Definition of the *attI1* site of class 1 integrons

Sally R. Partridge,^{1,2} Gavin D. Recchia,^{1,2} Carol Scaramuzzi,^{1,2}
Christina M. Collis,¹ H. W. Stokes² and Ruth M. Hall¹

Author for correspondence: Ruth M. Hall. Tel: +1 612 9490 5162. Fax: +1 612 9490 5005.
e-mail: ruth.hall@molsci.csiro.au

¹ CSIRO Molecular Science,
Sydney Laboratory,
PO Box 184, North Ryde,
NSW 1670, Australia

² School of Biological
Sciences, Macquarie
University Sydney,
NSW 2109, Australia

Integron-encoded integrases recognize two distinct types of recombination site: *attI* sites, found in integrons, and members of the 59-base element (59-be) family, found in the integron-associated gene cassettes. The class 1 integron integrase, *IntI1*, catalyses recombination between *attI1* and a 59-be, two 59-be, or two *attI1* sites, but events involving two *attI1* sites are less efficient than the reactions in which a 59-be participates. The full *attI1* site is required for high-efficiency recombination with a 59-be site. It is 65 bp in length and includes a simple site, consisting of a pair of inversely oriented *IntI1*-binding domains, together with two further directly oriented *IntI1*-binding sites designated strong and weak. However, a smaller region that contains only the simple site is sufficient to support a lower level of recombination with a complete *attI1* partner and the features that determine the orientation of *attI1* reside within this region. An unusual reaction between the *attI1* site and a 59-be appears to be responsible for the loss of the central region of a 59-be to create a potential fusion of two adjacent gene cassettes.

Keywords: *attI*, integron, integrase, site-specific recombination, gene cassettes

INTRODUCTION

Gene cassettes are small mobile elements that generally include only a single gene and a downstream recombination site, known as a 59-base element (59-be), which allows recognition and mobilization of cassettes (Hall *et al.*, 1991; Recchia & Hall, 1995; Collis & Hall, 1992a, b; Collis *et al.*, 1993; Hall & Collis, 1995, 1998). Gene cassettes are normally found associated with integrons. Integrons encode a site-specific recombinase (*IntI* integrase) and contain a specific site (*attI*), located adjacent to the *intI* gene in the integron (Stokes & Hall, 1989; Ouellette & Roy, 1987; Martinez & de la Cruz, 1990), and cassettes are inserted into the *attI* site. The simplest route that leads to incorporation of gene cassettes into integrons involves *IntI*-mediated site-specific recombination between the *attI* site in the integron and the 59-be in a circularized cassette (Hall *et al.*, 1991), and both integration and excision of cassettes by *IntI1* have been demonstrated experimentally (Collis & Hall, 1992a, b; Collis *et al.*, 1993).

Four classes of integron, each encoding a distinct *IntI* integrase, have been identified (Stokes & Hall, 1989;

Sundström & Sköld, 1990; Arakawa *et al.*, 1995; Clark *et al.*, 1997; Mazel *et al.*, 1998). However, only the reactions catalysed by the *IntI1* integrase encoded by class 1 integrons have been studied in detail using an *in vivo* experimental system devised by Martinez & de la Cruz (1990). As well as the integrative and excisive reactions between *attI1* and a 59-be (Martinez & de la Cruz, 1990; Recchia *et al.*, 1994; Hall *et al.*, 1999), *IntI1* can also catalyse both integrative and excisive recombination events between two 59-be (Martinez & de la Cruz, 1990; Hall *et al.*, 1991; Stokes *et al.*, 1997). Integrative recombination between two *attI1* sites has also been documented (Recchia, 1996; Hansson *et al.*, 1997). Recombination between secondary sites (2°rs) and 59-be (Francia *et al.*, 1993, 1997; Recchia *et al.*, 1994; Stokes *et al.*, 1997) or *attI1* (Hansson *et al.*, 1997) also occurs at a low frequency.

The architecture of the *attI1* site differs from that of the 59-be sites (Fig. 1). The 59-be family comprises a large number of sites that have diverse sequences and lengths, but share some common features (Hall *et al.*, 1991; Collis & Hall, 1992b; Stokes *et al.*, 1997). All include about 25 bp at each end that conform to consensus sequences that are imperfect inverted repeats of one another. The organization of each consensus region is similar to that of a simple site used by other integrases and comprises a pair of inversely oriented integrase-binding domains,

Abbreviations: 2°rs, secondary recombination site; 59-be, 59-base element; 5'-CS, 5' conserved segment; Ap, ampicillin; Cm, chloramphenicol; *IntI1*, integrase of class 1 integrons; Nx, nalidixic acid; Sm, streptomycin; Su, sulphamethoxazole; Tc, tetracycline; Tp, trimethoprim.

(a) *attI1/qacE* (b) *aadB/qacE* 59-be

Fig. 1. Sequence and structure of *attI1* and a 59-be. The extents of *attI1*, as determined in this study, and of the 59-be, defined by consensus, are indicated by filled bars. Further bases protected by *IntI1* and/or potentially involved in *IntI1* recognition (Collis *et al.*, 1998) are shown by open bars. Sequences related to the 7 bp core site are in bold type (1–4 in *attI1*; 1L, 2L, 1R and 2R in the 59-be) and their relative orientations are indicated with arrows. The extents of simple sites are also shown. The recombination crossover points are marked by vertical arrows and the region derived from the *qacE* cassette is in lower case. (a) The sequence of *attI1/qacE* shown is from bases 1219 to 1288 and 1880 to 1899 in Cameron *et al.* (1986). The experimentally determined strong and weak *IntI1*-binding sites (Collis *et al.*, 1998) and a pair of direct repeats, DR1 and DR2, are indicated. (b) The sequence of the *aadB/qacE* 59-be shown is from bases 1810 to 1899 in Cameron *et al.* (1986). Inverted repeats are underscored with arrows and an asterisk indicates the position of the extra C present in 2L as compared with 2R. The extents of the LH and RH 59-be consensus sequences as defined by Stokes *et al.* (1997) are also marked.

separated by a spacer of 7 or 8 bp (Stokes *et al.*, 1997; Fig. 1b). In contrast, the *attI1* sites of different integron classes do not share most of these features, nor do they share substantial sequence identity with each other (Recchia *et al.*, 1994; Collis *et al.*, 1998; Hall *et al.*, 1999). For *attI1*, 2 and 3 only one potential simple-site region can be detected by inspection of the sequences, whereas for *attI4* even this simple site is not obvious.

As the 59-be and the *attI1* sites represent two distinct types of recombination site, three formally distinct reactions, *attI1* × 59-be, 59-be × 59-be and *attI1* × *attI1*, are all catalysed by *IntI1*. The position of the recombination crossover (vertical arrows in Fig. 1) has been localized to between the G and TT in 1R of 59-be (Stokes *et al.*, 1997) and to the equivalent position in site 1 of *attI1* (Hansson *et al.*, 1997). *IntI1*-mediated recombination thus appears to involve only a single strand exchange and the product containing a Holliday junction is presumably resolved by subsequent processes such as replication or the action of a Holliday junction resolvase (Stokes *et al.*, 1997). In an integron that

contains cassettes, the 59-be of integrated cassettes will be composite sites in which the sequence following the G residue in 1R (lower-case letters in Fig. 1) comes from the adjacent cassette or module. Similarly, the *attI1* site will also be a composite site in which most of site 1 is derived from 1R in the 59-be of the first integrated cassette.

The length of the 5'-CS region (in upper case in Fig. 1a) that is required for maximal recombination activity has been delineated experimentally for the *attI1* site (Recchia *et al.*, 1994) using an assay that was shown to measure only recombination between *attI1* and a 59-be. Maximal activity of the *attI1/qacE* site was observed with fragments containing at least 64 bp of the 5'-CS, and smaller fragments which include 33 or 31 bp of the 5'-CS had greatly reduced activities (Recchia *et al.*, 1994). However, in a subsequent study where recombination between two *attI1* sites was measured (Hansson *et al.*, 1997), it was found that no more than 14 bp of the 5'-CS, i.e. only the simple-site region of the *attI1* site, was needed for maximal recombination efficiency with a

complete *attI1* site. More recent studies have examined the binding of IntI1 to DNA fragments that include all or part of the *attI1* region. As expected, two molecules of IntI1 bind to the simple-site region (Gravel *et al.*, 1998). However, IntI1 binds to two further regions located to the left of the simple site (Collis *et al.*, 1998; Gravel *et al.*, 1998). These binding sites (Fig. 1a), one of which is a strong IntI1-binding site and the other a weak binding site, overlap the direct repeats DR1 and DR2 (Collis *et al.*, 1998; Hall *et al.*, 1999).

In this study the differences in the reported length of the *attI1* site have been re-examined. The features of the *attI1* site essential for efficient recombination with either a 59-bp or a second *attI1* site have been assessed using conditions where only one of these reactions can occur. Rare recombinants have been used to more accurately locate the left-hand IntI1-binding domain of the *attI1* simple site.

METHODS

Bacterial strains. *Escherichia coli* [DH5 α *supE44* Δ *lacU169* (ϕ 80 *lacZ* Δ M15) *hsdR17* *recA1* *endA1* *gyrA96* *thi-1* *relA1*] was used for cloning. Strains UB5201 (F⁻ *pro met* *recA56* *gyrA*) and UB1637 (F⁻ *his* *lys* *trp* *recA56* *rpsL*) (de la Cruz & Grinsted, 1982) were used in conduction assays. Bacteria were

routinely cultured in LB medium or LB agar supplemented as appropriate with ampicillin (Ap; 100 μ g ml⁻¹), chloramphenicol (Cm; 25 μ g ml⁻¹), nalidixic acid (Nx; 25 μ g ml⁻¹), sulphamethoxazole (Su; 25 μ g ml⁻¹), or trimethoprim (Tp; 25 μ g ml⁻¹). Antibiotics were obtained from Sigma.

Plasmids. Plasmids used in this study are listed in Table 1. Fragments containing various lengths of the *attI1* recombination site were generated by PCR using pRMH232 as a template and the following primers: RH205, 5'-CGTTA-CGCCGTGGGTGCGACGTTTGAT-3' (*attI1* -78 to -53); RH206, 5'-GCTGTGAGCAATTATCAGCTGAGTGC-3' (*qacE* +7 to +32); RH211, 5'-GGAGCAGCAACGA-TGTTAC-3' (*attI1* -47 to -29), where bases in bold type indicate changes from the published sequence. These fragments were treated with the Klenow fragment of DNA polymerase I and cloned directly into the unique *EcoRV* site (GenBank accession no. X06403, position 1680; Rose, 1988) of pACYC184 (Cm^R Tc^R; Chang & Cohen, 1978; Table 1). The plasmids recovered were sequenced to confirm that they had the required inserts and to determine their orientation. Orientation 1 refers to plasmids in which the right-hand end of each composite site, as shown in Fig. 1, is closest to the origin of replication of pACYC184, while orientation 2 is the opposite. One clone in each orientation was retained. pRMH653 and pRMH654 were identified in this process and presumably arose by spontaneous deletion of part of the RH211/RH206 PCR product. pRMH653 includes only 36 bp

Table 1. Plasmids

Plasmid	Description	Position in pACYC184*	Relevant phenotype	Reference
R388	33 kb IncW plasmid containing In3 (cassettes <i>dfpB2-orfA</i>)	NA	Tp ^R Su ^R Tra ⁺ IntI1 ⁺	Avila & de la Cruz, (1988)
pSU2056	1176 bp <i>RsaI</i> - <i>Bam</i> HI fragment of In2 in pUC9	NA	Ap ^R IntI1 ⁺	Martinez & de la Cruz (1990)
pMAQ76	33 bp 5'-CS/198 bp <i>qacE</i>	1979- <i>EcoRV</i> (1)	Cm ^R	Recchia <i>et al.</i> (1994)
pMAQ79	31 bp 5'-CS/198 bp <i>qacE</i>	1986- <i>EcoRV</i> (1)	Cm ^R	Recchia <i>et al.</i> (1994)
pRMH232	256 bp 5'-CS/198 bp <i>qacE</i>	<i>Sph</i> I- <i>Hind</i> III (1)	Cm ^R	Collis <i>et al.</i> (1993)
pRMH251	64 bp 5'-CS/198 bp <i>qacE</i> from pRMH232	<i>EcoRV</i> (1)	Cm ^R	Recchia <i>et al.</i> (1994)
pRMH313	64 bp 5'-CS/198 bp <i>qacE</i> from pRMH232	<i>EcoRV</i> (2)	Cm ^R	This study
pRMH560	Cassette-free derivative of R388	NA	Su ^R Tra ⁺ IntI1 ⁺	This study
pRMH636	87 bp PCR fragment (RH219/RH206) of pMAQ79 in <i>Hind</i> III site of pUC19	NA	Ap ^R	Collis <i>et al.</i> (1998)
pRMH651	110 bp PCR fragment (RH205/RH206) of pRMH232	<i>EcoRV</i> (1)	Cm ^R	This study
pRMH652	79 bp PCR fragment (RH211/RH206) of pRMH232	<i>EcoRV</i> (2)	Cm ^R	This study
pRMH653	68 bp PCR fragment (RH211/RH206) of pRMH232	<i>EcoRV</i> (1)	Cm ^R	This study
pRMH654	45 bp PCR fragment (RH211/RH206) of pRMH232	<i>EcoRV</i> (1)	Cm ^R	This study
pRMH655	109 bp PCR fragment (RH205/RH206) of pRMH232	<i>EcoRV</i> (2)	Cm ^R	This study
pRMH658	79 bp PCR fragment (RH211/RH206) of pRMH232	<i>EcoRV</i> (1)	Cm ^R	This study
pRMH751	87 bp <i>Bsp</i> HI- <i>Hind</i> III fragment of pRMH636	<i>Bsp</i> HI- <i>Hind</i> III (1)	Cm ^R	This study
pRMH752	81 bp <i>Bsp</i> HI- <i>Sph</i> I fragment of pRMH636	<i>Sph</i> I- <i>Bsp</i> HI (2)	Cm ^R	This study

*Numbers represent positions in pACYC184 according to Rose (1988); numbers in parentheses represent the orientation of the 5'-CS/*qacE* fragment with respect to pACYC184 (see text for details). NA, Not applicable.

of the 5'-CS and 32 bp of *qacE*, while pRMH654 has 45 bp of the 5'-CS and none of the *qacE* sequence, with base 1 (G) of the 5'-CS adjacent to the A at position 1682 on the bottom strand of pACYC184 (accession no. X06403; Rose, 1988).

The plasmid pRMH636 contains an *attI1/qacE* fragment extending from -25 to +32, derived from pMAQ79, in the *HindIII* site of pUC19 (Collis *et al.*, 1998). The *BspHI* site present in the amplification primer RH219 (see Collis *et al.*, 1998) was used in conjunction with either the *HindIII* site or the *SphI* site in pUC19 to release the *attI1/qacE* fragment from pRMH636. These fragments were cloned into the appropriate sites in pACYC184 to give plasmids pRMH751 and pRMH752, in which the *attI1/qacE* sequence is present in opposite orientations. These plasmids were sequenced to confirm that they had the required inserts.

The plasmid pRMH560, a derivative of R388 which has lost both the *dfrB2* and *orfA* cassettes, was generated by *IntI1*-mediated excision of cassettes, as described previously (Collis & Hall, 1992a).

DNA procedures. Plasmid DNA was isolated using an alkaline lysis method (Birnboim & Doly, 1979) or a Wizard Miniprep kit (Promega). Restriction enzyme digests were carried out according to the manufacturers' instructions and fragments were separated on 1% (w/v) or 0.8% (w/v) agarose gels. *EcoRI*-digested bacteriophage SPP-1 DNA (Bresatec) was used as size markers. *BamHI*-digested R388 or pRMH560 were also used as markers for mapping cointegrates. DNA fragments for cloning were isolated from agarose gels using a GeneClean II kit (Bio101). Plasmid DNA for sequencing was annealed to primers according to the method of Jones & Schofield (1990). Double-stranded DNA sequencing was performed using a Sequenase kit, version 2.0 (United States Biochemical) with reaction mixtures containing dITP.

Conduction assays. Recombination efficiency was determined using a conduction (mating-out) assay (Martinez & de la Cruz, 1988, 1990; Hall *et al.*, 1991; Stokes *et al.*, 1997) to measure conduction of a pACYC184-based test plasmid (containing a cloned *attI1* site) from the donor strain UB1637 (RecA⁻ Sm^R) to the recipient strain UB5201 (RecA⁻ Nx^R). Donor cells containing the Tra⁺ plasmid R388 (Tp^R Su^R) or its cassette-free derivative pRMH560 (Su^R; Table 1) were first transformed with the pACYC184-based test plasmid. pSU2056 (Ap^R; Martinez & de la Cruz, 1990) was then introduced to supply *IntI1* integrase *in trans*. Single colonies of donor strains grown on LB agar were screened for Ap^R and Cm^R to confirm that each isolate assayed had a full complement of plasmids after purification.

For matings, 0.1 ml portions of stationary-phase cultures of donor and recipient cells were mixed, spread over the surface of an LB agar plate, and incubated overnight at 37 °C. Cells were then harvested and transconjugants selected on agar containing Tp and Nx (R388) or Su and Nx (pRMH560), while cointegrates were selected on plates containing Cm and Nx. The conduction frequency was expressed as the fraction of the total transconjugants (Tp^R or Su^R) that contained cointegrates (Cm^R). Where possible, three isolates of each donor strain were assayed on at least two occasions and the mean value calculated. For conduction frequencies below 10⁻⁶, where spontaneous mutation of the donor to Nx^R can contribute significantly to the number of Cm^R Nx^R colonies detected, all colonies were screened for Sm^R, which is characteristic of the donor. For a number of cointegrates, the site of insertion of the test plasmid was determined by restriction mapping of the cointegrate DNA with *BamHI* and *HindIII*, as described previously (Hall *et al.*, 1991; Recchia *et al.*, 1994; Stokes *et al.*, 1997).

RESULTS

Extent of the *attI1* site

The activity of cloned fragments containing all or part of the *attI1* recombination site was tested by measuring cointegrate formation resulting from recombination between the cloned site and a recombination site in the integron *In3* found in the conjugative plasmid R388. We have previously shown that when the *attI1* fragment is cloned in pACYC184 in one orientation, only products of recombination with the *orfA/qacE* 59-be, one of three sites in R388, can be recovered (Recchia *et al.*, 1994; Recchia, 1996). This method was used to establish a maximum and minimum for the functional length of *attI1* when the partner site is a 59-be (Recchia *et al.*, 1994) and here a wider range of lengths of the 5'-CS were examined in order to establish the role of the strong and weak *IntI1*-binding sites in *attI1* site activity (Table 2). In agreement with the earlier study, *attI1/qacE* derivatives with either 572 or 64 bp of the 5'-CS each showed a high recombination frequency (approx. 10⁻²). When all or part of the weak *IntI1*-binding site located at positions -56 to -43 (Fig. 1a) was removed, leaving only 47 bp (pRMH658) or 36 bp (pRMH653) of the 5'-CS, the recombination frequency was reduced 10-fold, consistent with a role for this site. Reducing the length of 5'-CS present to 31 bp (pMAQ79) or 25 bp (pRMH751) disrupts the strong *IntI1*-binding site at -37 to -21 and resulted in a further drop in activity of at least 25-fold. A further derivative (pMAQ76) with 33 bp of the 5'-CS retains most of the strong binding site and recombination was at an intermediate level. Thus, for the 59-be × *attI1* reaction, the strong binding site is critical for efficient *attI1* activity and the weak binding site further enhances the efficiency.

The level of recombination activity for the *attI1* fragments that contain only the simple site (pMAQ79 and pRMH751) was only slightly higher than that obtained with the vector alone. This background represents recombination between the *orfA/qacE* 59-be and 2°rs in the pACYC184-based test plasmid (Recchia *et al.*, 1994). These data confirm that, alone, the simple-site region of *attI1* is not an efficient recombination site when the partner site is a 59-be, the activity being equivalent to that of the total of the available 2°rs in the test plasmid. However, restriction mapping of 24 cointegrates containing pRMH751 (25 bp of 5'-CS) showed that, despite the low recombination efficiency, the majority of recombinants (19/24) had resulted from site-specific events involving the *attI1* fragment in pRMH751 and the *orfA/qacE* 59-be. This indicates that, for recombination with a 59-be, the simple-site region of *attI1* is sufficient for *IntI1* to recognize this site in preference to any of the available 2°rs sites.

A further plasmid, pRMH654, contains a fragment that includes bases -45 to -1 of the 5'-CS and hence the strong binding site, but has lost the part of the simple-site region that lies to the right of the recombination crossover (Fig. 1a). This plasmid supports recombination with a 59-be in R388 at a frequency of 7.4 × 10⁻⁵.

Table 2. Effect of length of 5'-CS/*qacE* on efficiency of *attI1* × 59-be recombination

Plasmid*	Length of 5'-CS/ <i>qacE</i> (bp)	Cointegration frequency			
		No. of assays	Range	Mean	%
pRMH232	572/198	4	6.3×10^{-3} – 3.8×10^{-2}	1.6×10^{-2}	106
pRMH651	78/32	6	4.4×10^{-3} – 1.2×10^{-2}	9.1×10^{-3}	61
pRMH251	64/198	6	3.6×10^{-3} – 2.8×10^{-2}	1.5×10^{-2}	100
pRMH658	47/32	6	6.6×10^{-4} – 2.0×10^{-3}	1.3×10^{-3}	9
pRMH653	36/32	6	6.5×10^{-4} – 2.5×10^{-3}	1.1×10^{-3}	7
pMAQ76	33/198	6	1.4×10^{-4} – 6.6×10^{-4}	3.9×10^{-4}	3
pMAQ79	31/198	6	2.9×10^{-5} – 7.7×10^{-5}	4.9×10^{-5}	0.3
pRMH751	25/32	6	2.1×10^{-5} – 5.2×10^{-5}	2.9×10^{-5}	0.2
pRMH654	45/0	7	3.5×10^{-5} – 1.5×10^{-4}	7.4×10^{-5}	0.5
pACYC184	–	6	5.0×10^{-6} – 2.5×10^{-5}	1.3×10^{-5}	0.08

* Donor strains also contain R388 and pSU2056.

Table 3. Effect of length of 5'-CS/*qacE* on efficiency of *attI1* × *attI1* recombination

Plasmid*	Length of 5'-CS/ <i>qacE</i> (bp)	Cointegration frequency			
		No. of assays	Range	Mean	%
pRMH655	78/31	5	1.0×10^{-4} – 7.9×10^{-4}	4.7×10^{-4}	100
pRMH313	64/198	1	1.3×10^{-4}	1.3×10^{-4}	28
pRMH652	47/32	6	2.1×10^{-4} – 6.1×10^{-4}	3.4×10^{-4}	72
pRMH752	25/32	5	2.2×10^{-5} – 7.5×10^{-5}	5.0×10^{-5}	11
pACYC184	–	5	2.8×10^{-7} – 2.3×10^{-6}	7.7×10^{-7}	0.16

* Donor strains also contain pRMH560 and pSU2056.

(Table 2), which is only slightly higher than that observed with pMAQ79 (–31 to +198) and pRMH751 (–25 to +32). This finding indicates that the presence of the strong binding site is at least as important in determining the efficiency of recombination with a 59-be site as the sequence of the core site (consensus GTTRRRY) surrounding the crossover point, which is replaced by GATCCCG in pRMH654. Mapping of 20 recombinants showed that in half of them recombination had occurred between *attI1* in pRMH654 and the *orfA/qacE* 59-be, indicating that this truncated *attI1* site is still recognized in preference to any 2°rs. Most of the remaining cointegrates appeared to be due to recombination between the *orfA/qacE* 59-be in R388 and 2°rs in pRMH654.

To investigate if the shorter length for *attI1* determined by Hansson *et al.* (1997) is due to the fact that they analysed the *attI1* × *attI1* reaction, the assay used here was modified to measure only recombination between two *attI1* sites. Recombinants formed via this reaction are not seen if the fragments are cloned in orientation 1 in pACYC184 but are recovered if the fragment is cloned in the opposite orientation (Recchia, 1996; C. M. Collis, G. D. Recchia & R. M. Hall, unpublished). Therefore fragments of *attI1/qacE* with 78, 64, 47 and 25 bp of the

5'-CS were recloned in the opposite orientation. To ensure that only *attI1* × *attI1* recombination could occur, a derivative of R388 that has lost the *dfrB2* and *orfA* cassettes (pRMH560), leaving only the *attI1/qacE* site, was used in place of R388 in the donor strains. The recombination frequency for fragments containing the full *attI1* site was at least 20-fold lower than for recombination of the equivalent fragments with a 59-be (Table 3), consistent with a lower efficiency for *attI1* × *attI1* events. A small decrease in activity was observed when the fragment length was shortened to include only 25 bp of the 5'-CS (Table 3). However, the recombination frequency for this fragment (pRMH752) was still 65-fold higher than that for the vector alone, which in this case represents recombination between the *attI1* site in pRMH560 and 2°rs in the vector. Restriction mapping of 12 cointegrates formed with pRMH752 revealed that, in all of them, recombination had occurred between the two *attI1* sites.

The *attI1* simple site

Because the left-hand segment of the *attI1* simple site (2 in Fig. 1a) does not contain a region that conforms precisely to the inverse (RYYYAAC) of the 7 bp core site consensus, it is not possible to accurately place this site

(a)		
R388	5'-CS/ <i>dfrB2</i>	GCAGCAGGGCAGTCGCCCTAA AA CAAAGTTAGGCAGCCGTT
B1	5'-CS/vector	GCAGCAGGGCAGTCGCCCTAA AT CTGTATCACATATTCTG
pACYC184	vector	GTGAGCGAGGAAGCGGAATAT AT CTGTATCACATATTCTG
B2	vector- <i>dfrB2</i>	GTGAGCGAGGAAGCGGAATAT AA CAAAGTTAGGCAGCCGTT
R388	5'-CS/ <i>dfrB2</i>	GCAGCAGGGCAGTCGCCCTAA AA CAAAGTTAGGCAGCCGTT
(b)		
R388	5'-CS/ <i>dfrB2</i>	GCAGCAGGGCAGTCGCCCTAA AA CAAAGTTAGGCAGCCGTT
B1	5'-CS/vector	GCAGCAGGGCAGTCGCCCTAA AT AAGTGATAATAAGCGGAT
pACYC184	vector	AACGCCTGGTGCTACGCCTGA AT AAGTGATAATAAGCGGAT
B2	vector- <i>dfrB2</i>	AACGCCTGGTGCTACGCCTGA AA CAAAGTTAGGCAGCCGTT
R388	5'-CS/ <i>dfrB2</i>	GCAGCAGGGCAGTCGCCCTAA AA CAAAGTTAGGCAGCCGTT
(c)		
R388	5'-CS/ <i>dfrB2</i>	GCAGCAGGGCAGTCGCCCTAA AA CAAAGTTAGGCAGCCGTT
B1	5'-CS/vector	GCAGCAGGGCAGTCGCCCTAA AT CTGTATTAAACGAAGCGCT
pACYC184	vector	GCTACCTGTGGAACACCTAC AT CTGTATTAAACGAAGCGCT
B2	vector- <i>dfrB2</i>	GCTACCTGTGGAACACCTAC AA CAAAGTTAGGCAGCCGTT
R388	5'-CS/ <i>dfrB2</i>	GCAGCAGGGCAGTCGCCCTAA AA CAAAGTTAGGCAGCCGTT
(d)		
(a)	AGCGGAATAT AT CTGTATCACATAT	
(b)	CTACGCCTGA AT AAGTGATAATAAGC	
(c)	GAACACCTAC AT CTGTATTAAACGAAG	
	* * *	
<i>attI1/dfrB2</i>	gtcgCCCTAA AA CAAAGTTAGGCagc	
	cagcGGGATT T TGAACAATCCGtcg	
	<div style="display: flex; justify-content: space-around; align-items: center;"> 2 → ← 1 </div>	

Fig. 2. (a–c) Sequences of junctions in rare cointegrates arising from recombination between the inverse core site (site 2 in Fig. 1a) of *attI1/dfrB2* and a 2°rs in pACYC184. Each example (a–c) shows the sequence of the 5'-CS/*dfrB2* junction in R388 and the relevant part of pACYC184. Amongst the five recombinants recovered, types (b) and (c) were found twice. The *attI1/dfrB2* core site (equivalent to 1 in Fig. 1a) is underlined, with the normal crossover point indicated by a vertical arrow. Boundary sequences found in the cointegrates are B1, the boundary between 5'-CS and pACYC184 sequence, and B2, the boundary between pACYC184 sequence and the *dfrB2* cassette. Colons indicate identical bases. Bases within the inverse core site and in pACYC184 to which the crossover can be confined are shown in bold type. The positions of these residues in pACYC184 (GenBank accession no. X06403) are: (a) 701 (bottom strand), (b) 3755–4 (bottom strand) and (c) 3487 (top strand). (d) A possible 2°rs consensus for recombination with site 2 of *attI1*. The regions in pACYC184 where recombination occurred are shown aligned with each other and with the *attI1/dfrB2* simple site. The bases to which the crossover can be confined are shown in bold type. Bases which are conserved in all three 2°rs are marked with asterisks. Core sites 1 and 2 of the *attI1/dfrB2* simple site are indicated by numbered arrows and bases that are outside the consensus region but predicted to be protected by bound IntI1 are in lower case.

on the basis of sequence alone. Five rare recombinants that arose independently by recombination between the inverse core site (designated 2 in Fig. 1a) of the *attI1* simple site and a 2°rs in the pACYC184 vector were recovered. The sequences of the boundaries in these recombinants (Fig. 2) permit the crossover position to be located to either side of the A at –7. The inverse core site shown in Fig. 1(a) has been placed in one of the two

possible positions on the basis of two criteria. The predicted core site shown differs from the consensus at fewer positions (2/7) than the alternative (3/7) and the spacer region (defined as the distance between the abnormal and normal crossover locations) is 7 bp, which coincides with the spacer length of 7 or 8 bp found in the RH simple site of 59-be (Stokes *et al.*, 1997), rather than 6 bp.

(Poirel *et al.*, 2000), the complete version of the cassette has not yet been found, but only a simple site which includes the *attI1* spacer sequence is present. Fig. 3(c) shows a schematic representation of how the substitution of the central region of the 59-be may have occurred. This involves a crossover between two sites that are only rarely the sites of strand exchange, namely 1L in the 59-be and 2 in *attI1*.

DISCUSSION

The discrepancy between the minimum length of the *attI1* site determined by Recchia *et al.* (1994) and by Hansson *et al.* (1997) was re-examined and the difference was traced to the nature of the reaction assayed. The biologically important reaction, i.e. *attI1* × 59-be, assayed here and by Recchia *et al.* (1994) is 100-fold more efficient than *attI1* × *attI1* recombination and requires the full 65 bp *attI1* site extending from -56 to +9 in Fig. 1. The less efficient *attI1* × *attI1* reaction described here and also by Hansson *et al.* (1997) requires only the simple-site region of *attI1* (Fig. 1a). The requirement for the longer *attI1* in the case of recombination with a 59-be site is consistent with *in vitro* binding studies that have shown that the longer region includes two further IntI1-binding domains (Collis *et al.*, 1998; Gravel *et al.*, 1998). It is likely that these two binding sites act as recombination enhancers by retaining the IntI1 recombinase in the vicinity of the simple site. As recombination of *attI1* with a 59-be is far more efficient than recombination between two *attI1* sites, and is also the biologically important reaction responsible for slotting gene cassettes into an integron, the *attI1* site should clearly be defined as the region that includes both the simple site and the two further IntI1-binding domains.

When Hansson *et al.* (1997) replaced R388Δ1, which is equivalent to pRMH560, with R388 and the *attI1* region tested included 36 or more than 200 bp of the 5'-CS, they observed a substantially higher recombination frequency than for the reaction with R388Δ1. This is presumably because the more efficient *attI1* × 59-be reaction can now occur as well as *attI1* × *attI1* recombination. These data are therefore not equivalent to any of the data presented here, where only one of these events is tested at one time. Shorter fragments containing 27, 20 or 14 bp of the 5'-CS tested by Hansson *et al.* (1997) were at least 30-fold less active. Although these results are in broad agreement with those presented here, the residual recombination level is higher than that described here and presumably represents *attI1* × *attI1* recombination, rather than the residual level of *attI1* × 59-be recombination.

Previous studies have shown that in recombination between two 59-be or between *attI1* and a 59-be, the predominant reaction involves the 1R site in the 59-be and site 1 in *attI1* (Martinez & de la Cruz, 1990; Hall *et al.*, 1991; Recchia *et al.*, 1994; Stokes *et al.*, 1997). Furthermore, only a single strand-exchange event occurs (Stokes *et al.*, 1997) and this feature distinguishes

reactions catalysed by the IntI1 integrase from site-specific recombination events catalysed by other integrases, where two strand-exchange events staggered by 6–8 bp normally occur (Sadowski, 1993; Hallet & Sherratt, 1997). The preferential recognition of site 1 in *attI1* and 1R in a 59-be indicates that features of both *attI1* and 59-be type sites ensure that the two sites participating in a recombination event are correctly oriented. Those features appear to lie entirely within the simple-site region of *attI1*, as recombinants arising from a crossover in site 2 of the *attI1* simple site, which can be identified by restriction mapping, were not recovered amongst 19 recombinants where the partner site was the orfA/*qacE* 59-be or 12 recombinants where the complete *attI1* site was the partner.

However, rare recombinants that arose via strand exchange within the 2R site in one 59-be and the 1R site in a second 59-be (Stokes *et al.*, 1997) or the 1L site of a 59-be and the *attI1* site 1 (Hall *et al.*, 1999) have been observed previously. In both cases, the sequences of the junctions within the recombinants revealed that only a single strand-exchange event had occurred. This indicates that strand exchange can occur, albeit with very low efficiency, at sites other than 1R in a 59-be but in both of these cases the crossover is in the normal location in the partner site. Such rare recombinants have been used to locate the 2R core site of 59-be (Stokes *et al.*, 1997).

A further type of rare recombinant that has arisen via recombination between the left-hand IntI1-binding domain (inverse core site) of the *attI1* simple site (2 in Fig. 1a) and a 2°rs in pACYC184 was recovered here. These recombinants allow a more precise definition of the *attI1* simple site than was possible from inspection of the sequence. However, the sequences of the regions in pACYC184 that are involved in the recombination events do not conform to the Ga/tTa/ca/t (Francia *et al.*, 1993) or Ga/tT (Recchia *et al.*, 1994) consensus for 2°rs that participate in reactions with 59-be. Although five bases were conserved in each of the three sites identified here (Fig. 2d), further examples are needed to establish if the conservation is significant. The reaction that gave rise to these recombinants is likely to be extremely rare as the frequency for all recombination events involving *attI1* and 2°rs observed here is extremely low (Table 3). It is approximately 20-fold lower than that for a 59-be with 2°rs (Table 2) and similar background frequencies were observed by Hansson *et al.* (1997). Only two 2°rs that recombined at the normal location in *attI1* (1 in Fig. 1a) have been located by sequencing and both include a region that is closely related to the 7 bp core site consensus (Hansson *et al.*, 1997). Further examples are needed to determine if the consensus for 2°rs that recombine with *attI1* is the same as that for 2°rs that recombine with 59-be. The fraction of 2°rs recombinants that arise via a crossover at the core site (1) or inverse core site (2) of the *attI1* simple site also remains to be determined.

The proposed route that leads to substitution of the *attI1* simple-site spacer for the bulk of a 59-be is also

likely to be extremely uncommon. The schematic model in Fig. 3(c) shows a requirement for an unusual recombination crossover between two core sites (the attI1 site 2 and 1L in the 59-be), neither of which is normally the location for strand exchange. Excision of a cassette from a class 1 integron via crossover between 1L in the 59-be and site 2 in attI1 would give rise in a single step to a circularized cassette containing the unusual hybrid simple site and leave behind the remainder of the 59-be fused to site 2 of attI1. Reintegration of this cassette into a complete attI1 site with the crossover at the normal position would lead to the configuration seen in the known examples. Alternatively, recombination between the 1L site of the 59-be in a cassette contained in one integron with the attI1 site 2 in a second integron would yield similar products in one step. It is likely that the simple site created as a consequence of these events can participate in IntI1-mediated recombination events. However, the efficiency of recombination with either the attI1 site or with a 59-be partner might be expected to be low (equivalent to pRMH751, i.e. -25 to +32 of 5'-CS) and this could provide a strong linkage between the cassette that ends with the simple site and the downstream cassette.

The fact that the architecture of attI1 differs from that of 59-be appears to play a role in ensuring that cassettes are preferentially integrated adjacent to the attI1 site of a class 1 integron. A similar effect is likely to pertain to the attI2, 3 and 4 sites, but the sequences of the known attI sites are not closely related and architecture of these sites has yet to be examined experimentally.

ACKNOWLEDGEMENTS

S. R. P. and C. S. were supported by a grant from the Australian National Health and Medical Research Council. We thank Mi-Jung Kim for analysis of one of the recombinants shown in Fig. 2.

REFERENCES

- Arakawa, Y., Murakami, M., Suzuki, K., Ito, H., Wacharotayankun, R., Ohsuka, S., Kato, N. & Ohta, M. (1995). A novel integron-like element carrying the metallo- β -lactamase gene *bla*_{IMP}. *Antimicrob Agents Chemother* **39**, 1612–1615.
- Avila, P. & de la Cruz, F. (1988). Physical and genetic map of the IncW plasmid R388. *Plasmid* **20**, 155–157.
- Birnboim, H. C. & Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res* **7**, 1513–1523.
- Cameron, F. H., Groot Obbink, D. J., Ackerman, V. P. & Hall, R. M. (1986). Nucleotide sequence of the AAD(2'') aminoglycoside adenylyltransferase determinant *aadB*. Evolutionary relationship of this region with those surrounding *aadA* in R538-1 and *dhfrII* in R388. *Nucleic Acids Res* **14**, 8625–8635.
- Chang, A. C. Y. & Cohen, S. N. (1978). Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the p15A cryptic miniplasmid. *J Bacteriol* **134**, 1141–1156.
- Clark, C. A., Purins, L., Kaewrakon, P. & Manning, P. A. (1997). VCR repetitive sequence elements in the *Vibrio cholerae* chromosome constitute a mega-integron. *Mol Microbiol* **26**, 1137–1143.
- Collis, C. M. & Hall, R. M. (1992a). Site-specific deletion and rearrangement of integron insert genes catalysed by the integron DNA integrase. *J Bacteriol* **174**, 1574–1585.
- Collis, C. M. & Hall, R. M. (1992b). Gene cassettes from the insert region of integrons are excised as covalently closed circles. *Mol Microbiol* **6**, 2875–2885.
- Collis, C. M., Grammaticopoulos, G., Briton, J., Stokes, H. W. & Hall, R. M. (1993). Site-specific insertion of gene cassettes into integrons. *Mol Microbiol* **9**, 41–52.
- Collis, C. M., Kim, M.-J., Stokes, H. W. & Hall, R. M. (1998). Binding of the purified integron DNA integrase IntI1 to integron- and cassette-associated recombination sites. *Mol Microbiol* **29**, 477–490.
- de la Cruz, F. & Grinsted, J. (1982). Genetic and molecular characterisation of Tn21, a multiple resistance transposon from R100-1. *J Bacteriol* **151**, 222–228.
- Francia, M. V., de la Cruz, F. & García Lobo, M. (1993). Secondary sites for integration mediated by the Tn21 integrase. *Mol Microbiol* **10**, 823–828.
- Francia, M. V., Avila, P., de la Cruz, F. & García Lobo, M. (1997). A hot spot in plasmid F for site-specific recombination mediated by Tn21 integron integrase. *J Bacteriol* **179**, 4419–4425.
- Gravel, A., Fournier, B. & Roy, P. H. (1998). DNA complexes obtained with the integron integrase IntI1 at the attI1 site. *Nucleic Acids Res* **26**, 4347–4355.
- Hall, R. M. & Collis, C. M. (1995). Mobile gene cassettes and integrons: capture and spread of genes by site-specific recombination. *Mol Microbiol* **15**, 593–600.
- Hall, R. M. & Collis, C. M. (1998). Antibiotic resistance in gram-negative bacteria: the role of gene cassettes and integrons. *Drug Resist Updates* **1**, 109–119.
- Hall, R. M., Brookes, D. E. & Stokes, H. W. (1991). Site-specific insertion of genes into integrons: role of the 59-base element and determination of the recombination cross-over point. *Mol Microbiol* **5**, 1941–1959.
- Hall, L. M. C., Livermore, D. M., Gur, D., Avoka, M. & Akalin, H. E. (1993). OXA-11, an extended-spectrum variant of OXA-10 (PSE-2) β -lactamase from *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* **37**, 1637–1644.
- Hall, R. M., Collis, C. M., Kim, M.-J., Partridge, S. R., Recchia, G. D. & Stokes, H. W. (1999). Mobile gene cassettes in evolution. *Ann N Y Acad Sci* **870**, 68–80.
- Hallet, B. & Sherratt, D. J. (1997). Transposition and site-specific recombination: adapting DNA cut-and-paste mechanisms to a variety of genetic rearrangements. *FEMS Microbiol Rev* **21**, 157–178.
- Hansson, K., Sköld, O. & Sundström, L. (1997). Non-palindromic attI sites of integrons are capable of site-specific recombination with one another and with secondary targets. *Mol Microbiol* **26**, 441–453.
- Jones, D. S. C. & Schofield, J. P. (1990). A rapid method for isolating high quality plasmid DNA suitable for DNA sequencing. *Nucleic Acids Res* **18**, 7463–7464.
- Martinez, E. & de la Cruz, F. (1988). Transposon Tn21 encodes a RecA-independent site-specific integration system. *Mol Gen Genet* **211**, 320–325.
- Martinez, E. & de la Cruz, F. (1990). Genetic elements involved in Tn21 site-specific integration, a novel mechanism for the dissemination of antibiotic resistance genes. *EMBO J* **9**, 1275–1281.

- Mazel, D., Dychinco, B., Webb, V. & Davies, J. (1998).** A distinctive class of integron in the *Vibrio cholerae* genome. *Science* **280**, 605–608.
- Ouellette, M. & Roy, P. H. (1987).** Homology of ORFs from Tn2603 and from R46 to site-specific recombinases. *Nucleic Acids Res* **15**, 10055.
- Poirel, L., Le Thomas, I., Naas, T., Karim, A. & Nordmann, P. (2000).** Biochemical sequence analyses of GES-1, a novel class A extended spectrum β -lactamase, and the class 1 integron In52 from *Klebsiella pneumoniae*. *Antimicrob Agents Chemother* **44**, 622–632.
- Recchia, G. D. (1996).** *Mobile gene cassettes and integrons: evolutionary and recombinational studies*. PhD thesis, Macquarie University Sydney, Australia.
- Recchia, G. D. & Hall, R. M. (1995).** Gene cassettes: a new class of mobile element. *Microbiology* **141**, 3015–3027.
- Recchia, G. D., Stokes, H. W. & Hall, R. M. (1994).** Characterisation of specific and secondary recombination sites recognised by the integron DNA integrase. *Nucleic Acids Res* **22**, 2071–2078.
- Rose, R. E. (1988).** The nucleotide sequence of pACYC184. *Nucleic Acids Res* **16**, 355.
- Sadowski, P. (1993).** Site-specific genetic recombination: hops, flips, and flops. *FASEB J* **7**, 760–767.
- Stokes, H. W. & Hall, R. M. (1989).** A novel family of potentially mobile DNA elements encoding site-specific gene-integration functions: integrons. *Mol Microbiol* **3**, 1669–1683.
- Stokes, H. W., O’Gorman, D. B., Recchia, G. D., Parsekhian, M. & Hall, R. M. (1997).** Structure and function of 59-base element recombination sites associated with mobile gene cassettes. *Mol Microbiol* **26**, 731–745.
- Sundström, L. & Sköld, O. (1990).** The *dhfrI* trimethoprim resistance gene of Tn7 can be found at specific sites in other genetic surroundings. *Antimicrob Agents Chemother* **34**, 642–650.
- Tolmasky, M. E. (1990).** Sequencing and expression of *aadA*, *bla*, and *tnpR* from the multiresistance transposon Tn1331. *Plasmid* **24**, 218–226.
-
Received 31 May 2000; revised 6 August 2000; accepted 14 August 2000.