The role of supercoiling in mycobacteriophage L5 integrative recombination

Carol E. A. Peña, J. Michelle Kahlenberg and Graham F. Hatfull

Department of Biological Sciences, University of Pittsburgh, Pittsburgh, PA 15260, USA and Slayter Box 1033, Denison University, Granville, OH 43023, USA

Received April 27, 1998; Revised and Accepted July 7, 1998

ABSTRACT

The genome of temperate mycobacteriophage L5 integrates into the chromosomes of its hosts, including Mycobacterium smearmatis, Mycobacterium tuberculosis and bacille Calmette-Guérin. This integrase-mediated site-specific recombination reaction occurs between the phage attP site and the mycobacterial attB site and requires the mycobacterial integration host factor. Here we examine the role of supercoiling in this reaction and show that integration is stimulated by DNA supercoiling but that supercoiling of either the attP or the attB substrate enhances recombination. Supercoiling thus facilitates a postsynaptic recombination event. We also show that, while supercoiling is not required for the production of a recombinogenic intasome, a mutant attP DNA deficient in binding of the host factor acquires a dependence on supercoiling for intasome formation and recombination.

INTRODUCTION

Mycobacteriophage L5 is a temperate phage whose hosts include the pathogen Mycobacterium smearmatis, the vaccine strain bacille Calmette-Guérin (BCG) and the non-pathogenic fast growing Mycobacterium smearmatis (1–4). The establishment of lysogeny involves integration of the L5 genome into the chromosome of its host (1,3,4). This site-specific recombination reaction occurs between an attachment site on the L5 phage DNA, attP, and an attachment site on the bacterial chromosome, attB (1,3,4). The integration reaction has been reconstituted in vitro and has been shown to require the L5-encoded integrase protein (Int-L5; 5) as well as a novel mycobacterially-encoded integration host factor (mIHF; 6,5).

The L5 attP region contains multiple sites to which Int-L5 can bind (7). These sites fall into two categories: core-type binding sites at the points of strand exchange and arm-type binding sites, which flank the core (P1–P7; Fig. 1). In DNase I footprinting of attP, mIHF protects regions just to the left and right of the core-type binding sites, but only when both Int-L5 and mIHF are present (6; C.E.A.P., J.M.K. and G.F.H., submitted). During L5 integration a recombinationally active intasome is assembled with these components, involving formation of intramolecular Int-L5 bridges between the core and P4/P5 of attP (C.E.A.P., J.M.K. and G.F.H., submitted). The mIHF protein, which by itself exhibits no binding preference for attP DNA (6), appears to promote or stabilize a DNA bend between the core and P4/P5, enabling intasome assembly. This intasome complex then recruits the M. smearmatis attB site to form the synaptosome, within which strand exchange occurs (C.E.A.P., J.M.K. and G.F.H., submitted). Even though a recombinationally active intasome can be formed with linear attP DNA (C.E.A.P., J.M.K. and G.F.H., submitted), integration is significantly stimulated by supercoiling of attP (5).

The topological state of DNA substrates is known to profoundly influence many site-specific recombination reactions (8-9). For example, co-integrate resolution by the Yö and Tn3 resolvases and DNA inversion by Gin and Hin and their relatives absolutely require supercoiled substrates, principally to promote formation of a specific synaptic topology within which strand exchange occurs (9,10). Supercoiling also contributes a torsional effect to a postsynaptic step of recombination in the Tn3 resolvase and Gin DNA invertase reactions, possibly by unwinding of the DNA double helices or providing energy by the release of supercoiling-induced tension upon cleavage (11). In addition, supercoiling has been shown to play more passive roles in resolution and inversion reactions, including aiding in binding of the recombination proteins to the DNA, alignment of the sites and increasing the local concentration of sites (8).

The topological requirements for phage integration have been studied extensively for phage λ (12,13) and are quite different from those for the resolvase and DNA invertase systems. In the λ system efficient integrative recombination requires supercoiling of the attP substrate both in vitro (13) and in vivo (14), but the random distribution of supercoils among the products demonstrates that the recombination sites synapse by random collision, rather than by the topological filtering typical of the resolvase systems (15). The principal role of supercoiling in the λ system appears to be to promote formation of a nucleosome-like intasome complex in which the attP DNA is wound around the integration proteins, such that protomers of integrase are correctly positioned for the capture of attB DNA (16,17). The requirement for supercoiling cannot be supplied by supercoiling of the attB DNA and the topological state of attB does not influence the efficiency of recombination (13).
Unlike the resolvase-like reactions, Λ integration does not have an absolute requirement for supercoiling; the requirement for attP superhelicity can be circumvented by performing the reactions with different ionic strength. Lower salt concentrations (≤100 mM) relax the requirement that attP be supercoiled (18). Excisive recombination of Λ also does not absolutely require supercoiling in vitro, but is stimulated by supercoiling of attL and attR when the salt concentration is >100 mM (using NaCl, KCl or NH₄Cl; 18). Below 100 mM there is no superhelicity requirement for excisive recombination and linear attL and attR substrates recombine efficiently (18,19).

Superficially, the processes of integration in Λ and L5 are similar; both systems use a complex attP site, a short attB site, a phage-encoded integrase and a host-encoded accessory factor (for reviews of Λ recombination see 20,21). However, the attachment sites of Λ and L5 share no sequence similarity, the binding sites in attP are organized quite differently and the integrases have little amino acid sequence similarity apart from the few critical active site residues. The host factors involved also have little sequence similarity and exhibit somewhat different mechanisms of action (6). These differences are perhaps not surprising, since phages L5 and Λ are themselves unrelated at the sequence level and the Escherichia coli and mycobacterial hosts are phylogenetically quite distinct. Moreover, E.coli and M.tuberculosis lie at the extremes of the spectrum of bacterial growth rate, with doubling times of 20 min and 24 h respectively (22). Phages infecting these bacteria are thus likely to encounter very different cellular environments, particularly with respect to DNA metabolism, and processes such as integration and excision can be expected to reflect such differences.

In this paper we show that L5 integrative recombination is not only stimulated by supercoiling of attP DNA, but can also be stimulated by supercoiling of attB DNA when the attP DNA is linear, suggesting that superhelicity enhances events in the recombination pathway after initial synthesis has occurred. Since L5 integrase does not appear to be able to relax superhelical plasmid DNA, cleavage of DNA at the crossover site may also not occur until after synthesis. Finally, we demonstrate that in the L5 system, mIHF effectively fills the role which supercoiling has been observed to play in intasome formation in other phage integration systems, such that a mutant site with base substitutions within a region of DNA proposed to interact with mIHF acquires a novel requirement for superhelicity of attP.

**Materials and Methods**

**Plasmid DNAs**

The 6202 bp plasmid pMH94 (3) and the 7763 bp plasmid pGL1 (7) both contain the wild-type attP site. The 4820 bp plasmid pMH12.1 is a pUC119 derivative containing a 1.7 kb SalI attB fragment from M.smegmatis (3).

Plasmids containing substitution mutations in the attP region were constructed from plasmid pGL1 as described previously (7) using the Muta-Gen Phagemid In vitro Mutagenesis system (Bio-Rad). The mutagenic oligonucleotides were designed to substitute the 5 bp at attP coordinates +61 to +65 (to make plasmid pMK1), the 10 bp from +41 to +50 (pMK2) or the 10 bp from +71 to +80 (pMK3), all between the core and P4 (see Table 1 and Fig. 4A for sequences). The substituted sequences introduced HindIII, EcoRV and ApaI restriction sites into plasmids pMK1, pMK2 and pMK3 respectively; plasmid DNA preparations were screened for the desired mutation by digestion with the appropriate enzyme. The identities of the mutants were confirmed by sequencing the attP regions using an ABI 310 automated sequencer (Perkin-Elmer).

**In vitro Integrative Recombination Reactions**

Recombination assays were similar to those described previously (5). Reactions were performed in a total volume of 20 μl and contained 10 mM Tris–HCl, pH 7.5, 25 mM NaCl (unless otherwise indicated), 1 mM dithiothreitol, 1 mM MgSO₄, 1 mM spermidine, 1 mM EDTA, ~0.024 pmol attP DNA, 0.024–0.06 pmol attB DNA, 0.14–0.71 pmol purified Int-L5 and 3.6 pmol purified mIHF (unless otherwise indicated). Reactions were incubated for 2 h at 37°C, stopped by addition of SDS to a final concentration of 0.1% and electrophoresed through a 0.7 or 0.8% agarose gel.

For experiments using linear DNAs, pMH94 was linearized using a unique BglII site; pGL1 and pMK2 using a unique EcoNI site; pMH12.1 using a unique HindIII site. A short, linear attB DNA was generated by annealing pairs of oligonucleotides (to give a 45 bp fragment) as described previously (23).

**Integrative Transformation Assays**

**In vivo** integrative transformation assays were performed as described previously (7). Approximately 0.1 μg (0.025 pmol) attP-containing plasmid (which also contains L5 int and lacks an origin of replication for mycobacteria) was electroporated into M.smegmatis strain mc²155 (4,24), recovered at 37°C, diluted plating on 7H10/ADC plates containing 0.5 μg/ml tetracycline and transformants counted after a 5 day incubation at 37°C.

**Native Gel Analysis of Intasome Complex Formation**

The conditions for intasome formation were similar to those used for in vitro recombination, with the exceptions that attP was provided as a short, linear DNA fragment, 1 μg salmon sperm DNA was added to each reaction and the total reaction volume was 10 μl. Approximately 3000 c.p.m. radiolabeled attP DNA was incubated with 0.0035 pmol Int-L5 and 0.37 pmol mIHF for 30 min on ice, reactions were electrophoresed through a native
either room temperature or 37°C. Plasmid pMH94 was incubated with 0.7–14.0 pmol Int-L5 at 7.5 or 8.0. Approximately 0.05 pmol of a large, linear attB DNA with a supercoiled attP plasmid results in efficient recombination similar to that observed previously. However, only a small amount of product was observed when both attP and attB were present on linear substrates, indicating that supercoiling of attP stimulates the reaction. Surprisingly, integration was also stimulated if attB, rather than attP, was the supercoiled substrate. This was unexpected, since supercoiling of the attB DNA does not substitute for supercoiling of attP in λ integration. Since integration of L5 is enhanced by supercoiling of either substrate, the stimulation must occur at a step involving both attP and attB and, since a putative synaptosome can form efficiently using linear attP with linear attB (in the presence of both Int-L5 and mHf), the stimulated process must be post-synaptic, either conversion of the initial synaptic complex into an activated form or a step in strand exchange. Supercoping therefore acts at a later step of integration for L5 than for λ, in which supercoiling is involved in formation of the intasome.

Since the supercoiling dependence of λ integrase-mediated recombination is influenced by salt concentration, we tested the salt dependence of L5 recombination. For λ, at relatively high salt concentrations (≥40 mM) supercoiling of attP is absolutely required, while at lower salt concentrations (≤25 mM) this requirement is relaxed. In contrast, L5 integrase-mediated recombination does not appear to be as sensitive to changes in salt concentration as λ integration; little or no difference in L5 recombination was observed between the 25 and 50 mM NaCl reactions when at least one of the substrates was supercoiled (Fig. 2C) and, while some inhibition of recombination between two linear substrates occurred in the presence of 50 mM NaCl (Fig. 2B), it was not observed when both attP and attB were present on linear substrates (Fig. 2B), indicating that supercoiling of attP stimulates the reaction. Surprisingly, integration was also stimulated if attB, rather than attP, was the supercoiled substrate (Fig. 2A). This was unexpected, since supercoiling of the attB DNA does not substitute for supercoiling of attP in λ integration. Since integration of L5 is enhanced by supercoiling of either substrate, the stimulation must occur at a step involving both attP and attB and, since a putative synaptosome can form efficiently using linear attP with linear attB (in the presence of both Int-L5 and mHf), the stimulated process must be post-synaptic, either conversion of the initial synaptic complex into an activated form or a step in strand exchange. Supercoping therefore acts at a later step of integration for L5 than for λ, in which supercoiling is involved in formation of the intasome.

RESULTS

Supercoiling requirements for integrative recombination

We noted previously that L5 integrative recombination between an attP DNA and a small, linear attB fragment is strongly stimulated in vitro by supercoiling of the attP substrate (3). To further test the supercoiling requirements of L5 integration, a series of similar in vitro recombination reactions was performed using different combinations of supercoiled and linear attP and attB substrates incubated with purified Int-L5 and mHf. Use of a large, linear attB DNA with a supercoiled attP plasmid results in efficient recombination similar to that observed previously (Fig. 2A; 5). However, only a small amount of product was observed when both attP and attB were present on linear substrates (Fig. 2B), indicating that supercoiling of attP stimulates the reaction. Surprisingly, integration was also stimulated if attB, rather than attP, was the supercoiled substrate (Fig. 2A). This was unexpected, since supercoiling of the attB DNA does not substitute for supercoiling of attP in λ integration. Since integration of L5 is enhanced by supercoiling of either substrate, the stimulation must occur at a step involving both attP and attB and, since a putative synaptosome can form efficiently using linear attP with linear attB (in the presence of both Int-L5 and mHf), the stimulated process must be post-synaptic, either conversion of the initial synaptic complex into an activated form or a step in strand exchange. Supercoping therefore acts at a later step of integration for L5 than for λ, in which supercoiling is involved in formation of the intasome.

Since the supercoiling dependence of λ integrase-mediated recombination is influenced by salt concentration, we tested the salt dependence of L5 recombination. For λ, at relatively high salt concentrations (≥40 mM) supercoiling of attP is absolutely required, while at lower salt concentrations (≤25 mM) this requirement is relaxed. In contrast, L5 integrase-mediated recombination does not appear to be as sensitive to changes in salt concentration as λ integration; little or no difference in L5 recombination was observed between the 25 and 50 mM NaCl reactions when at least one of the substrates was supercoiled (Fig. 2C) and, while some inhibition of recombination between two linear substrates occurred in the presence of 50 mM NaCl (Fig. 2B), it was not observed when both attP and attB were present on linear substrates (Fig. 2B), indicating that supercoiling of attP stimulates the reaction. Surprisingly, integration was also stimulated if attB, rather than attP, was the supercoiled substrate (Fig. 2A). This was unexpected, since supercoiling of the attB DNA does not substitute for supercoiling of attP in λ integration. Since integration of L5 is enhanced by supercoiling of either substrate, the stimulation must occur at a step involving both attP and attB and, since a putative synaptosome can form efficiently using linear attP with linear attB (in the presence of both Int-L5 and mHf), the stimulated process must be post-synaptic, either conversion of the initial synaptic complex into an activated form or a step in strand exchange. Supercoping therefore acts at a later step of integration for L5 than for λ, in which supercoiling is involved in formation of the intasome.

Since the supercoiling dependence of λ integrase-mediated recombination is influenced by salt concentration, we tested the salt dependence of L5 recombination. For λ, at relatively high salt concentrations (≥40 mM) supercoiling of attP is absolutely required, while at lower salt concentrations (≤25 mM) this requirement is relaxed. In contrast, L5 integrase-mediated recombination does not appear to be as sensitive to changes in salt concentration as λ integration; little or no difference in L5 recombination was observed between the 25 and 50 mM NaCl reactions when at least one of the substrates was supercoiled (Fig. 2C) and, while some inhibition of recombination between two linear substrates occurred in the presence of 50 mM NaCl (Fig. 2B), it was not observed when both attP and attB were present on linear substrates (Fig. 2B), indicating that supercoiling of attP stimulates the reaction. Surprisingly, integration was also stimulated if attB, rather than attP, was the supercoiled substrate (Fig. 2A). This was unexpected, since supercoiling of the attB DNA does not substitute for supercoiling of attP in λ integration. Since integration of L5 is enhanced by supercoiling of either substrate, the stimulation must occur at a step involving both attP and attB and, since a putative synaptosome can form efficiently using linear attP with linear attB (in the presence of both Int-L5 and mHf), the stimulated process must be post-synaptic, either conversion of the initial synaptic complex into an activated form or a step in strand exchange. Supercoping therefore acts at a later step of integration for L5 than for λ, in which supercoiling is involved in formation of the intasome.

Since the supercoiling dependence of λ integrase-mediated recombination is influenced by salt concentration, we tested the salt dependence of L5 recombination. For λ, at relatively high salt concentrations (≥40 mM) supercoiling of attP is absolutely required, while at lower salt concentrations (≤25 mM) this requirement is relaxed. In contrast, L5 integrase-mediated recombination does not appear to be as sensitive to changes in salt concentration as λ integration; little or no difference in L5 recombination was observed between the 25 and 50 mM NaCl reactions when at least one of the substrates was supercoiled (Fig. 2C) and, while some inhibition of recombination between two linear substrates occurred in the presence of 50 mM NaCl (Fig. 2B), it was not observed when both attP and attB were present on linear substrates (Fig. 2B), indicating that supercoiling of attP stimulates the reaction. Surprisingly, integration was also stimulated if attB, rather than attP, was the supercoiled substrate (Fig. 2A). This was unexpected, since supercoiling of the attB DNA does not substitute for supercoiling of attP in λ integration. Since integration of L5 is enhanced by supercoiling of either substrate, the stimulation must occur at a step involving both attP and attB and, since a putative synaptosome can form efficiently using linear attP with linear attB (in the presence of both Int-L5 and mHf), the stimulated process must be post-synaptic, either conversion of the initial synaptic complex into an activated form or a step in strand exchange. Supercoping therefore acts at a later step of integration for L5 than for λ, in which supercoiling is involved in formation of the intasome.
inhibition was not complete and thus supercoiling was not an absolute requirement for integration under high salt conditions.

**Int-L5 fails to display topoisomerase activity**

The experiments described above suggest that supercoiling primarily stimulates formation of a productive synaptosome or perhaps some subsequent step in the reaction. This raises the question as to whether formation of a productive synaptosome is a prerequisite for any chemical attack on the DNA by Int-L5. We therefore tested the ability of Int-L5 to relax negatively supercoiled DNA, a property that has been demonstrated for Int-λ (25). We did not observe any topoisomerase activity of Int-L5 under any of the conditions tested (Fig. 3). These conditions were similar to those used for in vitro recombination, but utilized different pH values (pH 7.5 and 8.0), temperatures (room temperature and 37°C) and integrase concentrations and lacked spermidine (which stimulates recombination but inhibits λ integrase-mediated topoisomerase activity; 25). Similar observations were made in reactions using DNA substrates with and without attP or attB, in the presence and absence of mIHF, for longer incubation times (up to 16 h), in the presence of spermidine and utilizing a buffer (50 mM Tris–HCl, pH 8.0, 5 mM EDTA, 100 mM KCl and 3 mg/ml BSA; 25) identical to that used in the Int-λ topoisomerase activity experiments (data not shown). While we note that the Int-λ-induced relaxation of supercoiled plasmids was readily detectable in a simple agarose gel in the experiments of Kikuchi and Nash (25), the addition of 0.75 µg/ml chloroquine to the agarose gel also did not reveal any changes in the topological state of supercoiled plasmids incubated with Int-L5 (data not shown). While we obviously cannot rule out the possibility that we have simply failed to find the appropriate conditions for Int-L5-mediated topoisomerase activity and its detection, all the variables which affect the topoisomerase activity of Int-λ were manipulated in an attempt to find favorable conditions. We note, however, that under similar conditions the integrase of the *Haemophilus influenzae* phage HP1 also fails to display topoisomerase activity (26).

**DNA supercoiling and the sequence requirements for mIHF action**

The experiments described above indicate that supercoiling acts relatively late in L5 recombination, as opposed to the earlier role that supercoiling plays in formation of the λ intasome complex. For L5, integration is strongly dependent on the presence of mIHF, which stimulates intasome formation (6). This host factor differs from *E. coli* IHF in that it has little or no sequence similarity to IHF and does not appear to bind specifically to attP DNA (6). However, when Int-L5 is present, mIHF stimulates formation of the intasome, of which it is an integral component, and occupies the DNA segment between the core and the P4/P5 pair of arm-type sites (6; see Fig. 1). The only sequence feature of note in this region is 5’-ATTTTTCTTTT, which, although part of a putative transcriptional terminator, may also facilitate bending of the DNA in this region to promote intasome formation. Thus, while mIHF does not bind alone with any preference for attP DNA, we cannot rule out that certain positions within the DNA may facilitate the binding of or orient mIHF within the intasome.

To probe the sequence requirements for mIHF binding, we constructed three mutant attP DNAs each with a substituted block of bases between the core and P4 and evaluated the effect of these mutations on recombination. All of the mutants substitute regions which are protected from DNase I cleavage when both Int-L5 and mIHF are present (6). Plasmid pMK1 contains a substitution of the 5 bp from +61 to +65, while plasmid pMK2 contains a substitution of the 10 bp from +41 to +50 (Table 1 and Fig. 4A). The third mutant, pMK3, substitutes 10 bp within the T-rich region, from +71 to +80 (see Table 1 and Fig. 4A). The effects of these substitutions on in vivo integration were determined using a transformation assay in which integration of a non-replicating plasmid DNA was required to produce antibiotic-resistant transformants. In this assay all three mutants behaved similarly to the wild-type substrate and all efficiently transformed *M. smegmatis* (Table 1).

**Table 1. Effect of inter-core/P4 region mutations on integration in vivo**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Coordinates of mutation</th>
<th>Wt sequence*</th>
<th>Mutant sequence*</th>
<th>Transformation frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGKL1</td>
<td>none</td>
<td>5′-CCCTC</td>
<td>5′-AAGCT</td>
<td>100.0</td>
</tr>
<tr>
<td>pMK1</td>
<td>+41 to +45</td>
<td>5′-CCCTC</td>
<td>5′-AAGCT</td>
<td>107.1</td>
</tr>
<tr>
<td>pMK2</td>
<td>+41 to +50</td>
<td>5′-CGGTCCAGAG</td>
<td>5′-GGCCGATATCC</td>
<td>89.3</td>
</tr>
<tr>
<td>pMK3</td>
<td>+71 to +80</td>
<td>5′-ATTTTTCTTT</td>
<td>5′-GGCCGATATCC</td>
<td>214.3</td>
</tr>
</tbody>
</table>

Transformation frequencies were determined by electroporation of plasmid DNA into *M. smegmatis*. The pGKL1 transformation frequency was defined as 100%.

*The sequence corresponds to the top strand shown in Figure 4A.

The three mutant attP DNAs were also tested as recombination substrates in vitro using a supercoiled attP plasmid and a short, linear attB fragment (Fig. 4B). Consistent with the in vivo experiments, none of the three mutants were substantially different from the wild-type attP DNA and all three showed a similar dependence on mIHF (Fig. 4B). These observations argue against a role of specific DNA sequences for mIHF binding. However, when the mutant substrates were tested as linear attP DNAs in conjunction with a linear attB substrate, no product was observed with pMK2 (linearized pMK1) and pMK3 behaved like linearized wild-type attP DNA, undergoing inefficient recombination with a linear attB
Figure 4. Characterization of an attP mutant which imposes a requirement for supercoiling of attP. (A) Sequence of the core–P4/P5 region of attP and mutants. The mutant sequences are shown below the wild-type sequence and correspond to the bottom strand (1, 2 and 3 indicate the substitutions in mutants pMK1, pMK2 and pMK3 respectively). The sequences of P4, P5 and the common core are boxed. The horizontal black bar shows the core region protected by Int-L5 and the larger arrow indicates a site of DNase I cleavage enhancement in footprinting of the intasome and synaptosome in situ (C.E.A.P., J.M.K. and G.F.H., submitted). The small arrowheads indicate the sites of strand exchange (23). (B) In vitro recombination reactions using attP substitution mutants. Reactions containing a 7763 bp supercoiled attP plasmid (either wild-type pGL1 or with mutations as indicated in Table 1 and (A)), a short 45 bp attB DNA, Int-L5 (as indicated) and varying amounts of mIHF (as indicated) were performed as in Figure 2A. The predicted size of the product is 7808 bp. The amounts of mIHF used were 3.7, 1.2, 0.37, 0.12 and 0.037 pmol (upper) and 3.7, 0.37 and 0.037 pmol (lower). The positions of the product, the supercoiled (sc) and relaxed circular (rel) states of the attP plasmid, the origin of electrophoresis (o) and sizes of the DNA marker (M) in kb are indicated. (C) Supercoiling of the attB substrate does not activate recombination with a linear pMK2 substrate. Recombination reactions were performed as in Figure 2A using the supercoiled 4820 bp attB plasmid pMH12.1 and a linearized 7763 bp attP DNA (either the wild-type pGL1 or the mutant pMK2, as indicated). The size of the predicted product is 12 583 bp. The positions of the supercoiled attB (sc attB), relaxed circular attB (rel attB), linear attP (lin attP), the product and several DNA markers in kb are indicated. (D) Native polyacrylamide gel analysis of intasome complexes assembled with attP substitution mutants. A radiolabeled 624 bp fragment of wild-type (pGL1) or mutant (pMK1, pMK2 or pMK3) attP DNA was incubated with purified Int-L5 and mIHF (as indicated) for 30 min on ice, followed by electrophoresis though a native polyacrylamide gel. The positions of free attP, the intasome and the origin of electrophoresis (o) are shown.

DNA; data not shown). Interestingly, recombination with linear pMK2 DNA is not stimulated by supercoiling of attB DNA (Fig. 4C), suggesting that pMK2 integration has acquired a specific requirement for supercoiling of attP. (We also note that the amount of attP plasmid present in the relaxed, circular state, presumably equivalent to a linear attP substrate, decreased in the wild-type pGL1 and mutant pMK3 reactions in Fig. 4B, but did not decrease in the pMK2 reaction; this further supports our conclusion that integrative recombination of pMK2 can only proceed using a supercoiled attP substrate.)

We have observed that Int-L5 and mIHF assemble with a short, linear attP DNA fragment to form an intasome which is capable of synapsing with attB DNA and undergoing recombination (C.E.A.P., J.M.K. and G.F.H., submitted). While recombination is relatively inefficient with these short, linear DNA substrates, the rate limiting step does not appear to be in intasome formation. The three mutant attP substrates, pMK1, pMK2 and pMK3, were therefore tested as short, linear DNA fragments for their ability to form an intasome in the presence of Int-L5 and mIHF (Fig. 4D). While pMK1 and pMK3 behaved similarly to wild-type attP, no band corresponding to the intasome was observed with pMK2 (Fig. 4D). A simple interpretation of these data is that the sequence change in pMK2 has disturbed the ability of mIHF to bind to this region, preventing mIHF from fulfilling its role of stimulating the formation of a tertiary complex. Since supercoiled pMK2 DNA is fully competent to undergo integrative recombination, supercoiling of attP can presumably compensate for this defect.
The observations presented here suggest that while integrative recombination of both L5 and \( \lambda \) is enhanced by DNA supercoiling, the stimulatory effect of superhelicity is exerted at different steps in the reaction pathway. In \( \lambda \), integration, supercoiling promotes a relatively early stage of the reaction, assembly of the recombinagenic intasome complex; in the absence of supercoiling, the \( \lambda \) arm-type sites are incompletely protected in footprinting experiments (17). In contrast, supercoiling appears to enhance a relatively late step in L5 integration, during or subsequent to the assembly of a productive synaptosome in which both DNA partners are present.

The specific step in the L5 recombination pathway that is stimulated by supercoiling is not clear. However, we have observed that attB DNA readily associates with the L5 intasome (assembled in vitro with linear attP DNA) to form a synaptic complex (C.E.A.P., J.M.K. and G.F.H., submitted), such that supercoiling does not appear to be required for synapsis per se. However, this quaternary complex is relatively long lived and the subsequent step in the reaction appears to be rate limiting when linear DNA substrates are involved; this is therefore a likely stage at which superhelicity exerts its effect. Supercoiling could specifically activate Int-mediated nucleophlic attack on the DNA, facilitate a step of strand exchange or stimulate conversion of a primary synaptic complex from an inactive into an active form. In situ footprinting experiments have shown that the P1 and P2 arm-type sites are only partially protected in the synaptosome observed in gel retardation experiments, even though both P1 and P2 are needed for recombination (C.E.A.P., J.M.K. and G.F.H., submitted). This phenomenon of partial arm-type site protection in the absence of supercoiling is subtly different from the observation in the absence of supercoiling of the \( \lambda \) attP site, Int-\( \lambda \) binds to the P1 and P3 arm-type sites weakly but does not produce a recombinagenic intasome (17). In contrast, the intasome formed using a linear L5 attP site (in which P1 and P2 are not protected at all) is a kinetic precursor to recombination (C.E.A.P., J.M.K. and G.F.H., submitted) and thus intasome formation is independent of supercoiling. However, supercoiling may facilitate occupancy of these sites and promote formation of Int-L5-mediated protein bridges between the P1 and P2 arm-type sites and the core-type binding sites in attB during formation of the synaptosome.

Int-L5 does not exhibit topoisomerase activity under any of the conditions tested (Fig. 3); a similar lack of topoisomerase activity was noted by Hakimi and Scocca (26) for the integrase of the \( H. influenzae \) phage HP1. However, Int-\( \lambda \) does relax negatively supercoiled DNA, even one lacking attP, and does so in the absence of attB DNA (25); chemical attack on DNA by Int-\( \lambda \) clearly does not require synopsis. The absence of such an activity in Int-L5 suggests that the catalytic functions of Int-L5 are more selective and that catalytic activity is dependent upon specific conditions or interactions encountered during recombination, possibly formation of a productive synaptic complex. This lack of activity in Int-L5, along with the fact that no Holliday junction intermediates were observed in recombination reactions using attB substrates constructed with inhibitive \( \alpha \)-thio-substituted dNTPs (23), leads us to believe that the activity of Int-L5 is very specific, perhaps conformationally activated and highly reversible.

Unlike in \( \lambda \), integration, formation of the L5 intasome appears to be independent of attP superhelicity. However, one of the three mutant attP substrates that were generated appears to have acquired a dependence specifically on supercoiling of attP for recombination. In this regard, mutant pMK2 has essentially become phenotypically equivalent to \( \lambda \) attP. One explanation is that this change in sequence affects the inherent flexibility of the inter-core/P4 region, preventing stable assembly of the bent intasome complex with linear attP DNA; supercoiling of attP may facilitate binding of pMK2 DNA, as it does in the \( \lambda \) system. However, no recognizable features of either the wild-type or mutant sequence in this region suggest the ability to impart or prevent DNA flexibility. In addition, if proper binding of the attP DNA could be influenced by such a sequence substitution, it is surprising that substitution of the nearby poly(dA:dT) stretch did not also influence recombination; pMK3, however, is clearly fully capable of both intasome formation (Fig. 4D) and recombination (data not shown) as a linear attP DNA.

A more likely explanation is that the dependence of pMK2 on attP supercoiling results from an interruption of normal binding of mIHF. The mIHF host factor does not bind with any preference for attP DNA (6), but in footprinting of the intasome in \( \lambda \) it protects most of the sequence between the core and P4 site of attP. However, a DNase I enhancement is seen approximately midway between the core-type Int-L5 binding site and the P4 site (see Fig. 4A; C.E.A.P., J.M.K. and G.F.H., submitted), consistent with one unit of mIHF (probably a homodimer) binding on each side of the enhancement. While this interaction between mIHF and attP DNA is strongly dependent upon the presence of Int-L5, specific base pairs within attP could form direct contacts with mIHF. Thus, the pMK2 mutant may be defective in binding mIHF at this particular location and require supercoiling to stabilize intasome formation.

The bases changed in mutant pMK2 overlap the sequence coding for the stem components of a likely transcriptional terminator (the putative stem is from +42 to +54, with its complement at +59 to +71) (3). These inverted repeats, as well as other identified loosely repeated sequences, are potential candidates for low affinity or Int-L5-induced recognition of attP by mIHF. Of the three sequences substituted, that changed in mutant pMK2 has the highest degree of conservation with its corresponding sequence in the attP region of the related mycobacteriophage D29 (3). Even though D29 is a lytic phage and does not form lysogens upon infection of mycobacteria, D29 is capable of integrating into the \( M. smegmatis \) genome (27) and D29 integration is also dependent on mIHF (M.Pedulla and G.F.Hatfull, unpublished observations). This further supports the idea that these specific base positions may be important for recognition of attP by mIHF, but that the strength of this interaction alone is insufficient to confer independent binding of mIHF to attP DNA. However, we note that pMK2 integration still needs mIHF for recombination (see Fig. 4), such that even if supercoiling completely substitutes for the requirement of mIHF binding at this site, it must play additional roles in integration.

The observations presented here suggest that Int-L5-mediated integration utilizes some torsional effect provided by supercoiling during a post-synaptic step of the reaction. Benjamin et al. have shown that supercoiling provides both a pre-synaptic conformational effect and a post-synaptic torsional effect to recombination by Tn3 resolvase (11). Such torsional effects have been suggested to contribute to helix unwinding (11), resulting in the necessary disruption of base pairing (8) or in correct positioning of the DNA cut sites relative to the recombinase active sites (e.g. the crystal
structure for the σ8 resolvase places the active site serines too far apart to make the correct attacks on DNA; 8,28–30). We note that in the crystal structure for Int-λ, the active site tyrosine can be positioned for either trans- or cis-cleavage with no distortion of the DNA helix (31). Furthermore, the crystal structure of Cre recombinase from phage P1 complexed with its DNA site reveals some distortion of the minor groove to accommodate protein–DNA contacts, but predominantly the DNA structure is consistent with B-form DNA; in addition, the double helix has partially denatured at the cut sites, consistent with the isomerization model of strand exchange (32). These observations suggest that, at least in the λ and P1 systems (which do not require supercoiling for post-synaptic steps; 33), supercoiling is not responsible for positioning of the first cut sites in proximity to the protein or initial strand separation. However, in order to model the completion of Cre/λ strand exchange, the torsion of the local DNA backbone must be changed in order for the DNA partners to interact with each other (32). In L5 recombination, supercoiling may serve to stimulate such a conformational change or further isomerization or conformational changes later in strand exchange.

The differences in the site-specific recombination reactions of mycobacteriophage L5 and coliphage λ may have evolved as specific adaptations to the differing lifestyles of their bacterial hosts. Upon infection of the rapidly dividing E.coli, phage λ utilizes host enzymes to circularize, supercoil and aid in integration of its own genome. In contrast, since mycobacteria grow extremely slowly and divide relatively infrequently, enzymes involved in specific stages of the cell cycle may not always be present and L5 may have evolved to be independent of them, at least for viral integration. With relatively little known about the cell cycle of the slow growing mycobacteria, such clues from the study of mycobacteriophages may reflect upon the biology of their hosts.

ACKNOWLEDGEMENTS

We thank M.Pedulla for helpful discussions, E.Shepard and D.Humphries for automated sequencing, D.Lever for excellent technical assistance and T.Harper for help in figure preparation. J.M.K. was supported by a grant from the Undergraduate Biological Sciences Education Program of the Howard Hughes Medical Institute. This work was supported by grant GM49647 from the National Institutes of Health.

REFERENCES