Group IIC intron mobility into *attC* sites involves a bulged DNA stem–loop motif

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ABSTRACT

Bacterial group IIC introns are a subclass of group II intron ribozymes that are typically located downstream from transcriptional terminators. Class IIC-*attC* introns constitute a monophyletic subset of subgroup IIC, which preferentially insert into site-specific recombination sequences for integron integrases (*attC*). *attC*s are a diverse family of nucleotide sequences composed of conserved inverted repeats that flank a variable, but palindromic, central region. In this study, we used both PCR and colony patch hybridization methods to determine the basis for recognition of the *attC*_{aadA1} stem–loop motif by the *Serratia marcescens* intron (*S.ma.I2*) in vivo. The quantitative results showed that mobility into the wild-type site occurs at a frequency of 18%, and is strongly biased by the orientation of the homing site relative to the direction of DNA replication. *S.ma.I2* mobility results into mutant *attC*_{aadA1} sites are consistent with recognition of stem–loop motifs in unwound DNA. The homing frequency results showed that, while the entire *attC* sequence is not necessary for recognition of the insertion site, short deletions of the *attC* stem–loop motif inhibited the intron mobility. Moreover, our data show that *S.ma.I2* requires a bulged base in the folded *attC* stem for high homing frequency. We demonstrate that the IBS1/IBS3 motifs and two bulge bases conserved among *attC*s determine *S.ma.I2* homing specificity for the *attC* bottom strand. These results suggest that class IIC-*attC* introns tolerate *attC* variation by recognition of a bulged hairpin DNA motif rather than a specific sequence.

Keywords: cassette; integron; resistance; retrohoming; ribozyme

INTRODUCTION

Group II introns are retroelements coding for a large structured catalytic RNA and a multidomain intron-encoded protein (IEP), which has maturase, reverse transcriptase (RT) and in some cases endonuclease activities (Lambowitz and Zimmerly 2004). They are found in organelles (from lower eukaryotes and higher plants), and in bacterial and archaeal genomes (Ferat and Michel 1993; Martinez-Abarca and Toro 2000; Bonen and Vogel 2001; Toro 2003; Pyle and Lambowitz 2006). Group II introns share very little sequence similarity but have a conserved intron RNA secondary structure, composed of six structural domains, which coevolved with the intron's RT (Toor et al. 2001). Phylogenetic analyses, based on IEP sequences, allow separation of group II introns into several lineages (chloroplast-like, mitochondrial-like, bacterial classes A–F) (Zimmerly et al. 2001;

Simon et al. 2008). Together, the IEP and the spliced intron RNA form a ribonucleoprotein (RNP) complex, which mediates intron mobility and invasion of DNA targets (Zimmerly et al. 1995; Matsuura et al. 1997; Cousineau et al. 1998). The intron RNA catalyzes excision (splicing) from RNA transcripts and integration (reverse splicing) into one strand of the new allele target. The IEP facilitates these reactions by stabilizing the intron RNA structure (maturase activity) and by cleaving the strand opposite to the reverse spliced intron (endonuclease activity) to generate the primer for reverse transcription of the intron RNA (Lambowitz and Zimmerly 2004). However, over half of the IEPs in bacteria lack the endonuclease domain (Toro 2003). It has been shown that nascent strands at DNA replication forks can also serve as primers for reverse transcription of the intron RNA (by the IEP's RT activity) in the absence of second strand cleavage (Martinez-Abarca et al. 2000; Jimenez-Zurdo et al. 2003; Munoz-Adelantado et al. 2003; Zhong and Lambowitz 2003). This mechanism of mobility shows a pronounced strand bias and dependence on replication. Homing site recognition uses both the IEP and base pairing of the intron RNA with a specific DNA sequence (Guo et al. 1997). In a first step, the RNP binds DNA nonspecifically and then

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Article published online ahead of print. Article and publication date are at <http://www.rnajournal.org/cgi/doi/10.1261/rna.1649309>.

searches for a small number of specific bases in the distal 5'-exon and 3'-exon regions of the target site via major groove interactions. These base interactions enhance local DNA unwinding and a conformational change associated with identification of the specific binding site (Singh and Lambowitz 2001). In a second step, two exon binding sites (EBS1 and EBS2), located in the intron RNA, are involved in 5' exon recognition through base pairing interactions with the target's intron binding sites (IBS1 and IBS2). Similarly, the 3' exon is recognized by either the δ - δ' or EBS3-IBS3 interactions (Jacquier and Michel 1987; Michel and Ferat 1995).

Bacterial class IIC introns are a phylogenetic subclass of group II introns that exhibit unique RNA structure (e.g., the EBS2 motif is missing; they have shorter EBS1-EBS3 motifs and a shorter catalytic domain 5) (Toor et al. 2001; Toor et al. 2008) and self-splicing properties (e.g., they generally self-splice in vitro through a hydrolytic pathway resulting in linear intron intermediates) (Podar et al. 1998). Furthermore, it was shown that a stem-loop motif located a few nucleotides upstream of the IBS1 was implicated in recognition of the 5' exon during both splicing and reverse-splicing events (Toor et al. 2006; Robart et al. 2007). The targeted stem-loop structures are mostly transcriptional terminators (Granlund et al. 2001), which show low levels of sequence identity but have conserved stem-loop structures. Interestingly, a specialized lineage within class C introns, named class IIC-*attC* introns, preferentially inserts into the inverted repeats of integron *attC* sites (Centron and Roy 2002; Quiroga et al. 2008).

attC sites (or 59-base elements) are site-specific recombination sequences located at the 3' end of integron gene cassettes (Hall et al. 1991). Integrator integrases mediate site-specific recombination of DNA sequences flanking open reading frames or structural genes (e.g., drug resistance genes) using two distinct sites, a proximal *attI* and a cassette-associated *attC* site. The key elements of integrons are: an integrase gene (*intI*), a promoter for expression of cassettes (P_c), and a recombination site (*attI*) (Fig. 1A; Stokes and Hall 1989; Mazel 2006). Alignment of different *attCs* shows that they vary in length from 57 to 141 base pairs (bp), and that their sequence similarities are primarily restricted to the boundaries, which correspond to two inverted integrase-binding repeats, 1L-2L separated by a spacer of 5 bp and 2R-1R separated by 6 bp (Fig. 1B; Francia et al. 1997; Stokes et al. 1997). The central part between the 2L and 2R repeats varies both in length and sequence but is an imperfect inverted repeat. It was shown by electrophoretic mobility shift assays (EMSA) that integrator integrase preferentially binds to single-strand DNA of the *attCs* (Francia et al. 1999). Interestingly, binding of integrase is strand specific for the bottom strand (bs) of the *attC*. It was suggested that formation of a cruciform (i.e., stem-loop structure on each strand) and imperfections in the inverted repeats (Fig. 1B) were of importance for *attC* site recognition by integrase and

for further recombination reactions (Hall et al. 1991; Stokes et al. 1997; Francia et al. 1999). A recent study demonstrates that integrase specificity for the *attC* bottom strand is due to the presence of two conserved asymmetrical bases (T and G) that bulge out from the folded DNA sequence (Fig. 1C; Johansson et al. 2004). The crystal structure of the VchIntIA-VCR_{bs} synapse (class 4 integrator integrase monomers bound to two antiparallel VCR_{bs} duplexes) showed that integrase binds its *attC* substrate as a dimer (MacDonald et al. 2006). The extrahelical base T is bound by *cis*-interaction with one integrase subunit, and is important for DNA site recognition, whereas the extrahelical base G is bound by *trans*-interaction with a second integrase subunit bound to the other VCR_{bs}. The *trans*-interactions are important features to hold the synaptic complex together during recombination.

To address how bacterial class IIC-*attC* introns insert specifically into the bottom strand of *attC* sites, we used the *S.ma.I2* intron in a two-plasmid mobility assay. *S.ma.I2* was found in a class 1 integrator, from the multiresistant clinical isolate *Serratia marcescens* SCH909, adjacent to the *aadB* (aminoglycoside resistance) gene cassette (Centron and Roy 2002). We generated several mutants of the *attC_{aadA1}* site from the *aadA1-qacE* cassette junction in Tn21 (Fig. 1B) in order to find stem-loop features that influence intron homing. We used this site because it is widespread among plasmid-borne cassettes and is almost a perfect palindromic sequence (Fig. 1C), but most importantly because of previous observations in vitro demonstrating specific binding of integrase to the bottom strand of *attC_{aadA1}* (Francia et al. 1999; Johansson et al. 2004; Bouvier et al. 2005). The quantitative results showed that mobility into the wild-type (WT) site occurs at a frequency (the fraction of homing site plasmids containing introns) of 18% and is strongly biased by the orientation of the homing site relative to the direction of DNA replication. *S.ma.I2* mobility results into mutant *attC_{aadA1}* sites are consistent with recognition of stem-loop motifs in unwound DNA, as in the *Bacillus halodurans* class IIC intron (Robart et al. 2007). The homing frequency results showed that, while the entire *attC* sequence is not necessary for recognition of the insertion site, short deletions of the *attC* stem-loop motif inhibited the intron mobility. Moreover, our data show that *S.ma.I2* requires a bulged base in the folded *attC* stem for high homing frequency. We demonstrate that the IBS1/IBS3 motifs and two bulge bases conserved among *attC* sites are determinants for *S.ma.I2* homing specificity for the *attC* bottom strand.

RESULTS

In vivo assay of mobility into the wild-type *attC_{aadA1}* site

To address how bacterial class IIC-*attC* introns insert specifically into the bottom strand of *attC* sites, we used

FIGURE 1. Integron structure and *attC* recombination sites. (A) General schematic representation of an integron. *intI*, integrase gene; P_i , integrase promoter; P_c , cassette promoter; *attI*, integrase recombination site (integrase specific); ORF, cassette open reading frame; *attC*, integrase recombination site (cassette specific). (B) Sequence of the *attC_{aadA1}* site. 2L-2R and 1L-1R boxes are inverted repeats; arrows, integrase recombination site and intron insertion site; Vienna output (dots and parentheses between the top-strand [ts] and bottom-strand [bs] sequences), base pairings from a potential single-stranded secondary structure based on MFOLD prediction. (C) Predicted stem-loop structures of the *attC* top- and bottom-strand sequences based on MFOLD predictions. IBS1 and IBS3, intron binding sites 1 and 3; asterisk (*), extrahelical bases T38 and G45 upon folding of the *attC* bottom strand.

an *Escherichia coli* two-plasmid mobility assay in which *S.ma.I2* inserted into WT or mutant *attC_{aadA1}* sites (Figs. 2, 3). For each target, oligonucleotides of both complementary DNA strands were annealed, cloned, and tested as homing sites (Supplemental Table 1). Intron mobility was screened using a qualitative PCR method in combination with a quantitative colony patch hybridization method to measure and compare intron mobility frequencies into WT and mutant *attC_{aadA1}* sites (Table 1; see Materials and Methods). Three targets were cloned in both orientations such that reverse transcription of the inserted intron RNA could potentially use either a nascent lagging (LAG) or leading (LEAD) DNA strand as a primer at the replication fork. These clones were tested in order to detect a probable strand bias for homing in our experiment. It was previously reported that different group II introns with IEPs lacking the endonuclease domain prefer either a nascent

lagging strand (Martinez-Abarca et al. 2004) or leading strand (Zhong and Lambowitz 2003) as a primer. Figure 4 shows the orientation of intron insertion using a highly sensitive PCR-based approach, whereas Figure 5 shows degrees of intron insertion (semiquantitatively) without information on the orientation. Mobility assays with the WT site show that *S.ma.I2* homes specifically into the *attC* bottom strand (Fig. 4). Sequencing of the PCR amplicon confirmed that *S.ma.I2* used the expected 5'-TTGT/T sequence (IBS1/IBS3, respectively) as its intron binding sites (data not shown) (Quiroga et al. 2008). However, comparison of intron mobility levels into the WT (LAG) and WT (LEAD) sites is consistent with a replication orientation bias (Fig. 5). Indeed, quantitative results suggest that reverse transcription of the inserted intron RNA preferred a nascent lagging (LAG) strand DNA as a primer over a leading (LEAD) strand DNA ($18.0 \pm 2.5\%$ and $<1.0\%$

FIGURE 2. Schematic of the two-plasmid mobility assay in *E. coli* (see the Materials and Methods section for a more detailed protocol). E_1 and E_2 , exon sequences; IEP, *S.ma.I2* intron encoded protein; Plac, lac promoter; Ap^R and Cm^R , ampicillin and chloramphenicol resistance genes; pMB1 and p15A, plasmid replication origins.

homing frequencies, respectively) (Table 1). Figure 4 shows that despite the low homing frequencies observed using the LEAD construct, mobility events into the WT (LEAD) site were amplified by PCR. The identities of the amplicons were confirmed by sequencing (data not shown). In order to find the structural determinants for recognition of the *attC_{aadA1}* site, we designed and cloned 26 *attC_{aadA1}* mutants and tested them as potential target sites for *S.ma.I2*, taking into account the effect of homing site orientation in pACYC184 (Fig. 3).

Mutation of the 5'-exon stem lengths

The *attC_{aadA1}* bottom strand is almost a perfect palindromic sequence except for the “spacer” bases (between the 1L-1R and 2L-2R boxes), the two conserved asymmetrical bases (T38 and G45) and the central loop (GAA) (Fig. 1C). According to the predicted secondary structure, we designed seven stem-loop mutants ($\Delta 1$ – $\Delta 7$), which are 5'-end truncations of the WT *attC_{aadA1}* bottom strand sequence (homing strand) (Fig. 3A). The homing results showed that the minimal target was a 44-bp *attC_{aadA1}* (mutant $\Delta 5$) that includes 39 bp of 5' exon (Fig. 4). The 5' exon sequence can fold into a stem (12 bp) and loop (3 nucleotides [nt]) motif, which is separated from the IBS1 sequence (5'-TTGT) by a 7-nt spacer (Fig. 3A). For this target, residual *S.ma.I2* mobility was $1.8 \pm 1.0\%$, whereas $18.0 \pm 2.5\%$ mobility was observed for the WT *attC_{aadA1}* (Table 1). Further

deletion resulted in the absence of detectable mobility into the homing site (Fig. 4). The homing frequencies showed that, while the entire *attC_{aadA1}* sequence is not necessary for intron mobility, even short deletions, like the 7-bp $\Delta 1$ mutant, inhibited the intron mobility ($8.0 \pm 0.7\%$). Interestingly, base deletions of the *attC* ranging from the 1R to the beginning of the 2R inverted repeats ($\Delta 1$ – $\Delta 4$) resulted in a $\sim 50\%$ (mean) diminution of the homing reaction in vivo. Additional deletions, including half ($\Delta 5$), or all, of the 2R site ($\Delta 6$ and $\Delta 7$), resulted in a $\sim 90\%$ diminution or a complete inhibition of the in vivo mobility, respectively.

Mutation of the stem pairings

For this mobility assay, we substituted five nucleotides in *attC_{aadA1}* in order to disrupt the stem before the central loop (Fig. 3B). It was shown that an identical stem mutation on *attC_{aadA7}* inhibits the recombination frequency by integrase (Bouvier et al. 2005). Here, intron mobility into the *attC* bottom strand was detected by PCR (Fig. 4) but quantitative results showed an important loss of efficiency ($<1.0\%$) (Table 1). This result was confirmed by the absence of PCR amplicon using two pACYC184-specific primers that flank the *attC* site (Fig. 5).

Mutation of IBS1 or IBS3

Retrohoming of group II introns is normally highly specific because of base pairings between the intron RNA and the DNA exons. Bacterial class IIC intron base pairings are limited to the short IBS1-EBS1 and IBS3-EBS3 interactions (Robart et al. 2007). Here, we designed three IBS1 motif mutants (IBS1-1 to IBS1-3) and one IBS3 mutant (IBS3) (Fig. 3C). PCR results confirmed the importance of a specific IBS1 sequence (5'-TTGT) and, to a lesser proportion, a specific IBS3 (T) base. Indeed, a weak PCR amplicon was obtained only for the first IBS1 mutant (IBS1-1) and a strong PCR amplicon for the unique IBS3 mutant (IBS3) (Fig. 4). Hybridization results confirmed the PCR results obtained with the IBS1-1 and IBS3 mutants, respectively, as the corresponding homing frequencies were $<1.0\%$ and $4.6 \pm 2.9\%$ (Table 1).

Deletion of the extrahelical bases T38 and G45

Quantitative results of mobility of *S.ma.I2* into *attC_{aadA1}* with mutated 5'-exon stems (above) showed that short

not essential for *attC* site recognition but enhances the mobility frequency. Sequencing of the PCR amplicon from the 5' exon-intron integration junction within the ΔT mutant (Fig. 4) confirmed the specificity of the intron for the homing site 5'-TTGT/T (data not shown). Deletion of the guanine at position 45 (G45) had no effect on target site recognition by the intron. The ΔG mutant had mobility frequencies similar to those of the WT (LAG) site. Deletion of both T38 and G45 bases ($\Delta T + \Delta G$ mutant) resulted in mobility frequencies comparable to ΔT alone, and a weak PCR amplicon corresponding to specific bottom-strand insertion was observed (Fig. 4).

TABLE 1. In vivo mobility frequencies of *S.ma*.I2 into WT and mutant *attC_{aadA1}* sites

Target <i>attC_{aadA1}</i>	Orientation ^a	Homing strand ^b	<i>S.ma</i> .I2 mobility frequency (%) ^c
WT	LAG	bs	18.0 ± 2.5
WT	LEAD	bs	<1.0
Δ1	LAG	bs	8.0 ± 0.7
Δ2	LAG	bs	9.4 ± 4.2
Δ3	LAG	bs	10.1 ± 3.8
Δ4	LAG	bs	9.7 ± 3.9
Δ5	LAG	bs	1.8 ± 1.0
Δ6	LAG	bs	<1.0
Δ7	LAG	bs	<1.0
Stem mut.	LAG	bs	<1.0
IBS1-1	LAG	bs	<1.0
IBS1-2	LAG	bs	<1.0
IBS1-3	LAG	bs	<1.0
IBS3	LAG	bs	4.6 ± 2.9
ΔT	LAG	bs	<1.0
ΔG	LAG	bs	19.6 ± 4.2
ΔT + ΔG	LAG	bs	<1.0
T38A	LAG	bs	7.8 ± 1.8
T38C	LAG	bs	15.2 ± 3.6
T38G	LAG	bs	14.0 ± 3.7
G45A	LAG	bs	12.3 ± 4.9
G45C	LAG	bs	24.2 ± 10.4
G45T	LAG	bs	13.3 ± 2.9
G45GG	LAG	bs	<1.0
T38A + G45C	LAG	bs	11.3 ± 3.5
T38G + G45T	LAG	bs	15.0 ± 6.5
IBS ts	LAG	bs and ts	<1.0
IBS ts	LEAD	bs and ts	<1.0
IBS + bulges ts	LAG	ts	<1.0
IBS + bulges ts	LEAD	ts	14.4 ± 6.0

^aOrientation of the *attC* bottom-strand sequence relative to the direction of DNA replication.

^b*S.ma*.I2 homing specificity (as detected by PCR on Fig. 4) for the *attC* bottom strand (bs) and/or top strand (ts).

^cThe percentage of recipient plasmids that received the intron. Mobility frequencies were determined by colony patch hybridization with an intron-specific probe and are the mean of positive hybridization signals ± standard deviation of at least three independent experiments in each case (see Materials and Methods).

Mutation of the extrahelical bases

Compilation of several integrase cassette *attC* sites and folding of the bottom-strand sequence into the most probable stem-loop motifs showed that the two asymmetrical bases in the stem are a conserved thymine and guanine (Stokes et al. 1997; MacDonald et al. 2006). In order to determine if *S.ma*.I2 homing involves a specific bulge base, nine different mutants of the extrahelical bases T38 and G45 were examined (Fig. 3E). We observed that substitution of T38 with an adenine (T38A mutant) resulted in a more than twofold decrease of the intron mobility frequency compared with WT (LAG) (Fig. 5; Table 1). However, such differences were not observed with the T38C and T38G mutants. Substitution of G45 with any

of the alternative bases (G45A, G45C, and G45T mutants) did not alter intron homing (Fig. 5). In the G45A mutant, additional mutations were made in order to prevent bulging of one of the two adjacent adenines (A46 or A47) instead of the G45A in the folded structure. In the G45GG mutant, another guanine was inserted next to the G45. This unusual mutant was tested as a homing site because it was previously shown that a GG bulge mutant of the *attC_{aadA1}* resulted in a complete loss of IntI1 integrase binding in vitro (Johansson et al. 2004). Strikingly, this mutant displayed <1.0% homing efficiency, whereas the ΔG mutant showed homing levels comparable to those of WT (LAG). Figure 4 shows that we successfully amplified homing events into the GG mutant bottom strand. Sequencing confirmed the specificity of the intron for the homing site 5'-TTGT/T (data not shown). Finally, intron mobility was tested using *attC_{aadA1}* mutants for which extrahelical bases were either changed for the complementary base (T38A + G45C) or exchanged between themselves (T38G + G45T). The T38A + G45C mutant showed a weak (~1.5-fold) decrease of homing frequency compared with WT (LAG) (most probably due to the T38A mutation), whereas no inhibition of the mobility was observed with the T38G + G45T mutant (Table 1).

Inversion of the intron binding sites with or without the extrahelical bases

Class IIC-*attC* introns identified so far are specifically inserted into *attC* bottom strands. Quantitative results show the unexpected importance of the T extrahelical base for a high level of mobility into *attC_{aadA1}* (above). A parallel can be drawn with integrase integrase, for which the T base dictates the position of the integrase monomer along *attC* bottom strands (MacDonald et al. 2006). It was shown that minor alterations of the *attC_{aadA1}*, involving nested inversions of both the bulge bases (T and G) and central loop, resulted in strong integrase binding to the top strand with the reciprocal loss of binding to the bottom strand (Johansson et al. 2004). Here, in order to change the *S.ma*.I2 bottom strand specificity, two minimal *attC_{aadA1}* inversions were examined (Fig. 3F). First, intron-binding sequences, IBS1 and IBS3 (5'-TTGT/T), were symmetrically moved from the *attC* bottom strand to the top strand in the “IBS ts” (LAG) mutant. This inversion leads to an >18-fold decrease of mobility compared with WT (LAG) (Table 1). Interestingly, Figure 4 shows two weak PCR amplicons corresponding to mobility events that occurred in either strand. Similar homing frequencies (<1.0%) and PCR results were obtained when the orientation of the target site was inverted relative to the direction of plasmid replication (“IBS ts” LEAD). Sequencing of the two PCR amplicons obtained from either LAG or LEAD mutant clones confirmed intron mobility into the new top strand IBS motifs, 5'-TTGT/T (IBS1/IBS3), but also into the

FIGURE 4. *S.ma.I2* in vivo mobility results into *attC_{aadA1}* mutants. Agarose gels of the PCR products using primer pair “a” or “b” in order to detect the orientation of intron insertion. The figure shows the 5' intron–exon integration junction (see Fig. 2 for primer position). “WT,” “IBS ts,” and “IBS + bulges ts” *attC_{aadA1}* were cloned in both orientations (LAG or LEAD) relative to the direction of DNA replication in pACYC184 (see Materials and Methods).

bottom strand sequence, 5'-GCGT/T (IBS1/IBS3) (Fig. 6) corresponding to the mutated wild-type IBS motifs (5'-TTGT/T → 5'-GCGT/T). Second, intron-binding sequences and bulge bases (T38 and G45) were symmetrically moved from the *attC* bottom strand to the top strand within the “IBS+bulges ts” (LAG) mutant (Fig. 3F). These mutations led to an important inhibition of mobility into the *attC* site (<1.0% homing efficiency), but strong PCR amplicons corresponding to specific top strand mobility events were observed (Fig. 4). Cloning of the same *attC* mutant into the opposite orientation (“IBS + bulges ts” LEAD), increased the intron mobility frequency to $14.4 \pm 6.0\%$, comparable to WT (LAG) (Table 1). This increase of mobility is related to the same replication orientation bias observed with the WT *attC* site. Similarly, PCR amplification of the target site shows an increase of mobility events relative to the “IBS + bulges ts” (LAG) mutant (Fig. 5). Figure 4 shows a switch in the intron specificity for the *attC* top strand and the absence of mobility events into the bottom strand. Sequencing of the PCR amplicons confirmed the top-strand homing and specificity of the insertion site into the new IBS motifs (Fig. 6). Therefore, moving the IBS1-IBS3 sites to the opposite strand of the *attC* site retains high-level mobility only when accompanied by the bulge bases and the correct orientation with regard to DNA replication.

DISCUSSION

In this study, we searched for stem-loop features of *attC_{aadA1}* that could explain the site specificity of class

IIC-*attC* introns. Our results, based on qualitative PCR and quantitative hybridization methods, indicate that target specificity of *S.ma.I2* occurs by recognition of short intron-binding sites (5'-TTGT/T as IBS1/IBS3, respectively) adjacent to a bulged hairpin structure within the *attC_{aadA1}* bottom strand. This hairpin structure implies that the inverted-repeat motifs (1L-1R and 2L-2R), conserved among *attC* sites, could form a robust double-strand DNA-like structure in vivo, which can be bound by nonspecific phosphate–backbone interaction or specific base contacts by the intron RNP amino acids. It was shown for several group II introns, that in the absence of second-strand cleavage, the intron RNA reverse splices at the DNA replication fork into either single-stranded or double-stranded DNA and then uses a nascent strand as primer for reverse transcription (Ichianagi et al. 2002; Zhong

and Lambowitz 2003; Martinez-Abarca et al. 2004). We found that homing of *S.ma.I2* displays a strong replication orientation bias and predominantly occurs using a nascent lagging DNA strand as a primer for reverse transcription of the inserted intron RNA. This bias reflects the absence of second-strand cleavage, because of the missing endonuclease domain of the IEP (Centron and Roy 2002), and suggests that *S.ma.I2* reverse splices into single-stranded DNA targets at the DNA replication fork (Ichianagi et al. 2002; Zhong and Lambowitz 2003; Coros et al. 2005). Together, these results support the hypothesis that the DNA replication fork (rather than transcription bubbles) is the most probable source of single-strand DNA, allowing the *attC* bottom strand to fold into a stem-loop motif used by class IIC-*attC* introns as a structural determinant for target site recognition. In addition to group II introns, other mobile elements, including the IS605 group of insertion sequences and transposon Tn7, which use different mobility pathways, demonstrate lagging-strand preference during transposition (Peters and Craig 2001; Barabas et al. 2008).

The crystal structure of the VchIntIA-VCR_{bs} complex confirms that the integrase binds the *attC* bottom strand as a DNA stem-loop with extrahelical bases (MacDonald et al. 2006). Our results suggest that *S.ma.I2* uses the conserved *attC* structure in order to compensate for the shortened sequence-specific IBS1-EBS1 interaction and the missing EBS2-IBS2 motifs, which are normally involved in 5' exon recognition. Therefore, there are two major determinants for *attC_{aadA1}* recognition by *S.ma.I2* in vivo. First, a four-base IBS1 (TTGT) and a one-base IBS3 (T) base pair to

FIGURE 5. *S.ma.I2* in vivo mobility results into *attC_{aadA1}* mutants. Agarose gels of the PCR products using PACYC-5' and PACYC.rev primers in order to detect variations in the degree of intron insertion for each mutant compared with the WT site (see Fig. 2 for primer position). The Figure shows *attCs* that were and were not targeted by *S.ma.I2*.

complementary intron RNA exon-binding sites (as predicted by its secondary structure), and are conserved among several targeted *attC* sites (Quiroga et al. 2008). We showed that mutations of the IBS1 and (to a lesser degree) IBS3 motifs strongly inhibited the homing reaction into *attCs* in vivo. Second, there is an imperfect stem-loop motif adjacent to the intron binding sites. Compilation of insertion sites of several subgroup IIC introns shows that a stem-loop structure is located a few bases (≤ 8) before the intron binding sites (Robart et al. 2007). Assays of homing into mutated *attC_{aadA1}* sites showed mobility into a minimal 5'-exon sequence folded into a 12-base stem and a three-base loop motif. The IBS motifs and the minimal 5'-exon sequence requirement identified in this study is consistent with the consensus structure for several class C targets (Robart et al. 2007). We showed that deletion of the 1R region of *attC* ($\Delta 1$) resulted in a $\sim 50\%$ diminution of the homing reaction in vivo (Table 1), while deletion of the region between 1R and 2R ($\Delta 2$ – $\Delta 4$) gave no

further loss of mobility. Additional deletions including half ($\Delta 5$) or the entire 2R site ($\Delta 6$ and $\Delta 7$) resulted in a $\geq 90\%$ inhibition of mobility. Further studies showed that mutations disrupting the stem before the central loop led to an important inhibition of intron mobility (Table 1). Interestingly, an identical stem mutation on *attC_{aadA7}*, which differs by two nucleotides from *attC_{aadA1}* (Johansson et al. 2004), leads to a 10-fold decrease of the class 1 integrase recombination frequency (Bouvier et al. 2005). Together, these results show that correct folding of the *attC* bottom strand is essential for substrate recognition, and suggest that *S.ma.I2* makes DNA–protein contacts with the same *attC* site determinants (1L-1R and 2L-2R subsites) as integrase (MacDonald et al. 2006; Johansson et al. 2009).

Sequence variation between the 2L and 2R repeats of several *attC* sites reveals a lack of absolute sequence requirement in the stem except for two conserved extrahelical bases (T and G) that are usually separated by a

FIGURE 6. (A) Alignment of the sequenced PCR products from the *S.ma.I2* mobility assays into the WT, “IBS ts,” and “IBS + bulges ts” *attC_{aadA1}* sites. Arrows indicate the intron homing site determined by PCR (Fig. 4). The thickness of the arrows corresponds to the mobility frequencies observed in Table 1. Asterisk (*), intron homing site; underlined bases, intron binding site; highlighted bases, difference from the WT sequence; dash, base deletion. (B) Predicted stem-loop structures of the *attC_{aadA1}* mutant *top*- and *bottom*-strand sequences based on MFOLD predictions. IBS1 and IBS3, intron binding sites 1 and 3; asterisk (*), extrahelical bases T38 and G45 upon folding of the *attC* *bottom* strand.

six-base spacer (Stokes et al. 1997). Mutation or deletion of the extrahelical base T results in inhibition of the homing reaction in vivo (Table 1). Interestingly, the crystal structure of the VchIntIA-VCR_{bs} synapse showed that the extrahelical base T is bound by *cis*-interaction with one integrase subunit and is important for DNA site recognition (MacDonald et al. 2006). Deletion of the T or G extrahelical bases in VCR_{bs} resulted in inhibition of excision frequency by VchIntIA integrase (MacDonald et al. 2006). Furthermore, it was demonstrated that a GG bulge mutant of the *attC_{aadA1}* resulted in a complete loss of IntI1 integrase binding in vitro (Johansson et al. 2004). This mutation also inhibits *S.ma.I2* homing into *attC_{aadA1}*, whereas deletion (or mutation for another base) of the G45 bulge has no effect on intron mobility. These results

show that the G45 bulge is not recognized by *S.ma.I2*, and suggest that the G45GG mutation resulted in a change of the *attC* structure, which potentially inhibited an important contact with the T38 bulge. Consequently, these results suggest that *S.ma.I2* makes an unexpected additional DNA–protein contact with the T bulge, in order to position the intron RNP along the DNA, facilitate the IBS–EBS pairings, and enhance the homing frequency.

Similarly, recognition of extrahelical bases from stem-loop motifs is also used by a particular class of DNA transposases coded by the IS605 group of insertion sequences. The transposases specifically bind to conserved 22–23 base imperfect inverted repeats located at the transposon ends. The crystal structure of the ISHp608 transposase from *Helicobacter pylori* with stem-loop DNA, from the folded

inverted repeats, shows how DNA/protein interactions direct the transposase toward its cleavage site (Ronning et al. 2005). The transposase interacts with two unpaired thymine bases (separated by a six-base spacer) in these stem-loops, forming the basis for discrimination between the top and bottom strand stem-loops. Interactions between the ISHp608 transposase and the extrahelical bases are both protein side-chain and backbone interactions. Here, we demonstrated that full homing of the intron to the *attC* top strand was possible when both bulge bases and intron binding sites were symmetrically moved to the top strand (Fig. 6). Therefore, the conserved extrahelical bases (most importantly the T bulge) from the folded *attC* sites and the IBS1/IBS3 (5'-TTGT/T) motifs are the determinants for directing the intron to the *attC* bottom strand. Finally, these results suggest that class IIC-*attC* introns and integron integrases have convergently evolved to recognize *attC* sites and to tolerate *attC* variation by recognition of a bulged hairpin DNA motif rather than a specific sequence.

MATERIALS AND METHODS

Plasmids and media

Plasmids are described in Supplemental Table 1. *E. coli* DH5- α (*supE44* Δ *lacU169* (ϕ 80*lacZ* Δ M15) *hsdR17* *recA1* *endA1* *gyrA96* *thi-1* *relA1*; Invitrogen) was grown in Luria-Bertani broth at 30°C or 37°C as indicated below. Antibiotics were used at the following concentrations: ampicillin (Ap), 100 μ g/mL; chloramphenicol (Cm), 68 μ g/mL. Isopropyl- β -D-thiogalactopyranoside (IPTG) was added to 1-mM final concentration.

Polymerase chain reaction procedures

Polymerase chain reaction (PCR) for plasmid assembly and other PCR reactions used the Phusion DNA polymerase (Finnzymes) according to the manufacturer's instructions. PCR primers (Supplemental Table 2) were synthesized using the DNA Synthesizer ABI-3900 from Applied Biosystems, Inc.

Nucleic acid folding

Target site DNA single-stranded secondary structures were determined using the MFOLD 3.2 program (Zuker 2003).

In vivo mobility assay

Intron mobility was determined by PCR amplification of the targeted sites using an *E. coli* two-plasmid assay in which *S.ma*.I2 cloned into pUC18 inserts into mutant *attC_{aadA1}* sites cloned into pACYC184 (Supplemental Table 1). pACYC184-based clones were obtained by cloning full-length complementary oligonucleotides (Supplemental Table 2) of each target site. Targets were cloned such that reverse transcription of the inserted intron RNA could potentially use either a nascent lagging (LAG) or leading (LEAD) DNA strand as a primer at the replication fork.

For each mobility assay, the intron donor (Ap^R) and recipient (Cm^R) plasmids were cotransformed into *E. coli* DH5- α compe-

tent cells (*recA1*⁻). One colony of each double transformant was grown in 5 mL of LB media in the presence of Ap and Cm at 30°C overnight. The preculture (100 μ L) was pelleted and inoculated in 5 mL of LB medium with both antibiotics at 37°C until O.D._{600 nm} = 0.5. Expression of the intron was then induced by addition of 1 mM IPTG, followed by incubation at 37°C for 3 h. Plasmid DNAs were extracted using a QIAprep kit (Qiagen). Mobility was screened by PCR using different combinations of primers (Fig. 2; Supplemental Table 2) and insertion sites were confirmed by sequencing.

Homing frequency was determined by colony patch hybridization. Purified plasmids (50–100 ng) from *S.ma*.I2 mobility assays, were digested with five units of PvuI. This enzyme cuts only once in the intron donor plasmid, outside of the intron sequence. *E. coli* DH5- α cells were transformed with the digested products and plated on LB/Cm plates to select for cells with recipient plasmids containing or lacking the intron. To calculate the intron mobility efficiency, measured by the percentage of recipient plasmids that received the intron, 100 isolated Cm^R colonies were patched onto LB/Cm plates. The patches were lifted onto nylon membranes and hybridized with 5'-end-labeled probe (*Sma*2.for) using [γ -³²P]ATP (3000 Ci/mmol; GE Healthcare). For each target site tested, positive colonies, randomly chosen, were cultured and the homing site-specificity confirmed by PCR amplification of the target site (using the PACYC-5' and PACYC.rev primers) and sequencing.

SUPPLEMENTAL MATERIAL

Supplemental material can be found at <http://www.rnajournal.org>.

ACKNOWLEDGMENTS

This work was supported by Canadian Institutes of Health Research (CIHR) Grant MT-13564 to P.H.R.

Received March 13, 2009; accepted May 4, 2009.

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RNA 2009 15: 1543-1553 originally published online June 9, 2009

Access the most recent version at doi:[10.1261/rna.1649309](https://doi.org/10.1261/rna.1649309)

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