamide gel. The appropriate lane was then excised and layered onto a Tris/Tricine/SDS gel and electrophoresed in the second dimension. The position of protein-DNA intermediates in the first dimension is marked along the bottom of the gel (the BL-BMC marked with a single asterisk contains two short substrates, while the one marked with a double asterisk contains a short and a long substrate). The position of free substrate and the two recombinant products is marked along the side of the gel. As electrophoresis proceeds in the first dimension, BMC complexes fall apart, and the single-DNA complexes and in the two BL-BMCs (Fig. 6; the faster BL-BMC is found at four distinct positions, in the UMC1 and DNA-IHF arm binding sites. In contrast, bottom strand footprints with the IntF protein; if so, the small amount of BL-BMCs assembled in these conditions could also represent post-strand exchange complexes rather than pre-strand exchange complexes. Two-dimensional analysis was performed on reactions with Int” at 0 °C, and with IntP at both 0 and 37 °C. As in Fig. 2, the 0 °C reaction with wild-type Int indeed shows formation of a small proportion of BL-BMCs, which are associated with recombinant products (data not shown). IntF also assembled BL-BMC species (Figs. 3–5) but, as expected, without any associated recombinant products (data not shown). Thus BL-BMCs indeed can form in the complete absence of strand exchange, although strand exchange leads to greater accumulation of these complexes.

**TABLE I**

| Incubation time (min) | Recombinant products in the BL-BMC (%) |
|----------------------|---------------------------------------|
| 5                    | 52.0 ± 1.7                            |
| 15                   | 75.4 ± 2.1                            |
| 30                   | 79.3 ± 0.2                            |
| 60                   | 85.4 ± 3.2                            |

**Synopsis Appears to Be the Slow Step in Bent-L Recombination**—As shown in Fig. 3, the rate of BL-BMC formation is slow compared with formation of UMC1 and UMC2. The appearance of BL-BMCs encompasses two steps, synopsis prior to strand exchange and strand exchange. Thus, it is important to distinguish whether strand exchange itself is fast or slow relative to synopsis. This was again tested by two-dimensional gel analysis; reactions incubated for 5, 15, 30, and 60 min were first electrophoresed using native polyacrylamide gel electrophoresis; individual lanes were then excised and electrophoresed through SDS-containing gels to measure the percentage of substrates and recombinant products associated with the respective BMCs formed. The results (Table I) indicate that the low percentage of BL-BMCs formed after 5 min already contain about 50% recombinant products, and nearly 80% after 15 min incubation. These data clearly show that the rate of strand exchange is fast relative to synopsis and suggest that synopsis prior to strand exchange may be the rate-limiting step in the bent-L reaction among the steps described in this work.

| Incubation time (min) | Recombinant products in the BL-BMC (%) |
|----------------------|---------------------------------------|
| 5                    | 52.0 ± 1.7                            |
| 15                   | 75.4 ± 2.1                            |
| 30                   | 79.3 ± 0.2                            |
| 60                   | 85.4 ± 3.2                            |

**Footprinting Analysis of Intermediates in the Bent-L Pathway and Comparison with SL-BMC Complexes**—The protein and DNA site requirements of the bent-L pathway predict that it depends both on bending by IHF at the \( H^+ \) site and on the absence of Int from the P’1 site (3). The actual configuration of individual protein-DNA intermediates was confirmed in this work by DNA footprinting analysis of specific complexes isolated by gel electrophoresis. In order to maximize the number of synaptic complexes available for analysis, they were assembled at their respective optimal temperatures, 25 °C for SL-BMCs and 37 °C for BL-BMC. “In gel” copper phenanthroline footprinting was performed as described previously (8, 16, 17) using both 1,10-phenanthroline copper (CuOP) and 50CuOP. The 50CuOP agent is reported to cut single-stranded regions of DNA more efficiently than does CuOP, as well as cutting distorted double-stranded DNA such as is found at the leading edge of transcription complexes (19). The top strand footprints of the att site with 50CuOP reveal that IHF is indeed present in the UMC1, UMC2, and BL-BMC complexes (Fig. 7A; a summary of the footprinting data is presented in Table II). The BL-BMC complex also shows enhanced cleavage at the −2 coordinate of the att core, which is the position of top strand cleavage. However, no strong Int footprints were seen on the arm binding sites. In contrast, bottom strand footprints with 50CuOP both confirm the presence of IHF at \( H^+ \) in UMC1, UMC2, and BL-BMC complexes and show strong Int footprints at the P’2 and P’3 arm sites for BL-BMC and UMC2 complexes (Fig. 7B and data not shown). As predicted, the P’1 arm site is free in all the \( attLtenP'1 \) intermediates but is occupied in the SL-BMC. The +4 position in the core, where bottom strand cleavage occurs, shows somewhat enhanced cleavage in the BL-BMC formed by Int” at 37 °C (Fig. 7C); this enhanced cleavage is not seen in the BL-BMCs formed with Int” at 0 °C, with IntF at 37 °C, or in the UMC1 or UMC2 species. It is also not seen in the SL-BMC. Other subtle differences in susceptibility to cleavage, for example at position 0 in the core, must reflect slight changes in the conformation of the protein-DNA intermediates. Footprints obtained with CuOP show the same
pattern as those with 5ØCuOP, except at the $-2$ position of the top strand and at the $+4$ position of the bottom strand, where the DNA is more susceptible to 5ØCuOP cleavage (data not shown).

The hypersensitive sites may be due to the disjoining of Int-DNA covalent complexes by 5ØCuOP. All of the Int-containing intermediates are associated with a small amount of slow moving SDS-resistant complexes (seen only on long exposures). These complexes contain covalently bound Int protein attached to the labeled DNA molecules and are sensitive to treatment with protease K prior to electrophoresis (data not shown).

Footprinting with dimethyl sulfate was performed in order to better characterize binding of Int to its sites both on the top and bottom strands. The data (shown for the top strand only; Fig. 7D) shows that, while Int binds the B core site in the UMC1, UMC2, and BL-BMC intermediates, the C' core site is free in the UMC1 complex, partially or unstably occupied in the UMC2 complex, and fully occupied in the BL-BMC. Thus, stable occupancy of the C' core site is not achieved until synapsis has occurred.

**DISCUSSION**

The simplest model for the procession of recombination substrates through the bent-L pathway can be sketched as follows (summarized in Fig. 8). Int and IHF bind to an attLtenP site and first assemble the UMC1 and the UMC2 intermediate. Most likely, an additional Int molecule loads onto the UMC1 complex to generate the UMC2 intermediate (Fig. 5). An alternate possibility is that the two complexes are assembled independently, with the UMC1 being a dead-end complex. Both UMC1 and UMC2 have Int bound at the B core site (next to the locus of top strand cleavage), and at the P'2 and P'3 arm sites, as well as IHF at the H' site. However, UMC1 intermediates have the C' Int core site free, while UMC2 intermediates have Int unstably bound at C'. The UMC2 intermediates proceed to BL-BMC, although it is not clear whether synapsis occurs by interactions of UMC2 with free attLtenP1 substrates or with

---

**TABLE II**

| Complex | Protein binding sites |
|---------|-----------------------|
| LtenP1  | UMC1 Occ Free Occ Occ Occ Occ |
| LtenP1  | UMC2 Occ Free/occ Occ Occ Occ Occ |
| LtenP1  | BMC Occ Occ Free Occ Occ Occ |
| L'      | BMC Occ Occ Free Occ Occ Occ |

---

3 A. Segall, unpublished results.

---

**Fig. 7. Footprinting analysis of bent-L intermediates.** A (top strand), B, and C (bottom strands) show in gel footprinting reactions with 5ØCuOP. D shows the top strand dimethyl sulfate footprints of DNA isolated from individual complexes (see “Experimental Procedures”). Hypersensitive sites are present in the BL-BMC/37 °C lane at position $-2$ in A and in the same complex at position $+4$ in C. The attLtenP1 site is referred to as LP1.
Fig. 8. Model for the assembly and disassembly of protein-DNA intermediates in the bent-L pathway and their relation to recombination. The model summarizes all study data. A rough indication of reaction rates (fast or slow) is proposed where data are available. Disassembly of complexes reflects dissociation in the presence of specific competitor DNA.

attLtenP' substrates bound with Int. BL-BMCs, the pre- and post-strand exchange synaptic intermediates (see below for further discussion), consist of two DNA molecules held together by Int and IHF and have both core binding sites stably occupied. As strand exchange takes place between the two substrates, the BL-BMCs accumulate. The BL-BMCs disassemble via UMC1 intermediates into IHF-DNA complexes (seen on two-dimensional gels for the products; Fig. 2, lanes 5, 7, and 8, Fig. 6, and data not shown), and free proteins and DNA substrates. If UMC2 complexes are also formed during the disassembly process, they constitute a very transient stage.

Among the steps of the reaction delineated in this study, synapsis is the best candidate for the rate-limiting step. Assembly of the precursor intermediates containing a single DNA substrate is fast (essentially complete by 5 min at either 0 or 37 °C). Accumulation of BMCs comprises (at least) two steps in the bent-L pathway, synapsis (UMC2 → BL-BMCsubstrates in Fig. 8) and strand exchange (BL-BMCsubstrates → BL-BMCproducts). Each of these steps has a forward and reverse rate constant. The forward rate of strand exchange to products is clearly faster than the reverse rate of strand exchange to substrates, since products accumulate in the BMC (Table I). In order for strand exchange rather than synapsis to be rate-limiting in this circumstance, the reverse rate of BL-BMCproducts to UMC2 intermediates would have to be faster than the forward rate of synapsis. This is extremely unlikely given the stability to DNA challenge of the pre-strand exchange synaptic complexes. At 0 and 37 °C, the most favorable temperatures for recombination, the reverse rate of BL-BMCs to UMC2 complexes accumulates. This is clearly not because UMC2 are intrinsically unstable at 37 °C, since they accumulate to very high levels when recombination is blocked by the absence of spermidine (Fig. 2, lane 6).

Recombination is more efficient at 30 °C and above than at or below 25 °C, implying that at least one reaction step is cold-sensitive. BL-BMC intermediates are stable to DNA challenge both at 0 and at 37 °C once formed, but synapsis may be cold-sensitive. Another possibility is a step after synapsis, presumably one associated with strand exchange. Cold sensitivity has classically been associated with protein-protein interactions and the "hydrophobic effect." While it would not be surprising if a rearrangement in protein-protein contacts must accompany, or perhaps drive, strand exchange, this remains to be investigated.

Finally, a surprising discovery was the difference in DNA binding abilities of the Intα and IntF proteins (Figs. 3–5). It has been assumed that, except for their catalytic properties, these two proteins bind DNA in an identical fashion. The difference in their ability to assemble the UMC2 intermediates, in particular, puts this assumption into question. IntF is not defective in protein-DNA interactions generally; it assembles SL-BMCs efficiently (8), and, like Intα, it assembles stable (albeit fewer) BL-BMC complexes. The involvement of the active site tyrosine in complex stability is being investigated further. Nevertheless, information obtained from experiments carried out with the IntF protein will have to be interpreted cautiously before it is extended to the wild-type Int protein.

Fig. 8. Model for the assembly and disassembly of protein-DNA intermediates in the bent-L pathway and their relation to recombination. The model summarizes all study data. A rough indication of reaction rates (fast or slow) is proposed where data are available. Disassembly of complexes reflects dissociation in the presence of specific competitor DNA.

attLtenP' substrates bound with Int. BL-BMCs, the pre- and post-strand exchange synaptic intermediates (see below for further discussion), consist of two DNA molecules held together by Int and IHF and have both core binding sites stably occupied. As strand exchange takes place between the two substrates, the BL-BMCs accumulate. The BL-BMCs disassemble via UMC1 intermediates into IHF-DNA complexes (seen on two-dimensional gels for the products; Fig. 2, lanes 5, 7, and 8, Fig. 6, and data not shown), and free proteins and DNA substrates. If UMC2 complexes are also formed during the disassembly process, they constitute a very transient stage.

Among the steps of the reaction delineated in this study, synapsis is the best candidate for the rate-limiting step. Assembly of the precursor intermediates containing a single DNA substrate is fast (essentially complete by 5 min at either 0 or 37 °C). Accumulation of BMCs comprises (at least) two steps in the bent-L pathway, synapsis (UMC2 → BL-BMCsubstrates in Fig. 8) and strand exchange (BL-BMCsubstrates → BL-BMCproducts). Each of these steps has a forward and reverse rate constant. The forward rate of strand exchange to products is clearly faster than the reverse rate of strand exchange to substrates, since products accumulate in the BMC (Table I). In order for strand exchange rather than synapsis to be rate-limiting in this circumstance, the reverse rate of BL-BMCproducts to UMC2 intermediates would have to be faster than the forward rate of synapsis. This is extremely unlikely given the stability to DNA challenge of the pre-strand exchange synaptic complexes. At 0 and 37 °C, the most favorable temperatures for recombination, the reverse rate of BL-BMCs to UMC2 complexes accumulates. This is clearly not because UMC2 are intrinsically unstable at 37 °C, since they accumulate to very high levels when recombination is blocked by the absence of spermidine (Fig. 2, lane 6).

Recombination is more efficient at 30 °C and above than at or below 25 °C, implying that at least one reaction step is cold-sensitive. BL-BMC intermediates are stable to DNA challenge both at 0 and at 37 °C once formed, but synapsis may be cold-sensitive. Another possibility is a step after synapsis, presumably one associated with strand exchange. Cold sensitivity has classically been associated with protein-protein interactions and the “hydrophobic effect.” While it would not be surprising if a rearrangement in protein-protein contacts must accompany, or perhaps drive, strand exchange, this remains to be investigated.

Finally, a surprising discovery was the difference in DNA binding abilities of the Intα and IntF proteins (Figs. 3–5). It has been assumed that, except for their catalytic properties, these two proteins bind DNA in an identical fashion. The difference in their ability to assemble the UMC2 intermediates, in particular, puts this assumption into question. IntF is not defective in protein-DNA interactions generally; it assembles SL-BMCs efficiently (8), and, like Intα, it assembles stable (albeit fewer) BL-BMC complexes. The involvement of the active site tyrosine in complex stability is being investigated further. Nevertheless, information obtained from experiments carried out with the IntF protein will have to be interpreted cautiously before it is extended to the wild-type Int protein.

Finally, a surprising discovery was the difference in DNA binding abilities of the Intα and IntF proteins (Figs. 3–5). It has been assumed that, except for their catalytic properties, these two proteins bind DNA in an identical fashion. The difference in their ability to assemble the UMC2 intermediates, in particular, puts this assumption into question. IntF is not defective in protein-DNA interactions generally; it assembles SL-BMCs efficiently (8), and, like Intα, it assembles stable (albeit fewer) BL-BMC complexes. The involvement of the active site tyrosine in complex stability is being investigated further. Nevertheless, information obtained from experiments carried out with the IntF protein will have to be interpreted cautiously before it is extended to the wild-type Int protein.

In summary, the bent-L pathway intermediates described above divide the recombination pathway into several well-defined steps. This is useful both in characterizing λ site-specific recombination in general and in identifying distinctions between recombination via different pathways. Int mutant proteins are being analyzed for their ability to perform each of these steps in order to further determine structure/function relationships of Int. In addition, the geometry of the synaptic intermediates is being determined.

Interestingly, the UMC2 complex acts as a checkpoint irreversible; if strand exchange is blocked or slowed down, this precursor to the synaptic complex accumulates. Such a change, albeit subtle, in the conformation of the complex may drive the reaction forward. The conformational change may drive the reaction forward by stabilizing the post-strand exchange intermediate. This is clearly not because UMC2 are intrinsically unstable at 37 °C, since they accumulate to very high levels when recombination is blocked by the absence of spermidine (Fig. 2, lane 6).

Recombination is more efficient at 30 °C and above than at or below 25 °C, implying that at least one reaction step is cold-sensitive. BL-BMC intermediates are stable to DNA challenge both at 0 and at 37 °C once formed, but synapsis may be cold-sensitive. Another possibility is a step after synapsis, presumably one associated with strand exchange. Cold sensitivity has classically been associated with protein-protein interactions and the “hydrophobic effect.” While it would not be surprising if a rearrangement in protein-protein contacts must accompany, or perhaps drive, strand exchange, this remains to be investigated.

Finally, a surprising discovery was the difference in DNA binding abilities of the Intα and IntF proteins (Figs. 3–5). It has been assumed that, except for their catalytic properties, these two proteins bind DNA in an identical fashion. The difference in their ability to assemble the UMC2 intermediates, in particular, puts this assumption into question. IntF is not defective in protein-DNA interactions generally; it assembles SL-BMCs efficiently (8), and, like Intα, it assembles stable (albeit fewer) BL-BMC complexes. The involvement of the active site tyrosine in complex stability is being investigated further. Nevertheless, information obtained from experiments carried out with the IntF protein will have to be interpreted cautiously before it is extended to the wild-type Int protein.

In summary, the bent-L pathway intermediates described above divide the recombination pathway into several well-defined steps. This is useful both in characterizing λ site-specific recombination in general and in identifying distinctions between recombination via different pathways. Int mutant proteins are being analyzed for their ability to perform each of these steps in order to further determine structure/function relationships of Int. In addition, the geometry of the synaptic intermediates is being determined.

4 A. Burgin and H. Nash, personal communication.
Acknowledgments—I thank Howard Nash, Lea Jessop, Forest Rohwer, and Geoffrey Cassell for their thoughtful comments on the manuscript, and Alex Burgin for very helpful discussions.

REFERENCES
1. Landy, A. (1993) Curr. Opin. Genet. Dev. 3, 699–707
2. Nash, H. A. (1996) in Escherichia coli and Salmonella: Cellular and Molecular Biology (Neidhardt, F. C., Umbarger, H. E., Ingraham, J. L., Lin, E. C. C., Low, K. B., Magasanik, B., Reznikoff, W. S., Riley, M., and Schaechter, M., eds) pp. 2363–2376, American Society for Microbiology Press, Washington, D. C.
3. Segall, A. M., and Nash, H. A. (1996) Genes Cells 1, 453–463
4. Moitoso de Vargas, L., Pargellis, C. A., Hasan, N. M., Bushman, E. W., and Landy, A. (1988) Cell 54, 923–929
5. Tirumalai, R. N., Healey, E., and Landy, A. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 6104–6109
6. Richet, E., Abcarian, P., and Nash, H. A. (1988) Cell 52, 9–17
7. Kim, S., and Landy, A. (1992) Science 256, 198–203
8. Segall, A. M., and Nash, H. A. (1995) EMBO J. 14, 4567–4576
9. Burgin, A. B., Jr., and Nash, H. A. (1995) Curr. Biol. 5, 1312–1321
10. Echols, H. (1970) J. Mol. Biol. 47, 575–583
11. Kikuchi, A., Flamm, E., and Weisberg, R. A. (1985) J. Mol. Biol. 182, 30–38
12. Landy, A., and Weisberg, R. (1983) in Lambda II (Hendrix, R. W., Roberts, J. W., Stahl, F. W., and Weisberg, R. A., eds) pp. 211–250, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
13. Nash, H. A., and Robertson, C. A. (1989) EMBO J. 8, 3523–3533
14. Numrych, T. E., Gumport, R. I., and Gardner, J. P. (1990) Nucleic Acids Res. 18, 3953–3959
15. Schagger, H., and von Jagow, G. (1987) Anal. Biochem. 166, 368–379
16. Mazumder, A. (1993) in Footprinting of Nucleic Acid Complexes (Revzin, A., ed) pp. 45–73, Academic Press, San Diego, CA
17. Sigman, D. S., Kuwahara, M. D., Chen, C.-H. B., and Bruice, T. W. (1991) Methods Enzymol. 208, 414–433
18. Maxam, A. M., and Gilbert, W. (1980) Methods Enzymol. 65, 499–560
19. Thederahn, T., Spassky, A., Kuwahara, M. D., and Sigman, D. S. (1990) Biochem. Biophys. Res. Commun. 168, 756–762
20. Pargellis, C. A., Nunes-Duby, S. E., Moitoso de Vargas, L., and Landy, A. (1988) J. Biol. Chem. 263, 7678–7685
21. Numrych, T. E., Azaro, M. A., and Landy, A. (1995) Curr. Biol. 5, 139–148
22. Guo, F., Gopaul, D. N., and Van Duyne, G. D. (1997) Nature 389, 40–46