**REVIEW
ARTICLE****Gene cassettes: a new class of mobile element**Gavin D. Recchia^{1,2} and Ruth M. Hall¹

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Keywords: gene cassette, integron, site-specific recombination, antibiotic-resistance genes

Overview

Gene cassettes form a diverse group of small mobile elements that already includes over 40 members. Usually, each cassette contains only a single gene and a specific recombination site. At present, the genes found in cassettes are mostly antibiotic-resistance genes, but it is likely that gene cassettes represent a more general modular gene-packaging mechanism. These gene cassettes are normally found integrated at a specific site in an integron, where they are often part of multi-cassette arrays. Three classes of integron are currently known, all of which appear to be able to integrate the same set of cassettes. However, cassettes are also found rarely at non-specific sites. They move by conservative site-specific recombination catalysed by an integrase encoded by the recipient integron, and thus form a family of mobile elements that are more akin to integrating phage and plasmids than to transposons. Cassettes do not, in general, include a promoter and are transcribed from a promoter in the integron. The multi-component cassette-integron system is thus a natural cloning and expression system.

Historical perspective

Mobile gene cassettes were first identified as the various integrated antibiotic-resistance genes found in integrons. In the early 1980s, studies using restriction mapping and heteroduplex analysis revealed that different sets of antibiotic-resistance genes were sometimes found in the same location in otherwise closely related plasmids such as R388 and pSa (Ward & Grinstead, 1982) or transposons such as Tn21, Tn2603 and Tn2424 (Schmidt & Klopfer-Kaul, 1984; Tanaka *et al.*, 1983; Meyer *et al.*, 1983). As the sequences of antibiotic-resistance genes began to be determined in the mid-1980s, several of them including *dhfrB2* (*dhfrII*) from R388, *aadA2* from pSa, *aadA1* from Tn21, *oxa1* from Tn2603 and *oxa2* from R46 were found to be flanked by identical sequences, indicating that they were all integrated at the same site within a common

structure (Cameron *et al.*, 1986; Hall & Vockler, 1987; Ouellette *et al.*, 1987). Over the ensuing years, many further genes determining resistance to aminoglycosides (gentamicin, tobramycin, amikacin, kanamycin, streptomycin and spectinomycin), trimethoprim, chloramphenicol and β -lactam antibiotics were also found in the same site and a second site in the transposon Tn7 was identified (see Hall *et al.*, 1991). There are several cassettes encoding distinct determinants of resistance to each of these antibiotics or antibiotic families. Further cassettes include open reading frames (ORFs), whose functions have not yet been identified (see Hall *et al.*, 1991). At the present time, over 40 distinct cassettes have been identified.

These integrated cassettes are often part of an array that contains two or more cassettes, and as sufficient sequences became available it was possible to determine accurately the boundaries of the gene cassettes (Hall *et al.*, 1991) and to deduce that they were discrete genetic units, integrated at a unique site in larger mobile units that were named integrons (Stokes & Hall, 1989). A gene encoding a product related to integrases of the λ integrase family of site-specific recombinases was identified in the conserved sequences located 5' to the cassettes (5'-CS) in members of the major integron family (Hall & Vockler, 1987; Ouellette & Roy, 1987), and this integrase was shown to catalyse site-specific recombination (Martinez & de la Cruz, 1988, 1990). Subsequent studies demonstrated that the gene cassettes were indeed mobile elements that can be excised from or integrated into an integron by this integrase (Collis & Hall, 1992a, b; Collis *et al.*, 1993; Bunney *et al.*, 1995).

Diversity of cassette-encoded genes

The gene cassettes for which sequences are available are listed in Table 1. Cassettes are generally identified by the name of the gene they encode, or in the case of cassettes containing ORFs whose function is unknown, the ORFs have been assigned letters in the order of their identi-

Table 1. Gene cassettes

Gene cassette*	Protein	GenBank/EMBL accession no.†	Position of cassette‡,§	Length of cassette (bp)§	Length of coding region (bp)¶	Length of 59-base element (bp)§
Resistance to β-lactams						
Class A β -lactamases						
<i>blaP1</i>	PSE-1/CARB-2**	Z18955	102–1145	1044	915	111
<i>blaP2</i>	–	D13210	163–1206	1044	915	111
<i>blaP3</i>	CARB-4	U14749	129–> 1151	> 1023	867	> 92
Class B β -lactamase						
<i>bla_{IMP}</i>	IMP-1	D50438	1179–2058	880	741	127
Class D β -lactamases						
<i>oxa1</i>	OXA-1	J02967	1289–2292	1004	831	90
<i>oxa2</i>	OXA-2	M95287	1439–2314	876	828	70
<i>oxa3</i>	OXA-3	L07945	40–> 900	> 861	828	> 56
<i>oxa5</i>	OXA-5	X58272	63–977	915	804	106
<i>oxa7</i>	OXA-7	X75562	115–988	874	801	65
<i>oxa9</i>	OXA-9	M55547	2269–3225	957	840	69
<i>oxa10</i>	OXA-10 (PSE-2)	U37105	102–1021	920	801	111
Resistance to aminoglycosides						
Aminoglycoside adenyltransferases						
<i>aadA1a</i>	AAD(3'')	X12870	1290–2145	856	792	60
<i>aadA1b</i>	AAD(3'')	M95287	2315–3170	856	792	60
<i>aadA2</i>	AAD(3'')	X68227	145–1000	856	780	60
<i>aadB</i>	AAD(2'')	L06418	1287–1877	591	534	60
Aminoglycoside acetyltransferases						
<i>aacA1††</i>	AAC(6')-Ia	M18967	713–> 1487	> 778	558	–††
<i>aacA4</i>	AAC(6')-Ib	M55547	820–1456	637	555	70
<i>aacA</i> (orfB)	AAC(6')-Id	X12618	896–1421	526	450	72
<i>aacA7</i>	AAC(6')-II	U13880	299–889	591	459	112
<i>aacA</i>	AAC(6')-IIa	M29695	682–1309	628	555	60
<i>aacA</i>	AAC(6')-IIb	L06163	507–1159	653	543	97
<i>aacC1</i>	AAC(3)-Ia	X15852	1304–1880	577	465	109
<i>aacC</i>	AAC(3)-Ib	L06157	609–> 1106	> 498	465	> 34
Resistance to chloramphenicol						
Chloramphenicol acetyltransferases						
<i>catB2</i>	CATB2	M80188	298–1036	739	633	72
<i>catB3</i>	CATB3	U13880	890–1604	715	633	60
<i>catB5</i>	CATB5	X82455	2–> 678	> 677	633	> 25
Chloramphenicol exporter						
<i>cmlA</i>	CmlA	U12338	3227–4775	1549	1260	70
Resistance to trimethoprim						
Class A dihydrofolate reductases						
<i>dfrA1</i>	DHFR Ia	X00926	216–792	577	474	95
<i>dfrA5</i>	DHFR V	X12868	1287–1854	568	474	87
<i>dfrA7</i>	DHFR VII	X58425	573–1189	617	474	134
<i>dfrA12</i>	DHFR XII	Z21672	302–885	584	498	90
<i>dfrA14</i>	DHFR Ib	S76821	216–> 738	> 523	483	> 43
Class B dihydrofolate reductases						
<i>dfrB1</i>	DHFR IIa	U36276	573–1057	485	237	57
<i>dfrB2</i>	DHFR IIb	J01773	707–1090	384	237	57
<i>dfrB3</i>	DHFR IIc	X72585	5854–6261	408	237	57
Resistance to streptothricin						
Streptothricin acetyltransferase						
<i>sat</i>	SAT-2	X15995	247–830	584	525	60

Table 1 (cont.)

Gene cassette*	Protein	GenBank/EMBL accession no.†	Position of cassette‡,§	Length of cassette (bp)§	Length of coding region (bp)¶	Length of 59-base element (bp)§
Resistance to antiseptics and disinfectants						
Quaternary ammonium compound exporter						
<i>qacE</i>	QacE	X72585	4947–5533	587	333	141
Unidentified ORFs						
orfA		J01773 + X12869‡‡	1091–1167 7–430	501	435	69
orfC		X17477	1150–1656	507	378	60
orfD		M95287	4047–4366	320	291§§	60
orfE		U12338	2109–2370	262	246§§	60
orfF		Z21672	886–1205	320	291§§	60

* Cassettes are named after the gene they encode. However, the gene nomenclature used here may differ from that found in the original publications, and in many cases the genes have not previously been assigned names. As an agreed numbering system for the *aacA* and *aacC* genes is not currently available, several of these genes are not numbered. The *dfrA* and *dfrB* genes encode proteins belonging to two distinct families. The ORFs with no known function were assigned letters in the order of their publication.

† The first or the first accurate complete cassette sequence has been used in each case. Incomplete sequences have been used only where no complete cassette sequence is available.

‡ Positions of cassettes are frequently not or incorrectly identified in the published sequence and database entries.

§ >, Database entry ends before the end of the cassette.

¶ The first in-frame initiation codon within the cassette boundary has been assumed to be the start codon.

** The genes encoding the PSE-4 and CARB-3 β -lactamases each differ by only one nucleotide from the *blaP1* sequence.

‡‡ The *aacA1* gene is part of a cassette that also contains an ORF, orfG, followed by a 59-base element (A. Gravel, R. Parent & P. H. Roy, personal communication).

‡‡ The complete sequence of the orfA cassette is not present in any single database entry and has therefore been compiled from two separate entries.

§§ Coding region extends beyond the cassette boundary.

fication and the cassettes are identified by these names (see Hall *et al.*, 1991). Table 1 lists the boundaries of the cassettes, many of which were not identified or were incorrectly identified in the primary publication of the sequence. The extent of each gene, assuming that the first in-frame initiation codon within the cassette boundary is the start codon, is also listed in Table 1. The genes listed account for a significant proportion of the antibiotic-resistance genes found in Gram-negative bacteria of the *Enterobacteriaceae* family and the genus *Pseudomonas*. For most antibiotics, several quite distinct resistance genes are found, and in some cases the mechanism by which resistance is conferred is different. For example, resistance to chloramphenicol by acetylation of the antibiotic (*catB* genes) and by efflux of the antibiotic from the cells (*cmlA* gene) are both represented, and for the aminoglycosides, modification of the antibiotic by acetylation (*aacA* and *aacC* genes) and adenylation (*aadA* and *aadB* genes) are both represented. The β -lactamases encoded by cassettes fall into three distinct families, class A (*blaP* genes), class B metallo β -lactamases (*bla_{IMP}* gene) and class D (*oxa* genes), and the dihydrofolate reductases conferring trimethoprim resistance are also from two unrelated protein families (*dfrA* and *dfrB* genes). Partial sequences of several further potential cassettes (not listed) are found flanking some known cassette sequences, and further cassettes are

likely to be identified in the future. Though at present the genes found in cassettes are mostly antibiotic-resistance genes, this bias is likely to reflect the origins of the genes studied, which are from antibiotic-resistant clinical isolates.

Structure of gene cassettes

Cassettes vary considerably in total length from 262 to 1549 bp (see Fig. 1, Table 1). This is largely because of differences in the size of the gene, as in most cassettes little non-coding sequence flanks the gene. Despite the diversity in the size and function of the genes they contain, cassettes share common features (Hall *et al.*, 1991). Each cassette includes a single gene that covers most of the cassette length and a recombination site known as a 59-base element located downstream of the gene (Fig. 1). Though the 59-base elements differ substantially in length from 57 to 141 bp (see Table 1) they share common features (see below). In many cases, a 59-base element was not identified in the primary publications of the sequences of the cassettes, but in all cases where the complete cassette sequence is available a 59-base element has been subsequently identified (Hall *et al.*, 1991; G. D. Recchia & R. M. Hall, unpublished observations) and their precise locations are listed in Table 1.

Fig. 1. For legend see facing page.

Fig. 1. Structure of gene cassettes. Arrows represent the extent of the gene coding regions. Filled boxes denote complete 59-base elements and striped boxes are 59-base elements for which the complete sequence is currently not available. Restriction sites are: B, *Bam*HI; Bg, *Bgl*II; C, *Cl*aI; E, *Eco*RI; Ev, *Eco*RV; H, *Hind*III; Hc, *Hinc*II; K, *Kpn*I; N, *Nco*I; Nd, *Nde*I; P, *Pst*I; PI, *Pvu*I; PII, *Pvu*II; Sa, *Sac*I; S, *Sal*I; Sm, *Sma*I; Sp, *Sph*I; St, *Stu*I; X, *Xba*I; Xh, *Xho*I.

Fig. 2. Cassette mobility. Integrase-mediated integration of a circular gene cassette into an integron (left-hand side) and at a non-specific location (right-hand side). The *IntI* integrase is encoded within the 5'-conserved segment (5'-CS) of the integron. The integron site required for integration of cassettes, *attI*, is represented by a stippled box. The secondary (non-specific) site is represented by X. P_{ant} is the promoter in the integron 5'-CS which is used for transcription of integrated cassette genes. At a non-specific location the integrated cassette gene can only be expressed if a suitably oriented promoter (P) is present. *IntI* is able to catalyse the excision of a gene cassette from an integron, but excision from a non-specific location is unlikely to occur as two specific recombination sites are not present.

While gene cassettes are most commonly found in an integrated form either in an integron or at a non-specific location, they can also exist free as covalently closed circular molecules (Collis & Hall, 1992b). Free circular cassettes are formed by excision of the cassette from an integron, and are likely to be important intermediates in the dissemination of cassettes (see Fig. 2).

Because of the nature of the site-specific recombination event that leads to integration of a free circular cassette, a small part of the cassette-associated 59-base element is found at the start of the linearized cassette and the remainder is downstream (at the 3' end) of the gene (Fig. 3). The conserved triplet GTT, which is part of the core site GTTRRRY (R = purine, Y = pyrimidine) found at the 3' end of 59-base elements, can be found at both ends of integrated cassette sequences, though only one GTT is part of the cassette (Hall *et al.*, 1991). This led initially to some ambiguity in defining the precise boundaries of cassettes, which has now been resolved experimentally by determining the precise location of the crossover in recombination events involving one partner with a mutation in the GTT (D. O'Gorman, H. W. Stokes & R. M. Hall, unpublished observations). The crossover occurs between the G and the first T, and thus the first six bases

Fig. 3. Boundaries of gene cassettes. Vertical arrows indicate the position of the recombination crossover and hence the boundaries of a gene cassette in a linear array. The core sites (GTTRRRY) found at each end of the integrated cassette are shown. The solid line and arrow above the cassette indicate the extent of the 59-base element associated with the free, circular form of the cassette, and the dashed line and arrow denote those bases making up the 3' end of the functional composite 59-base element in the integrated form. The inverse core site (RYYAAC) at the 5' end of the 59-base element is also shown. Short dashes represent the central region of the 59-base element (not to scale).

of each integrated cassette are derived from the 59-base element and have the sequence TTRRRY. The last base of the integrated cassette sequence is conserved and is a G residue (see Fig. 3).

The cassette-associated recombination sites

An essential feature of gene cassettes is the presence of a recombination site known as a 59-base element that is located downstream of the gene coding region (Hall *et al.*, 1991). Generally, each gene cassette possesses a unique 59-base element, and both the length and sequence of 59-base elements can vary considerably (see Table 1 and Fig. 4). However, despite this heterogeneity, all 59-base elements share several features. They are bounded by an inverse core site (RYYAAC) at the end closest to the 3' end of the gene coding region and a core site (GTTRRRY) at the other end, and are related to a consensus sequence that covers approximately the first and last 20 bases (Collis & Hall, 1992b). Most 59-base elements have a central axis of symmetry as the consensus regions are imperfect inverted repeats. All 59-base elements also contain inverted repeat sequences in their central region, and it is this region that varies in length.

Within the 59-base element family, a few groups of closely related elements can be identified. Members of each group are of similar length and share considerable but not complete sequence identity with one another. The largest group of 59-base elements includes those first identified by Cameron *et al.* (1986) and now contains 12 members, several of which are associated with completely unrelated genes, e.g. the *aadA* genes, *aadB*, *catB3* and *orfD*. Most of the 59-base elements in this group differ from the 60 base consensus for the group (Hall *et al.*, 1991) by only a few bases. However, over half of the known 59-base elements, apart from conforming to the consensus sequences for all 59-base elements at their outer ends, are not otherwise closely related to any other known 59-base element. Nonetheless, several 59-base elements with quite different sequences and lengths have been shown to function as recombination sites recognized by the integron integrase (*IntI*) (Martinez & de la Cruz, 1990; Hall *et al.*, 1991;

Locations of integrated cassettes

Most of the cassettes listed in Table 1 were found integrated at a specific site in an integron of the type shown in Fig. 6(a). Integrons of this class (class 1) are mobile elements that include the gene for the *IntI* integrase and the cassette integration site *attI* (see below for details). Most of the class 1 integrons whose structures have been examined in detail are defective transposons or transposon remnants (Hall *et al.*, 1994; H. J. Brown, H. W. Stokes & R. M. Hall, unpublished observations), though Tn402 is an integron that is an active transposon (Shapiro & Sporn, 1977; Rådström *et al.*, 1994). The cassettes are located at a unique position within the integron-associated recombination site, and several cassettes can be present simultaneously. Integrated cassettes are always in the same orientation, with the 5' end of the gene closest to the integron segment that contains the *intI* gene (see Fig. 6a), and this segment is designated the 5'-conserved segment (5'-CS). Class 1 integrons are the most prevalent class of integron found in clinical isolates and most contain a sulphonamide-resistance gene, *sulI*, in a conserved region found 3' to the integrated cassettes and known as the 3'-conserved segment (3'-CS).

Cassettes have also been found at a unique site near the left end of the transposon Tn7 and its close relatives (see Hall *et al.*, 1991; Sundström *et al.*, 1991; Tietze & Brevet, 1991; Young *et al.*, 1994). The sequences flanking these

cassette arrays are not related to those flanking the cassette arrays described above. However, the general organization of the left end of Tn7 (Fig. 6b) is similar to that of the class 1 integrons described above. A putative but defective *intI* gene (*intI2* is interrupted by a termination codon), whose product is 40% identical to that of *IntI1*, is located 5' to the first cassette, at the left end of Tn7 (Hall & Vockler, 1987; A. Pelletier & P. H. Roy, GenBank/EMBL accession no. L10818), and Tn7 and its relatives therefore form a second class of integron.

Recently, a third class of integron has been identified (Arakawa *et al.*, 1995). In this case, the available sequence data have revealed a new *intI* gene (*intI3*) located 5' to the *bla*_{IMP} cassette which was first found in a class 1 integron (Osano *et al.*, 1994). The putative integrase (*IntI3*) is 61% identical to the *IntI1* integrase (Arakawa *et al.*, 1995). Whether this integrase is also located within a transposon or defective transposon remnant, as are the class 1 and 2 integrases, remains to be established.

The cassette array found in Tn1331 (Tolmasky, 1990) includes the *aacA4* and *aadA1-oxa9* cassettes flanked by approximately 100 bp of the 3'-CS of class 1 integrons and a few bases that may derive from the 5'-CS. This fragment of an integron has become incorporated into a Tn3-like transposon to give rise to Tn1331.

Rarely, known cassettes have also been found outside an integron context and these cassettes (*aadB* and *dfrA14*) are

Fig. 6. Locations of gene cassettes. Cassettes are represented by solid lines and their associated 59-base elements are represented by filled circles. (a) Some naturally occurring class 1 integrons. The *intI1* gene encoding the DNA integrase responsible for cassette movement is contained in the 5'-CS, and the 3'-CS found downstream of the integrated cassettes includes the sulphonamide-resistance gene, *sulI*. The *attI* recombination site is represented by a striped box. The origins of these integrons are: In0, pV51; In1, R46; In2, Tn21 (NR1); In3, R388; In4, Tn1696; In6, p5a; In7, pDGO100; In30, pBWH301. (b) The left end of Tn7 and its close relatives. The putative *intI2** gene, located 5' to the integrated cassettes, is interrupted by a termination codon in Tn7, Tn1825 and Tn1826 (Hall & Vockler, 1987; A. Pelletier & P. H. Roy, personal communication) and in Tn4132 (S. J. Rosser & H.-K. Young, personal communication). Transposition genes present downstream of the cassettes are also indicated.

located at non-specific sites (Recchia & Hall, 1995; H.-K. Young, personal communication). It appears that this is the result of recombination catalysed by *IntI* but involving a secondary recombination site (Recchia & Hall, 1995), and it is likely that further 'wild' cassettes will be found in the future. Indeed, one further potential example of a gene cassette located at a non-specific location is the *dfrA6* gene (Wylie & Koornhof, 1991). Though the sequences flanking the *dfrA6* gene do not resemble any known integron or gene cassette sequences, the DHFRVI protein belongs to the same subfamily as DHFR I, -V and -VII, all of which are encoded by cassette-associated genes, and a potential 59-base element containing inverted repeats and showing some similarity to the consensus for the outer ends of 59-base elements can be identified 3' to the *dfrA6* gene (see Sundström *et al.*, 1993). However, it remains to be proven that *dfrA6* is in fact part of a cassette.

Cassette movement

Some of the gene cassettes listed in Table 1 have been found in integrons of more than one class and it seems reasonable to conclude that all cassettes can be integrated into each of the three known integron types. However, to date all experimental work on cassette movement described below has been carried out with class 1 integrons.

To date, only one protein, the integron-encoded integrase *IntI1*, has been shown to be required for the movement of gene cassettes. *IntI1* is believed to be a member of the λ integrase family on the basis of similarities between the conserved domains shared by members of this family of site-specific recombinases (Ouellette & Roy, 1987; Stokes & Hall, 1989; Abremski & Hoess, 1992). *IntI1* has been shown to catalyse both integrative and excisive recombination events in *in vivo* experimental systems (Martinez & de la Cruz, 1990; Hall *et al.*, 1991; Recchia *et al.*, 1994). *IntI1* has also been shown to be essential for the excision, integration and reassortment of gene cassettes (Collis & Hall, 1992a, b; Collis *et al.*, 1993). The specific recombination sites known to be recognized by *IntI1* are the cassette-associated 59-base elements (Martinez & de la

Cruz, 1990; Hall *et al.*, 1991) and the integron-determined integration site, *attI*, which is largely contained within the 5'-CS and is not obviously related to the 59-base elements (Recchia *et al.*, 1994) (Fig. 7).

Cassette integration and excision occurs via a conservative site-specific recombination mechanism (Fig. 2). In its simplest form, cassette integration involves a single site-specific recombination event between the 59-base element of a free circular cassette and the integron *attI* site (Collis *et al.*, 1993). Cassette excision is the reverse of integration, though in this case the sites involved may be either *attI* and a 59-base element or two 59-base elements (Hall & Collis, 1992a, b). *IntI1*-mediated excision of a cassette from an integron regenerates the circularized form of the cassette (Collis & Hall, 1992b). Other routes involving two site-specific recombination events can also lead to the reciprocal acquisition and loss of cassettes from the arrays found in integrons (see Hall & Collis, 1995), and these routes may even predominate under normal circumstances. When free circular cassettes were integrated experimentally into integrons containing both *attI* and one or more cassettes and hence one or more 59-base elements, a strong preference for insertion at *attI* was observed (Collis *et al.*, 1993). In other experimental systems this preference has not been observed. Rather, events involving two 59-base element recombination sites have generally predominated (Martinez & de la Cruz, 1990; Hall *et al.*, 1991; Collis & Hall, 1992a). While this type of event is clearly important to effect the excision of any cassette that is not located in the first position (i.e. adjacent to the 5'-CS and thus *attI*) in a cassette array, the reasons for the different site preferences in different experimental systems may reflect constraints intrinsic to these systems and this requires further investigation.

Occasionally, recombination can occur between a 59-base element and a secondary (or non-specific) site (Francia *et al.*, 1993; Recchia *et al.*, 1994; Recchia & Hall, 1995) and lead to the integration of a cassette at the non-specific location (Fig. 2). This type of event, though it occurs at very low frequencies (Francia *et al.*, 1993; Recchia *et al.*, 1994) is likely to be important in the dissemination of

Fig. 7. Comparison of the sequences of *attI* and a representative 59-base element (*aadB*). For *aadB*, solid lines indicate the extent of the 59-base element determined by consensus (60 bases). The *attI* site is from class 1 integrons, and the solid and dashed lines indicate the estimated minimum and maximum extent of sequences required for full site activity (40 and 70 bases, respectively). Horizontal arrows denote regions of inverted repeatedness in each site. The core sites are shown in bold and are boxed, and vertical arrows show the position of the recombination crossover. The inverse core site (GCCTAAC) of the *aadB* 59-base element is also boxed. No inverse core site has as yet been identified in the *attI* sequence.

cassette-associated genes and in the evolution of plasmid and bacterial genomes. Excision of cassettes from secondary sites is unlikely to occur as these cassettes are not flanked by two fully active recombination sites (Recchia & Hall, 1995), and integration of a cassette at a secondary site thus leads to the permanent acquisition of a new gene.

Expression of cassette genes

The known gene cassettes do not generally include promoter signals. Indeed in many cases less than 10 bases separate the first in-frame initiation codon from the 5' boundary of the cassette. Exceptions are the *cmlA* cassette that includes both a promoter and translational attenuation signals (Stokes & Hall, 1991) and is described in more detail below, and the *qacE* cassette which includes a weak promoter (Guerineau *et al.*, 1990). Cassettes are normally found integrated at *attI* in only one of the two possible orientations, and this orientation-specificity is presumably determined by some feature of the recombination sites. As a consequence, the genes are all in the same orientation, which allows their expression from a promoter, P_{ant} , in the integron 5'-CS (see Fig. 2). In class 1 integrons, four variants of this promoter are known (Stokes & Hall, 1989; Bunney *et al.*, 1995). These variants differ in strength over at least a 20-fold range (Lévesque *et al.*, 1994; Collis & Hall, 1995), and the strongest has an activity equivalent to or greater than that of the *tac* promoter (Lévesque *et al.*, 1994; C. Tomaras, H. W. Stokes & R. M. Hall, unpublished observations). In a few integron variants a second promoter, P_2 , has been created by the insertion of three bases between otherwise poorly spaced -35 and -10 signals. P_2 is a strong promoter (Lévesque *et al.*, 1994) and increases the level of resistance expressed when the weakest version of P_{ant} is present (Collis & Hall, 1995).

Integration of multiple cassettes at *attI* creates new operons, as all cassette-encoded genes, whether in the first cassette or in promoter-distal cassettes, are dependent on P_{ant} for their expression. Recent studies have demonstrated the existence of long transcripts covering several cassettes (Collis & Hall, 1995). However, the majority of transcripts are shorter. All originate at P_{ant} but end prematurely at discrete points that appear to correspond to the ends of cassettes. These observations raise the possibility that the 59-base elements, which generally include inverted repeats, either act as transcription terminators or as processing signals for endonucleolytic cleavage of transcripts (Collis & Hall, 1995). This effect leads to differences in the levels of transcripts from promoter proximal and promoter distal genes. These differences reflect differences in the level of resistance conferred by the same cassette gene when its cassette is first or located downstream of other cassettes, and in general the first cassette is expressed at the highest level (Collis & Hall, 1995).

When cassettes are integrated at secondary sites, expression is dependent on the presence of a suitably oriented promoter in the target. In the case of the *aadB* cassette found integrated at a secondary site in a plasmid

equivalent to RSF1010, a promoter is present and gentamicin resistance is expressed (Recchia & Hall, 1995). However, it is clearly possible that in other cases the cassette gene could remain silent if no promoter were available.

Translational initiation signals, i.e. potential ribosome-binding sites (RBSs), have been identified preceding several cassette-associated genes and are located within the cassette boundaries. However, in other instances, particularly when the first in-frame initiation codon is located close to the cassette boundary, no obvious RBS is present. In some of these cases further potential in-frame initiation codons are present but whether these are used is not known as, in general, the sequences of the N-terminus of the protein products have not been determined. However, for the *aadA2* gene where the first in-frame initiation codon, an ATG located 10–12 bp from the cassette boundary, is not preceded by an RBS, evidence consistent with the use of a GTG initiation codon 12 bp downstream has been reported (Bito & Susani, 1994) and this GTG is preceded by an RBS. For several cassette genes, the gene coding region is placed such that when the cassette is in the first position in an integrated array the gene is fused in-frame with an ORF of 69 codons in the 5'-CS (see e.g. Cameron *et al.*, 1986), and this could affect the length of the polypeptide product. However, it is unlikely that translation of these genes is initiated within the 5'-CS as RBSs are not found in association with any of the potential initiation codons within this ORF. However, in certain integrons a duplication of 19 bp of the 5'-CS sequence alters the sequence such that translation can potentially be initiated within the 5'-CS at an ATG that is preceded by an RBS leading to a short N-terminal extension of the protein encoded by the first cassette (Wohlleben *et al.*, 1991), and the product of the *aacC1* gene has an N-terminal sequence that indicates that translation commences at this position. However, the *aacC1* gene is also expressed when the cassette is located downstream in a cassette array, indicating that a suitable initiation site is also present within the cassette (Collis & Hall, 1992a). Furthermore, the level of expression of the *aacA4* and *aadA2* genes, measured by the level of antibiotic resistance conferred, does not appear to differ when the cassette abuts a 5'-CS that does or does not contain the 19 bp duplication (C. M. Collis & R. M. Hall, unpublished observations).

Expression of the *cmlA* gene

The *cmlA* gene cassette found in the integron In4 of Tn1696 is unusual in that it includes both a promoter and translational attenuation signals (Stokes & Hall, 1991), and the promoter has been shown to be responsible for the expression of the *cmlA* gene (Stokes & Hall, 1991; Bissonnette *et al.*, 1991). Expression of *cmlA* is inducible by sub-inhibitory levels of chloramphenicol and the region upstream of *cmlA* includes a leader peptide and inverted repeats capable of forming alternate stem-loop structures (Stokes & Hall, 1991). These features are similar to those found upstream of the inducible *cat* and *ermC* genes of Gram-positive bacteria which are known to

be regulated by translational attenuation (for reviews see Lovett, 1990; Weisblum, 1995).

Origins of gene cassettes

The origins of the cassette-associated genes and of the cassettes themselves are fascinating questions. Based on the features of gene cassettes, namely that they contain only a single gene with little flanking non-coding sequences and a unique 59-base element, we have previously speculated that cassettes may originate from transcripts that are converted to DNA (Hall *et al.*, 1991). This would presumably involve the action of an as-yet-unidentified reverse transcriptase. With this scenario, the cassette-associated recombination sites (59-base elements) could either be part of the processed transcript, e.g. a transcription terminator, or added to it prior to its conversion to DNA. Alternatively, the 59-base element could be acquired at a later stage.

Though there is no real evidence to support this proposition, the burgeoning of available DNA sequences for both cassettes and other resistance genes, and for the genomes of plasmids, transposons and bacteria, now permits the examination of some questions that may ultimately shed light on the origins of gene cassettes. For example, several sets of cassette genes are quite closely related, with 60–95% identity between individual pairs of DNA sequences within the coding regions. In some of these groups, for example *aadA1* and *aadA2*, the same level of relatedness extends to the cassette boundaries, thus including the 59-base elements, and these cassettes may have diverged from a common ancestral cassette. However, in other cases quite different 59-base elements are associated with each of the genes in the set. For example, the *dfrA1*, *dfrA5* and *dfrA7* genes are between 62 and 67% identical but the 59-base elements are 95, 87 and 134 bases long, respectively, and are not obviously related (Sundström *et al.*, 1993) except that they all contain the features common to 59-base elements and the first and last 20 bases all show similarity to the consensus for these regions (Hall *et al.*, 1991; Collis & Hall, 1992b). The *catB2* and *catB3* cassettes also contain quite different 59-base elements (Bunny *et al.*, 1995). This is consistent with a separate origin for the gene and the 59-base element in each cassette. The fact that quite closely related 59-base elements are also found associated with unrelated genes supports this conclusion. The largest group of 59-base elements includes those associated with the *aadA* genes, *aadB*, *catB3*, *orfD* and *aacA* (*Ila*) genes and each of these 59-base elements differs from the consensus for this group (Hall *et al.*, 1991) by only a few bases. Other cases in which closely related 59-base elements are associated with different genes have also been found; for example, the *aacA4* and *aacA* (*orfB*) genes encode proteins belonging to distinct protein families but are associated with very similar 59-base elements (see Hall *et al.*, 1991).

If cassettes are indeed constructed from separate pools of genes and 59-base elements, then when potential progenitors of these genes, e.g. chromosomal equivalents of cassette genes, are identified they would not be expected

to be associated with downstream sequences related to the 59-base elements found in the final cassette. Only one case of potential progenitors for cassette-associated genes has been identified to date. These are an ORF (of unknown function) in the chromosome of *Pseudomonas aeruginosa* which shares 67.5 and 69.5% identity with the *catB2* and *catB3* genes, respectively, and the *catB1* gene in the chromosome of *Agrobacterium tumefaciens* which shares 66.0% and 64.8% identity with *catB2* and *catB3*, respectively (Bunny *et al.*, 1995). In both cases the similarity is confined to within the gene coding regions.

Gene cassettes are not transposons

Though gene cassettes are mobile elements, they are distinct from transposons in a number of ways. Firstly, they do not include any genes encoding proteins that catalyse their movement. The integrase is encoded by the integron. Secondly, cassettes are not bounded by inverted repeats, nor are they flanked by a duplication of target sequences. Thirdly, the mechanism used for mobilization of cassettes is distinct from transposition, as it involves conservative site-specific recombination. Gene cassettes thus form a family of mobile elements that are more akin to integrating phage (e.g. λ) and plasmids than to transposons. However, cassettes also form a distinct subgroup of the family of mobile elements that utilize conservative site-specific recombination for movement. Most other mobile elements of this family encode the site-specific recombinase (integrase) responsible for their movement whereas gene cassettes do not. In addition, integrating phage encode both phage proteins and replication functions, integrating plasmids encode replication functions, and the conjugative transposons (which integrate by conservative site-specific recombination and thus are not strictly speaking transposons) encode conjugation functions. In contrast, gene cassettes include only a single gene.

Further distinguishing features of the gene-cassette-integron system are that all known gene cassettes are recognized by the same integrase, despite differences in the precise sequence of their 59-base element recombination sites, and that three known integrases, each with different sequences, appear to be able to recognize the same family of cassettes.

Evolutionary implications

The gene-cassette-integron system is a multi-component system that functions as a natural cloning and expression system and is able to create arrays of genes of enormous diversity. Over 40 gene cassettes and three quite distinct classes of integron have so far been identified and it seems likely that further cassettes and further integrons will be identified in the future. That the majority of gene cassettes identified thus far encode antibiotic-resistance determinants is most likely a reflection of intense selective pressure and the fact that they all originate from clinical isolates. Granted the diversity of the resistance genes, it seems reasonable to assume that genes other than anti-

biotic-resistance genes may also be contained in cassettes. If this is so, the gene-cassette-integron system may be of much more widespread significance, and provide a general mechanism for the dissemination of modular gene units. The notion that genomes are composed of discrete modules (Shapiro, 1993) is an attractive one, and gene cassettes provide clear evidence for the existence of a modular gene-packaging system. This system is thus likely to be an important factor not only in facilitating the spread of antibiotic-resistance genes, but also in the natural engineering of bacterial genomes. The ability of integrons to create new operons (cassette arrays) that can then be fixed in place by deletion of the 59-base elements has obvious potential for the evolution of stable new operons in plasmid and bacterial genomes. The integration and consequent fixation of gene cassettes at secondary recombination sites is also a potential general mechanism for moving genes to new locations.

Acknowledgements

We wish to thank Paul Roy for critical reading of the manuscript and Lars Sundström, Paul Roy and Hilary-Kay Young for making available unpublished data. G.D.R. is supported by a CSIRO Postgraduate Scholarship.

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