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The role of supercoiling in mycobacteriophage L5 integrative recombination

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ABSTRACT

The genome of temperate mycobacteriophage L5 integrates into the chromosomes of its hosts, including *Mycobacterium smegmatis*, *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 post-synaptic recombination event. We also show that, while supercoiling is not required for the production of a recombinagenic 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 tuberculosis*, the vaccine strain bacille Calmette-Guérin (BCG) and the non-pathogenic fast growing *Mycobacterium smegmatis* (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; 5,6).

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.smegmatis 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 γδ and Tn*3* 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 post-synaptic step of recombination in the Tn*3* 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).

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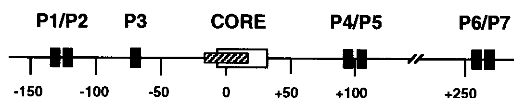

Figure 1. The *attP* region of mycobacteriophage L5. The seven arm-type integrase binding sites are shown as black boxes (the P4/P5 pair of sites spans +90 to +110) and the area of Int-L5 protection at the core is indicated by a horizontal striped rectangle (spanning approximately –18 to +18). The 43 bp common core sequence found in both *attP* and the *M.smegmatis attB* site is shown as a white box and spans positions –4 to +38. Coordinate 0 refers to the central base pair between the cut sites, indicated by small arrowheads in Figure 4A.

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 (≤25 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 NH_4Cl; 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 synapsis 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 synapsis. 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 *Sal*I *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-Gene 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 *Hin*dIII, *Eco*RV and *Apa*I 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 mg/ml BSA, 10 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 *Bgl*II site; pGL1 and pMK2 using a unique *Eco*NI site; pMH12.1 using a unique *Hin*dIII 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 mc^{2}155 (4,24), recovered at 37°C, dilutions plated 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

5% polyacrylamide gel in 1× TBE (100 mM Tris, 84 mM borate, 1 mM EDTA) and products visualized by autoradiography. DNA segments containing *attP* were generated by cutting plasmids pGL1, pMK1, pMK2 and pMK3 with *Bam*HI to give 624 bp fragments. DNA fragments were radiolabeled by end-fill with Klenow as described previously (7).

Assay for topoisomerase activity

Buffer conditions were similar to those used for *in vitro* recombination assays, with the exceptions that no spermidine was added and the pH was either 7.5 or 8.0. Approximately 0.05 pmol plasmid pMH94 was incubated with 0.7–14.0 pmol Int-L5 at either room temperature or 37°C for 2 h. The reactions were stopped by addition of SDS to a final concentration of 0.1%, electrophoresed through a 0.8% agarose gel in the absence of ethidium bromide and stained with ethidium bromide for visualization after electrophoresis.

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 (5). 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 mIHF. 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 mIHF; C.E.A.P., J.M.K. and G.F.H., submitted), the stimulated process must be a post-synaptic event, either conversion of the initial synaptic complex into an activated form or a step in strand exchange. Supercoiling 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 (18), 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),

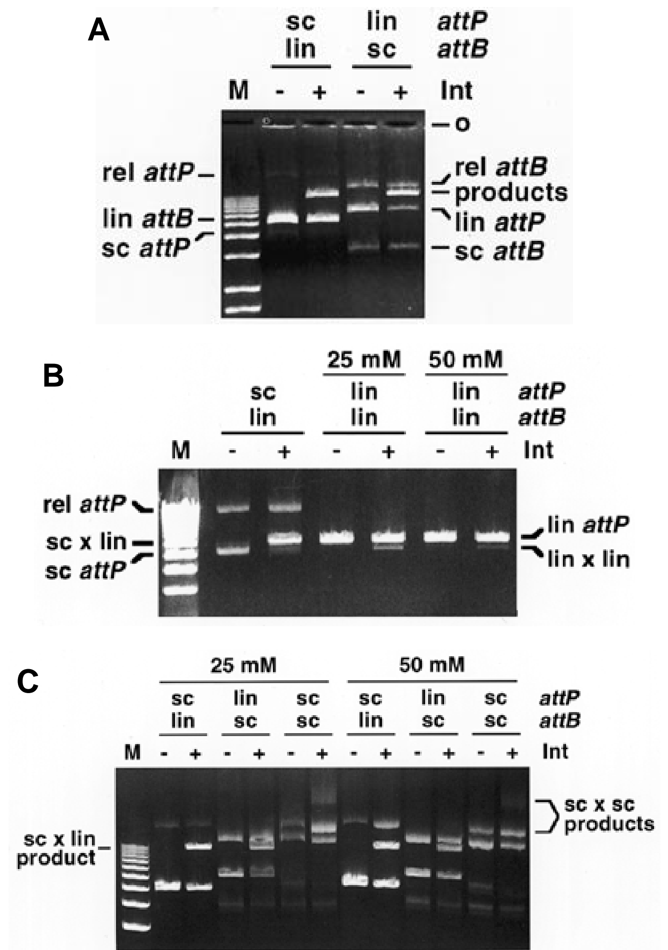

Figure 2. Supercoiling requirements for integrative recombination. (**A**) *In vitro* recombination reactions using supercoiled and linear substrates. A supercoiled (sc) *attP* plasmid was incubated with a linearized (lin) *attB* plasmid and a linear *attP* plasmid with a supercoiled *attB* plasmid (as indicated) for 2 h at 37°C in the presence of Int-L5 (as indicated) and mIHF. The reactions were stopped by addition of SDS and electrophoresed through an agarose gel. The predicted size of the product of integrative recombination between the 6202 bp pMH94 *attP* plasmid and the 4820 bp pMH12.1 *attB* plasmid is 11 022 bp. The positions of the supercoiled and linearized *attP* and *attB* plasmids, the products and relaxed circular (rel) *attP* and *attB* plasmids are indicated. The sizes of the 1 kb DNA markers (M) are (first eight bands, from the bottom up) 1.6, 2.0, 3.1, 4.1, 5.1, 6.1, 7.1 and 8.1 kb. O, origin of electrophoresis. (**B**) *In vitro* recombination reactions using linear substrates at different salt concentrations. A 6202 bp linearized pMH94 *attP* plasmid and a 45 bp *attB* DNA fragment were incubated with Int-L5 (as indicated) and mIHF at 25 or 50 mM NaCl (as indicated), similarly to the reactions in (A). The predicted sizes of the recombinant products are 5496 and 746 bp. A control reaction using a supercoiled *attP* plasmid and the 45 bp *attB* DNA at 25 mM NaCl is also shown. The positions of the linearized *attP* plasmid (lin *attP*), the 5496 bp product of the linear *attP* × linear *attB* (lin × lin) reaction, the supercoiled *attP* (sc *attP*), the 6247 bp linear product of the supercoiled *attP* × linear *attB* (sc × lin) reaction and relaxed circular *attP* (rel *attP*) are shown. The sizes of the 1 kb DNA ladder (M) are (first four bands, from the bottom up) 3.1, 4.1, 5.1 and 6.1 kb. (**C**) *In vitro* recombination reactions using supercoiled and linearized substrates at different salt concentrations. The substrates used are the same as in (A), with the addition of a supercoiled *attP* × supercoiled *attB* reaction. Reactions were performed as in (A) at 25 and 50 mM NaCl, as indicated. The predicted products from the supercoiled *attP* × linear *attB* and linear *attP* × supercoiled *attB* reactions are 11 022 bp linear DNAs (sc × lin product); the products from the supercoiled *attP* × supercoiled *attB* reaction should be the same length, but circularized and in different topological states (sc × sc products). Sizes of the 1 kb DNA marker (M) are (from the bottom up) 3.1, 4.1, 5.1, 6.1, 7.1, 8.1, 9.2, 10.2, 11.2 and 12.2 kb.

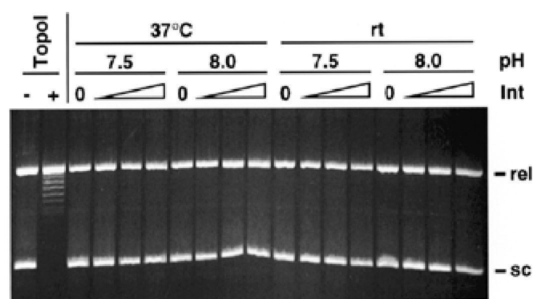

Figure 3. Assay for topoisomerase activity in Int-L5. A supercoiled 6202 bp *attP* plasmid, pMH94, was incubated for 2 h with increasing concentrations of Int-L5 at varying temperature and pH. The amounts of Int-L5 used were 0.7, 7 and 14 pmol. A control reaction in which topoisomerase I (Gibco BRL) was incubated with plasmid pMH94 is shown. The supercoiled (sc) and relaxed circular (rel) states of pMH94 are indicated.

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′-ATTTTTCTTT, 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*

Plasmid	Coordinates of mutation	Wt sequence*	Mutant sequence*	Transformation frequency (%)
pGL1	none			100.0
pMK1	+61 to +65	5′-CCCTC	5′-AAGCT	107.1
pMK2	+41 to +50	5′-CTGGTCAGAG	5′-GACCGATATC	89.3
pMK3	+71 to +80	5′-ATTTTTCTTT	5′-GCGCGGGCCC	214.3

Transformation frequencies were determined by electroporation of plasmid DNA into *M.smegmatis*. The pGL1 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*

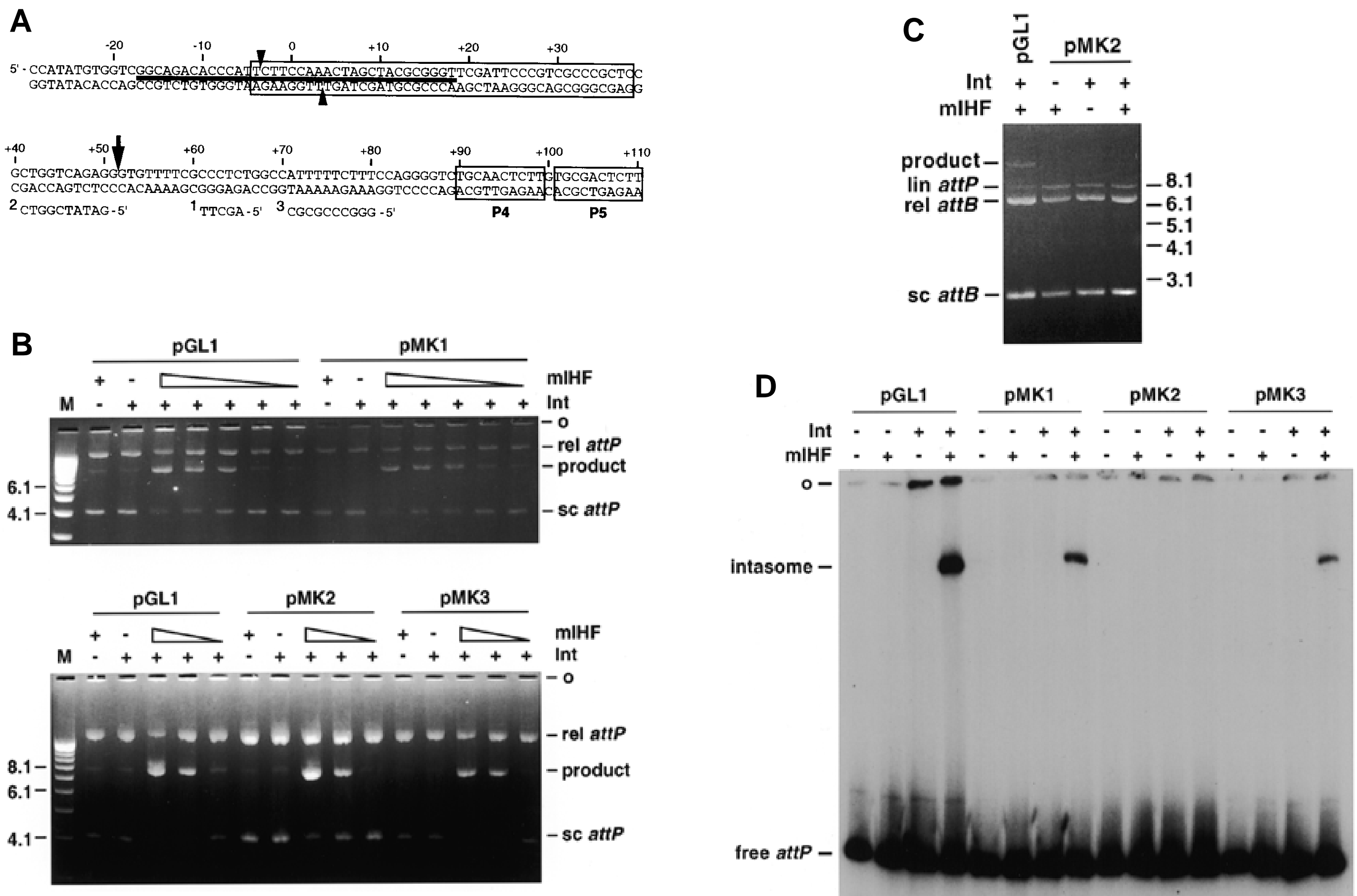

Figure 4. Characterization of an *attP* mutant which imposes a requirement for supercoiling of *attP*. (**A**) Sequence of the core–P4/P5 region of *attP* and mutants. The mutant sequences are shown below the wild-type sequence and correspond to the bottom strand (1, 2 and 3 indicate the substitutions 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.

DISCUSSION

The observations presented here suggest that while integrative recombination of both L5 and λ is enhanced by DNA supercoiling, the stimulatory effect of superhelicity is exerted at different steps in the reaction pathway. In λ integration, supercoiling promotes a relatively early stage of the reaction, assembly of the recombinagenic intasome complex; in the absence of supercoiling, the λ arm-type sites are incompletely protected in footprinting assays (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 nucleophilic 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 λ system that, in the absence of supercoiling of λ *attP*, Int-λ binds to the P1 and P′3 arm-type sites weakly but does not produce a recombinogenic 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-λ 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-λ clearly does not require synapsis. 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 α-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 λ 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 λ *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 bending of pMK2 DNA, as it does in the λ 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 bending 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 situ* 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 Tn*3* 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 γδ 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 accomodate 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/*loxA* 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.

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