Characterization of the *attP* site of the integrative element pSAM2 from *Streptomyces ambofaciens*

Alain Raynal, Annick Friedmann, Karine Tuphile, Michel Guerinéau and Jean-Luc Pernodet

Author for correspondence: Alain Raynal. Tel: +33 1 69 15 62 10. Fax: +33 1 69 15 45 44.
e-mail: alain.raynal@igmors.u-psud.fr

Laboratoire de Biologie et Génétique Moléculaire, Institut de Génétique et Microbiologie, UMR CNRS 8621, Bât. 400, Université Paris-Sud, F-91405 Orsay Cedex, France

pSAM2 is integrated into the *Streptomyces ambofaciens* chromosome through site-specific recombination between the element (*attP*) and the chromosomal (*attB*) site. The 43 kDa integrase protein encoded by pSAM2 catalyses this recombination event. Tools have been developed to study site-specific recombination in *Escherichia coli*. *In vivo* studies showed that a 360 bp fragment of *attP* is required for efficient site-specific recombination and that *int* can be provided *in trans*. pSAM2 integrase was purified and overexpressed in *E. coli* and *Int* binding at the *attP* site was studied. DNaseI footprinting revealed two sites that bind integrase strongly and appear to be symmetrical with regard to the core site. These two P1/P2 arm-type sites both contain a 17 bp motif that is identical except at one position, GTCACGCAG(A/T)TAGACAC. P1 and P2 are essential for site-specific recombination.

Keywords: actinomycetes, integrase, site-specific recombination, attachment site

INTRODUCTION

As well as their complex life cycle and industrial importance, *Streptomyces* spp. are also remarkable due to the accessory genetic elements they harbour. Integrative elements, which have only been found in actinobacteria, are present as integrated chromosomal copies. During mating with a strain lacking them, these integrative elements are able to excise, to transfer and to integrate at a specific site in the chromosome of their new host. Most integrative elements have similar functions to plasmids, such as pock formation associated with transfer, and are able to replicate autonomously. Such elements have been isolated from various actinobacteria belonging to the genera *Streptomyces*, *Saccharopolyspora*, *Micromonospora* and *Amycolatopsis*. The integration and excision processes have been studied in some of these elements [pSAM2 (Boccard *et al.*, 1988), SLP1 (Omer & Cohen, 1986), pMEA100 (Madon *et al.*, 1987), pSG1 (Cohen *et al.*, 1985), pIJ408 (Sosio *et al.*, 1989), pSE211 (Brown *et al.*, 1990), pSE101 (Brown *et al.*, 1994) and pMEA300 (Vrijbloed *et al.*, 1994)] and shown to involve site-specific recombination.

pSAM2 is an 11 kb element, originally isolated from *Streptomyces ambofaciens*, which produces the macro-lide antibiotic spiramycin (Pernodet *et al.*, 1984). pSAM2 can replicate, is self-transmissible, elicits the lethal

zygosis reaction (pock formation) and mobilizes chromosomal markers (Smokvina *et al.*, 1991). Furthermore, pSAM2 has a recombination system that is very similar to that of temperate phages. pSAM2 is integrated into the chromosome through site-specific recombination between the element (*attP*) and the chromosomal (*attB*) sites. These regions share a 58 bp segment extending from the anticodon loop to the 3' end of a tRNA^{Pro} gene (Mazodier *et al.*, 1990; Boccard *et al.*, 1989).

In a previous study (Raynal *et al.*, 1998), the pSAM2 site-specific recombination system was reconstructed in *Escherichia coli* and the chromosomal *attB* site was characterized by use of two compatible plasmids, one carrying the *attP* site and expressing the pSAM2 integrase and the other carrying various fragments of the chromosomal *attB* region. With this system, it was shown that *Int* is the only *Streptomyces* protein required for site-specific integration and that *Int* and *Xis* are both required for site-specific excision. The *attB* region required for efficient site-specific recombination was shown to be 26 bp long and centred around the anticodon loop. Comparison of this 26 bp region with *attP* suggested, according to the lambda model (Campbell, 1992), that B and B', and C and C', core-type *Int* binding sites consist of 9 bp imperfect inverted repeats separated by a 5 bp overlap region.

This study aimed to characterize the *attP* site with respect to its minimal size and to the integrase binding sites.

METHODS

Strains, plasmids and media. *E. coli* DH5 α (Hanahan, 1983) was used for cloning experiments and plasmid DNA propagation. pCRII (Invitrogen) was used for cloning PCR products, and pBluescript (Stratagene) was used for cloning experiments. pMCL200, a low-copy-number p15A derivative (Nakano *et al.*, 1995), was used for the construction of *attP*-containing plasmids. Integrase was overexpressed by use of a pET-21a(+) vector in *E. coli* BL21(DE3) (Novagen). The *attB*-containing plasmids, pOSattB and pOSint1 Δ P, a pOSint1 derivative expressing *int* (Raynal *et al.*, 1998) and deleted for the *attP* site (see Fig. 1), were used for *in vivo* recombination experiments.

Bacteria containing plasmids were routinely grown on LB agar supplemented with 100 μ g ampicillin (Ap) ml⁻¹, 40 μ g streptomycin (Sm) ml⁻¹, 20 μ g chloramphenicol (Cm) ml⁻¹. Liquid LB was supplemented with 50 μ g Ap ml⁻¹, 20 μ g Sm ml⁻¹, 10 μ g Cm ml⁻¹.

Construction of *attP*-containing plasmids. The complete *attP* region was isolated from pTS33 (Smokvina *et al.*, 1990) as an

AvrII–*Bgl*II fragment whose *AvrII* extremity was blunt-ended by Klenow treatment. The resulting fragment was cloned into *EcoRV*/*Bam*HI-digested pMCL200 vector generating pOS-*attP*pSAM2. The minimal *attP* site and parts of this site (a, b, c) (Fig. 2) were cloned either by subcloning or by PCR amplification. pOSattPbc was derived from pOSattPpSAM2 after *Xba*I–*Dra*III deletion in the 5' region followed by a *Sall*–*Sall* deletion in the 3' region. Deletion was performed by generating blunt ends with Klenow treatment, followed by ligation.

*attP*min, *attP*ab and *attP*b were generated by PCR amplification using the PminX/PminE, PminX/PB3 and PB5/PB3 primer pairs, respectively (Fig. 2). (Oligonucleotides: PminX, 5'-GGACGGCATCCTCGAGCGGGGTCC-3'; PminE, 5'-CGCGAATTCGTCGACGAGCAGGCCGGTGAGTG-3'; PB5, 5'-GCGATCGATCTCACCTGGTGTCTCTGTC-3'; PB3, 5'-CGCGAATTCCTACTGGCCCAAGGTCGAGC-3'; restriction sites present in the primers are underlined.) The amplified fragments were cloned into pMCL200 as *Xho*I–*Eco*RI, *Xho*I–*Eco*RI and *Cla*I–*Eco*RI fragments, respectively. PCR was performed on a TECHNE thermocycler (Fisher) using *Taq* DNA polymerase (Amersham Pharmacia Biotech). Synthetic oligonucleotides were provided by Genosys Biotechnologies.

Construction of the integrase expression plasmid. To overproduce and to purify the pSAM2 integrase, the *int* gene was placed under the control of the T7 promoter of the *E. coli* plasmid pET-21a(+) (Novagen) in which the basal expression is very low in the absence of IPTG induction. The *int* gene was PCR amplified from pOSint1 (Raynal *et al.*, 1998) with the Int5B/IntTag primer pair. A *Bam*HI site was introduced immediately upstream of the *int* ATG start codon by use of the Int5B (5'-TTTGGATCCATGGCCAAGCGACGTAGC-3') synthetic sequence.

The IntTag sequence (5'-GTGCTCGAGTCGCGCCGGTCCCCGCTTG-3') hybridizes to the 3' end of the integrase coding sequence. The cloning of a *Bam*HI–*Xho*I amplified fragment in pET-21a(+) led to an in-frame fusion, providing a His-Tag coding sequence at the C-terminus of the integrase. pET-21int was obtained in DH5 α and then introduced into BL21(DE3) to overproduce integrase after IPTG induction.

Purification of integrase. The *int* gene was cloned in the expression vector, pET21a+, under the control of the IPTG-regulated T7 promoter, yielding pET21-int. A His-tag was added to the C-terminal end of the integrase. Five millilitres of a 37 °C overnight preculture of *E. coli* BL21(DE3) containing pET-21int were inoculated into 500 ml LB medium containing 100 μ g ampicillin ml⁻¹. The culture was grown at 22 °C with shaking to an OD₆₅₀ of 0.45. IPTG was then added (1.0 mM) and the cells were grown for a further 5 h at the same temperature and harvested by centrifugation. After washing once in 50 mM Tris, 100 mM NaCl, pH 8, the pellet was resuspended in 5 ml of the same buffer and frozen at –20 °C before lysis. Lysozyme (1 mg ml⁻¹) was added and the sample was lysed for 30 min at room temperature. After centrifugation the pellet was resuspended in the same buffer containing 0.1% sodium deoxycholate. The resulting suspension was denatured by sonication and centrifuged at 13000 g for 10 min. The resulting supernatant was further purified, as described by Novagen, and the soluble fraction was found to contain only a small amount of integrase. Therefore, integrase was purified from inclusion bodies present in the pellet following the last centrifugation step. The pellet was resuspended in binding buffer (5 mM imidazole,

Fig. 1. Structures of *attP*- and *attB*-containing plasmids. pOSint1 Δ P is an integrase-producing plasmid deleted for the *attP* site; replicative at 37 °C, Ap^R. pOSattP shows a schematic structure of all the *attP*-containing plasmids. Replicative at 37 °C, Cm^R. pOSattB is a plasmid carrying a 400 bp *Sma*I–*Sma*I fragment including the chromosomal *attB* site; replicative at 30 °C, Sm^R. As a first step *attP*-containing plasmids were introduced into the strain expressing integrase. As a second step, an *attB*-containing plasmid was introduced into the resulting strain in order to select Sm^R colonies at 42 °C.

Fig. 2. Schematic representation of the *attP* site located immediately downstream of the *Int* coding sequence. The *AvrII*–*BglII* fragment was isolated from pSAM2. All the restriction sites used in subcloning experiments are represented. *attPpSAM2*, *attPmin*, *attPab*, *attPbc* and *attPb* were cloned in pMCL200. *attPmin* was cloned in pBluescript KS+ for footprint experiments. The shaded bars represent the 58 bp identity segment; the circles at the end of the shaded bars (core site) show the localization of the recombination point; the black arrows indicate the position and orientation of the oligonucleotides used for PCR amplification.

0.5 M NaCl, 20 mM Tris/HCl pH 7.9) containing 6 M urea (final concentration). All further purification steps (loading, washing and elution) were performed with buffer containing 6 M urea. Proteins were eluted in 300 mM imidazole, 0.5 M NaCl, 20 mM Tris/HCl pH 7.9, 6 M urea. Fractions (1 ml) were eluted from the His-bind resin column, and the A_{595} revealed that integrase was in fractions 2, 3 and 4. Urea was gradually removed by successive dialysis to a final urea concentration of 1.5 M to allow protein refolding. A 20% (v/v) glycerol solution containing 0.6 mg integrase ml⁻¹ was split into aliquots and stored at –20 °C. SDS-PAGE was carried out in 10% acrylamide gels according to Sambrook *et al.* (1989). Proteins were detected by Coomassie blue staining.

DNase I footprinting. DNase I footprinting experiments were performed by use of the Promega Core Footprinting System. The probes consisted of fragments carrying a 3' and a 5' protruding end isolated from the cloned *attP* minimal site. The 5' end was radio-labelled by filling in with the Klenow fragment of DNA polymerase I and [α -³²P]CTP. The labelled fragment was loaded onto an agarose gel and purified by use of the GFX Gel Band Purification Kit (Amersham Pharmacia Biotech). Binding reactions were carried out in 25 mM Tris/HCl pH 8.0, 6.25 mM MgCl₂, 0.5 mM EDTA, 0.5 mM DTT, 200 mM KCl and 10% (v/v) glycerol buffer. Purified integrase (10 µg) was incubated with 50000 c.p.m. of labelled probe at 25 °C for 1 h. Reactions were treated with 1.5 U DNase I (RQ1 DNase Promega) for 1 min at 25 °C and the resulting product was analysed on a 6% sequencing gel.

RESULTS

Tripartite system used for the study of *attP* in *E. coli*

pSAM2 site-specific recombination is functional in *E. coli* and requires the *attP* and *attB* sites and integrase production (Raynal *et al.*, 1998). In these experiments, the three partners were carried by three different compatible plasmids. *int*, carried by pOSintAP, was expressed under the control of the IPTG-inducible *ptrc* promoter on an Ap^R multicopy plasmid, derived from

pTrc99A (Amann *et al.*, 1988) and replicating with a *ColE1* replication origin. The *attB* site was carried by pOSattB, as previously described (Raynal *et al.*, 1998). This low-copy-number plasmid, constructed from pGB2ts (Clerget, 1991), a pSC101 derivative (Churchward, 1984), confers Sm^R and replicates at 30 °C but not at 42 °C. The pSAM2 *attP* site was carried by pMCL200, a low-copy-number p15A derivative (Nakano *et al.*, 1995), conferring Cm^R. The *attP* and *attB* sites were carried by vectors sharing no sequence identity. To test the functionality of the pSAM2 site-specific recombination system, we aimed to select co-integrates arising from site-specific recombination between *attB* and *attP*.

An *E. coli* DH5 α strain harbouring pOSintAP for the production of pSAM2 integrase was first transformed by pOSattP. The resulting clones were selected as Ap^R/Cm^R colonies at 37 °C. The plasmids used are stably maintained in *E. coli*, at least in the presence of a selection pressure, and before transformation with the third plasmid, recipient cells retained the two other plasmids. These strains were further transformed by pOSattB. After 4 h expression in the presence of IPTG (5 $\times$ 10⁻⁴ M) to allow expression of the Sm^R phenotype, incubation was continued overnight at 30 °C in the presence of both Sm (20 µg ml⁻¹) and IPTG. Finally, Sm^R clones were selected at 30 °C and 42 °C, on plates containing Sm (40 µg ml⁻¹) and IPTG. As pOSattB, conferring Sm^R, cannot replicate at 42 °C, the growth of Sm^R clones at 42 °C should indicate the formation of pOSattB–pOSattP co-integrates by site-specific recombination. Sm^R colonies obtained at 42 °C were replica plated to test for Cm^R at 42 °C. All the tested Sm^R colonies isolated at 42 °C were Cm^R. In all cases, restriction analysis on plasmids isolated from these strains confirmed that they were co-integrates resulting from recombination between *attB* and *attP*, even when the recombination efficiency was very low. In the

Table 1. Recombination between *attP* and *attB* on plasmids in *E. coli*

All the plasmids containing the 'native' *attP* site and its derivatives are presented, with the origin of the inserts (subcloning or PCR amplification) and their sizes. The estimated recombination efficiencies between pOSattP and pOSattB plasmids are given as percentages. These values are means obtained with three independent experiments. In each experiment, 100 % represented more than 1500 colonies.

<i>attP</i> -containing plasmid	Cloned fragment	Insert origin	Insert size (bp)	Recombination (%)
pOSattPpSAM2	<i>Bgl</i> II– <i>Ave</i> II	Subcloned	829	94
pOSattPmin	<i>Xho</i> I– <i>Eco</i> RI	PCR	361	92
pOSattPbc	<i>Dra</i> III– <i>Sal</i> I	Subcloned	269	0.37
pOSattPab	<i>Xho</i> I– <i>Eco</i> RI	PCR	195	0.19
pOSattPb	<i>Cla</i> I– <i>Eco</i> RI	PCR	115	2.5

absence of the *int* gene, no Sm^R colonies were obtained at 42 °C. The co-integrates were therefore due to site-specific recombination promoted by Int.

As Cm^RSm^R colonies growing at 42 °C were only obtained if co-integrates were formed, the ratio of Sm^R colonies isolated in non-permissive (42 °C) and permissive (30 °C) conditions for pOSattB replication allowed us to measure the relative frequency of recombination. With pOSattPpSAM2, harbouring a 829 bp fragment containing the *attP* region, this ratio was 94 %, confirming that pSAM2 site-specific recombination is very efficient in *E. coli* (Table 1) even when Int acts *in trans* on *attP* sites. Intermolecular recombination between *attP* and *attB* was assayed in our experiments and the conclusions reached about the sequences required do not necessarily apply for the excision reaction.

Identification of the minimal *attP* functional site

Given the proposed similarity between the organization of the pSAM2 *attP* and the *attP* sites of other temperate phages (Boccard *et al.*, 1989), we expected to find arm-type Int binding sites on both sides of the core-type Int binding site. Therefore we amplified, as a putative minimal *attP* site, a fragment extending about 200 bp either side of the *attP* core region (Fig. 2). This 360 bp fragment (*attP*min) was tested for *in vivo* site-specific recombination in the same way as *attP*pSAM2 and gave 92 % of co-integrates. Thus, this fragment contains all the sites required for efficient recombination. This *attP*min site was further divided into three regions, a, b and c: b containing the core binding site, a and c containing the putative arm-type sites (Fig. 2). Various fragments corresponding to regions a and b (*attP*ab), b alone (*attP*b) and b and c (*attP*bc) were tested for their ability to recombine. Although *attP*b contained the 58 bp identity segment, shared by *attP* and *attB*, and the core site where recombination takes place, it did not recombine efficiently, demonstrating that sequences present in a and/or c are required. The results obtained with *attP*ab and *attP*bc (Table 1) clearly showed that the

a and c regions are involved in the recombination event. The *attP*min site was further analysed for *in vitro* binding of the integrase.

Purification of integrase

Before purifying integrase, the functionality of the His-tagged integrase was studied *in vivo*. As for the study of the *attP* site, we used pOSattPmin and pOSattB to provide recombinant sites and pET21-int to produce the His-tagged integrase in recombination experiments performed in the expression strain BL21(DE3). The frequency of recombination was 85 % with pET21-int, compared to 92 % with pOSint1ΔP in DH5α. These results clearly show that the His-tagged integrase encoded by pET21-int was functional and bound efficiently to its target sequences.

Integrase was purified from inclusion bodies using His-bind resin. After the purification of these inclusion bodies, integrase was recovered under denaturing conditions (6 M urea) followed by partial renaturation. Approximately 2 mg purified integrase was obtained from 500 ml culture. Analysis of the final product by 10 % SDS-PAGE showed a unique band at 43 kDa, corresponding to the purified integrase (Fig. 3).

Study of Int binding sites in *attP* by footprinting analysis

To determine the precise location of the Int binding sites, we investigated the ability of Int to protect *attP* against DNaseI cleavage. Firstly, the *Xho*I–*Eco*RI fragment corresponding to the *attP*min region was cloned into pBluescript KS+. The *Asp*718I–*Dra*III (104 bp) and *Nco*I–*Pst*I (165 bp) fragments were isolated from the resulting plasmid and labelled at their 5' protruding end, respectively *Asp*718I and *Nco*I. *Asp*718I and *Pst*I sites were provided by the multiple cloning site of the vector. After purification on agarose gel, the resulting labelled fragments were used to analyse binding on the top strand of the a and c regions (Fig. 2). In the same way, the *Bam*HI–*Dra*III fragment (226 bp) labelled at

Fig. 3. Purification of overexpressed His-tagged Int from *E. coli*. The fractions from each purification step were analysed on 10% SDS-PAGE and Coomassie blue stained. Lanes: 1, Molecular mass markers; 2, total *E. coli* crude extract; 3, purified inclusion bodies after 0.45 μ m filtration; 4, soluble fraction; 5/6, fractions 2 and 3 eluted from the His-bind column.

the *Bam*HI end was used to analyse the protected sequence on the bottom strand of the *c* region of the *attP* site. After integrase binding and DNaseI digestion, the final products were analysed on a 6% sequencing gel. The results showed two domains of 28 nt protected from the DNaseI digestion, located in the a and c region of the *attP* site (Fig. 4). These domains show the same protection patterns: strongly protected 6 nt, 9 nt and 7 nt motifs separated by two blocks of three weakly or not protected nucleotides. This weak protection at certain positions may be due to the use of a high K^+ concentration (200 mM), which increases specificity but decreases the affinity of protein–DNA binding.

In accordance with the nomenclature used in the lambda system, these two protected domains were designated as arm-type sites. Comparison of the two arm-type sites in the protected regions revealed a common 17 nt sequence with a single mismatch: GTCACGCAG(A/T)TAGACAC. This sequence is probably the target of Int. This sequence was absent in the central (b) region of *attP*min and was only found twice in the complete *attP*min region. These two arm-type sites were called P1 and P2. Several attempts have been made to investigate the putative binding of integrase at or around the core site, but no binding was observed in our experimental conditions, on a fragment carrying the core site alone or both the core site and the P1 defined site, even in low K^+ concentration (50 mM), which increases the binding affinity. This observation was not really surprising and confirmed that although the core binding site is essential for recombination, it binds Int more weakly than the arm sites (Dorgai *et al.*, 1998; Tirumalai *et al.*, 1998).

Fig. 4. DNaseI footprinting of *attP*–Int complexes. 1, DNaseI footprinting of the complex between Int and *attP* DNA 5' labelled (*Bam*HI site) at the 3' end of the *attP* site. Only the bottom strand is presented. 2, DNaseI footprinting of the complex between Int and *attP* DNA 5' labelled (*Asp*718I site) at the 5' end of the *attP* site. The top strand is presented. – and + indicate respectively absence or presence (10 μ g) of integrase. Due to the presence of compression in the sequencing gel of the *attP* region, because of 21 bp GC-rich inverted repeats, the size marker presented here is the pBluescript KS+ vector sequenced starting from the T3 universal primer. The 17 nt repeat (16/17) sequence found in both the A and B regions is boxed; these repeat sequences are considered as arm-type sites called P1 and P2 (see Fig. 5).

If we represent the pSAM2 minimal *attP* site with a coordinate 0 at the centre of our previously described core site, the 17 bp repeat sequences lie between

Fig. 5. Schematic representation of the entire region including both the Int coding sequence and the *attP* site. P1 and P2 arm-type sites are indicated.

positions $-100/-116$ in the 5' region and $+102/+118$ in the 3' region, represented as P1 and P2 in Fig. 5. Thus, the two defined arm sites, which strongly bind integrase, appear to be symmetrical with regard to the core site at which the recombination event occurs.

DISCUSSION

In this work we have shown that a 360 bp region of the pSAM2 *attP* site is required for efficient recombination between *attB* and *attP*. This region contains the core-type site and two arm-type-binding sites, P1/P2, for the Int protein.

As pSAM2 site-specific recombination is efficient in *E. coli* (Raynal *et al.*, 1998), no *Streptomyces*-specific protein, besides Int, and Int together with Xis for excision, seems to be required for this process. However, the involvement of some *E. coli* proteins, e.g. histone-like proteins, cannot be excluded.

pSAM2 integrase resembles other integrases in its C-terminal part (Nunes-Düby *et al.*, 1998) and this part is most probably involved in the catalytic activity. For the N-terminal part of pSAM2 Int, possibly involved in arm-binding activity, no obvious conserved region was detected after comparison with other integrases. pSAM2 integrase appears to have a strong affinity for arm-type sites, but no integrase binding could be detected on the

core-type site, even in low KCl concentrations, which decrease binding specificity. It seems that the binding of Int to arm-type sites is a prerequisite for proper recognition of the core binding site during integration.

The overall organization of the pSAM2 *attP* site can be compared to those of other phages such as P22, P2, lambda and L5 (Pena *et al.*, 1997) (Fig. 6). In all these cases, the arm-type sites are located between $-60/-130$ and $+60/+120$ nt on both sides of the core site. For pSAM2, only two arm-type sites, consisting of 17 nt direct repeat sequences with a single mismatch, are required. In lambda (Ross & Landy, 1982), P2 (Yu & Haggard-Ljungquist, 1993), P22 (Smith-Mungo *et al.*, 1994) and mycobacteriophage L5 (Pena *et al.*, 1997), the sequences of the arm-type sites are shorter (about 10 nt). For lambda and L5 phages, arm-type binding sites consist of four or five repeats of shorter sequences, but some of them do not appear to be essential, for example P'1/P2 for lambda (Bauer *et al.*, 1986), P3/P6-7 for L5 (Pena *et al.*, 1997). There is little or no sequence similarity between the arm-type sites of these four phages. Nevertheless, the site we described for pSAM2 shares five nucleotides with the lambda consensus (GTCAC). In the five arm-type binding sites described for lambda, the nucleotides TCA are perfectly conserved in the consensus sequence. In addition, arm-type mutants that contain a C-to-T transition, located in the lambda P1, P'2 or P'3 arm-type site, exhibit a 10- to 100-fold reduction in integrative recombination (Bauer *et al.*, 1986). In contrast to phage lambda, for L5, P2 and P22, arm-type sites are arranged in pairs and it was suggested for L5 that Int might bind co-operatively to pairs of sites. However, recent results (Pena *et al.*, 1999, 2000) clearly indicate that the formation of site-specific recombination complexes in L5 is quite different to that in lambda.

The structural organization of the pSAM2 *attP* appears to be simpler than that of the other *attP* sites studied to

Fig. 6. Comparison of *attP* sites. The structures of the *attP* sites of pSAM2, L5, P22, P2 and lambda are presented, centred on the aligned core sites. The positions of arm-type and core-type integrase binding sites are shown and their relative orientation indicated by an arrow.

date. It only contains two arm-type binding sites, but they are longer and are both essential for recombination.

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