11-1-2008

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Note

A Simple Polymerase Chain Reaction-Based Method for the Construction of Recombinase-Mediated Cassette Exchange Donor Vectors

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ABSTRACT

Here we describe a simple method for generating donor vectors suitable for targeted transgenesis via recombinase-mediated cassette exchange (RMCE) using the ΦC31 integrase. This PCR-based strategy employs small attB "tails" on the primers used to amplify a sequence of interest, permitting the rapid creation of transgenes for *in vivo* analysis.

SEVERAL recent advances in transgenesis have made use of site-specific recombinases (SSRs) to achieve the integration of transgenes into predetermined locations in the genome (reviewed by BRANDA and DYMECKI 2004; VENKEN and BELLEN 2007). The capacity to target multiple insertions to a common location greatly facilitates comparisons of different transgenes by controlling for position effects. In one powerful approach called recombinase-mediated cassette exchange (RMCE; reviewed by WIRTH *et al.* 2007), a selectable marker flanked by SSR recognition sequences is first integrated into the genome by other methods to create a genomic target "cassette." Subsequently, a vector carrying a donor cassette consisting of a sequence of interest flanked by SSR recognition sequences that are compatible with those of the target cassette is introduced in the presence of the relevant SSR. Recombination events on both sides of the donor and target cassettes result in a clean exchange of the target sequence for the donor cassette, yielding integration of the sequence of interest and not the plasmid backbone (Figure 1). Importantly, RMCE events can be monitored simply by scoring the phenotype produced by the selectable marker in the target cassette, which is lost from the genome during the exchange event, permitting the integration of unmarked sequences (SEIBLER *et al.* 1998; FENG *et al.* 1999; BATEMAN *et al.* 2006).

We recently developed an RMCE strategy for Drosophila (BATEMAN *et al.* 2006) based on the ΦC31 integrase, which catalyzes recombination between attP and attB

recognition sites (reviewed by BRANDA and DYMECKI 2004). Our original strategy called for a sequence of interest to be subcloned into a vector that contained attB sequences; once constructed, this plasmid would be co-injected along with mRNA encoding the integrase into embryos bearing a genomic target—in our case a *mini-white* gene flanked by inverted attP sites—and integration events would be scored following several straightforward genetic manipulations (BATEMAN *et al.* 2006). Notably, because the process of obtaining integrants via embryonic injection is quite simple, we have found that the construction of donor vectors represents a rate-limiting step in obtaining transgenes for *in vivo* analysis. We therefore sought to simplify the subcloning steps involved in donor vector construction. Specifically, we were curious as to whether the size of the attB sequences used in our donor constructs could be reduced, allowing them to be more easily manipulated *in vitro*. Our original scheme made use of 285-bp "full-length" attB sites, but prior *in vitro* analysis has shown that attB fragments as small as 35–40 bp are competent for recombination with a full-length attP (GROTH *et al.* 2000). Thus, we asked whether 40-bp attB (attB40) sites could support RMCE in our system.

AttB40 sites function in Drosophila: For our initial test, we constructed the donor vector pBS-yin(B40XC), consisting of an intronless *yellow* gene flanked by inverted attB40 sites in the plasmid pBluescript (pBS). In constructing this donor, we subcloned the attB40 sites using complementary oligonucleotides with overhanging "sticky" ends that permitted ligation with restriction sites in pBS. We then used pBS-yin(B40XC) as a donor vector for RMCE via two methods. First, we co-injected the vector and mRNA encoding the ΦC31 integrase into

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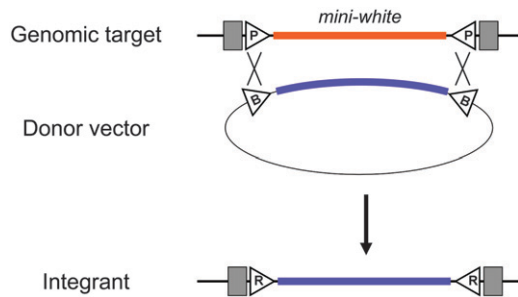
FIGURE 1.—Site-specific integration via RMCE in Drosophila. The target cassette, a *mini-white* gene (orange line) flanked by inverted attP sites and borne on a *P* element, was integrated at several locations in the genome via standard *P*-element-mediated transgenesis (SPRADLING and RUBIN 1982). In this study, we used the target P[attP.w+.attP] (GenBank accession EU761203), a derivative of pUASTP2 (BATEMAN *et al.* 2006) in which the UAS and TATA sequences have been removed; in prior unpublished presentations we have referred to this target as "PUASTP2.1" (see http://www.pairing.org/RMCE for further details). Target positions were determined by inverse PCR as previously described (BATEMAN *et al.* 2006). Introduction of a donor vector carrying a sequence of interest (blue line) flanked by inverted attB sites allows exchange of the cassettes in the presence of integrase, resulting in integration of the donor cassette. Shaded boxes, *P*-element ends; open triangles, att sites. Features are not drawn to scale.

embryos homozygous for an RMCE target in a *yellow⁻ white⁻* background as described previously (BATEMAN *et al.* 2006). Among the progeny of surviving injectees, we were able to detect flies that had lost the white⁺ eye color produced by the *mini-white* gene in the target cassette and had gained yellow⁺ pigmentation, consistent with successful RMCE integration events. Although rates of integration by this method were relatively low (2–11%), they were consistent with control experiments using the donor vector pCiB-yin, an intronless *yellow* gene flanked by full-length attB sites in the plasmid pCar4 (BATEMAN *et al.* 2006) (Table 2). Thus, attB40 sequences are competent for recombination with full-length attP sites in Drosophila.

We next wanted to determine whether the efficiency of RMCE could be improved by using a genomic source of the ΦC31 integrase that was recently described (BISCHOF *et al.* 2007). In this case, females carrying an integrase source on the X chromosome were crossed to males carrying an RMCE target on chromosome II, and donor vectors were injected into the progeny embryos (Figure 2). For convenience, we screened only male progeny in the subsequent generations, as the X-encoded integrase source is present in males of the G_0 generation and absent in males of the F_1 (Figure 2). As judged by scoring F_1 progeny that had lost the white⁺ eye coloration and had gained yellow⁺ pigmentation, we obtained high integration rates of the yellow donor cassette into an RMCE target at position 36F (Table 1). Furthermore, the rate of integration using attB40 sites is comparable to that using the full-length attB sites of
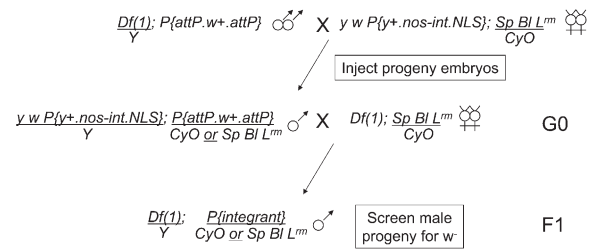

FIGURE 2.—RMCE using a genomic source of the ΦC31 integrase. The integrase is supplied via a *nanos-integrase-NLS* fusion, marked with a *yellow⁺* transgene, that is carried on the X chromosome (BISCHOF *et al.* 2007), while the target cassette is carried on chromosome II. In the G_0 generation, males are backcrossed singly to two or three virgin females carrying the CyO balancer and dominant markers on the second chromosome, and their progeny are screened for candidate insertion events wherein the chromosome carrying the RMCE cassette has lost the *mini-white* gene.

pCiB-yin (27% *vs.* 30%, respectively), indicating that there is no appreciable loss of integration efficiency using attB40-based donor cassettes in Drosophila.

A PCR-generated donor cassette supports RMCE: Having demonstrated the utility of attB40 sites in Drosophila, we next sought to establish a simple method for the production of donor vectors. Our strategy was to incorporate the attB40 sequence into oligonucleotide primers used to amplify a sequence of interest. We reasoned that the placement of attB40 "tails" at the 5′-ends of both primers in a PCR reaction would produce a linear DNA fragment consisting of the sequence of interest flanked by inverted attB40 sites (Figure 3A), and simply subcloning this fragment into any plasmid should

TABLE 1

40-bp attB sequences are fully functional in Drosophila

Target position	Donor gene	Donor attB size	Integrase source	Embryos injected	Vials with RMCE	% RMCE
37B	*yellow*	285 bp	mRNA	100	1/42	2
37B	*yellow*	40 bp	mRNA	100	3/28	11
53F	*yellow*	40 bp	mRNA	100	1/44	2
36F	*yellow*	285 bp	Genomic	100	6/20	30
36F	*yellow*	40 bp	Genomic	100	3/11	27

The donor vector pBS-yin(B40XC) was constructed using an intronless *yellow* gene and the attB40 sequence 5′-CGGGTGCCAGGGCGTGCCCTTGGGCTCCCCGGGCGCGTAC-3′ (subcloning details are available upon request) and represented the 40-bp donor. For the 285-bp donor, we used the plasmid pCiB-yin (BATEMAN *et al.* 2006). For experiments using an mRNA source of the integrase, donor constructs (~350 ng/μl) and mRNA (~1000 ng/μl) were prepared and injected into embryos homozygous for the RMCE target as described (BATEMAN *et al.* 2006). For experiments using a genomic source of the integrase, donor constructs (~400 ng/μl) were injected according to the scheme in Figure 2. RMCE events were detected by screening for white⁻ eyes and yellow⁺ bodies in the F_1 generation. "Vials with RMCE" refers to the number of vials containing at least one F_1 fly that was scored as positive, with the total number of vials scored given on the right.
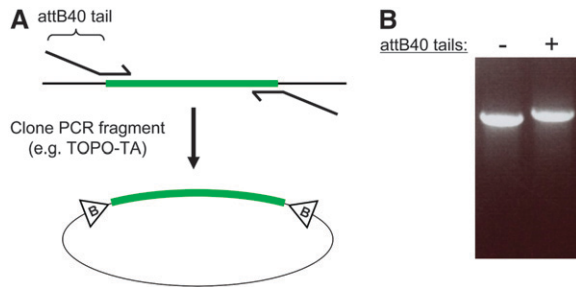
FIGURE 3.—AttB40 tails can be used to generate donor vectors via PCR. (A) Scheme for generating a donor vector via PCR. The attB40 sequence 5′-CGGGTGCCAGGGCGTGCCC TTGGGCTCCCCGGGCGCGTAC-3′ was added to the 5′-ends of the primers hsp70GFP5 (5′-CCGCTTTGGTACCTTTGCT A-3′) and hsp70GFP3 (5′-GCAGGTCGACCAATTCGAG-3′), which were subsequently used to amplify the GFP gene from the plasmid RINheXho-GFP (WITTKOPP *et al.* 2002). The resulting fragment was gel purified using a kit from Qiagen and subcloned using Invitrogen's TOPO-TA cloning kit to create the donor vector pTA-attB40GFP. (B) Ethidium-bromide-stained gel of PCR products that were generated using primers with (right lane) and without (left lane) attB40 tails.

TABLE 2

A PCR-generated attB40 donor cassette supports RMCE

Target position	Donor gene	Donor attB size	Integrase source	Embryos injected	Vials with RMCE	% RMCE
37B	*GFP*	285 bp	Genomic	275	9/19	47
37B	*GFP*	40 bp	Genomic	500	17/35	48
53F	*GFP*	285 bp	Genomic	50	1/5	20
53F	*GFP*	40 bp	Genomic	375	6/24	25

Donor constructs ($\sim$350 ng/μl) were injected according to the scheme in Figure 2. The vector piB-GFP (BATEMAN *et al.* 2006) represented the 285-bp donor, while the 40-bp donor was represented by the vector pTA-attB40GFP (see Figure 3). Insertions were verified via PCR as previously described (BATEMAN *et al.* 2006) using a primer pair internal to the GFP gene (RNXG-6, 5′-ATGGCATGGACGAGCTGTA-3′, and RNXG-8, 5′-GCAGTGCAGCTTTTTCCTTT-3′) and primers flanking the predicted recombination junctions (lac4, 5′-ACTGTGCGTTAGGTCCTGTTCATTGTT-3′, and ry1, 5′-CCTTAGCATGTCCGTGGGGTTTGAAT-3′ in the *P*-element ends flanking the cassette and RNXG-2, 5′-CAGCTGCGCT TGTTTATTTG-3′, and RNXG-9, 5′-GTGGTTTGTCCAAACT CATCAA-3′ in the GFP gene). These primers also allowed us to assess the orientation of the integrated GFP gene relative to the chromosome. Of note, in three of eight cases where we generated lines from multiple flies originating from a single vial, we observed some lines with one insertion orientation and other lines with the opposite orientation, indicative of multiple integration events in the germline of the G_0 parent of that vial.

create a donor vector suitable for RMCE without the need for preexisting attB sites in the plasmid backbone.

To test the feasibility of this approach, we PCR amplified a *GFP* gene from a plasmid source using primers that either possessed or lacked attB40 tails. Using standard reaction conditions and an unmodified *Taq* enzyme, we saw little difference in the quality of the reaction in the presence or the absence of the attB40 tails as assayed on ethidium-bromide-stained agarose gels (Figure 3B), indicating that attB40 sequences do not adversely affect the PCR reaction. Next, we gel purified and subcloned the band containing the attB40 tails using commercially available kits (see Figure 3 legend) and used the resulting plasmid as a donor vector for RMCE. Using the genomic source of integrase as described above (Figure 2), we achieved high rates of integration into two RMCE target sites located at polytene positions 37B and 53F (Table 2). Importantly, these rates closely matched the integration efficiency produced by a donor vector carrying full-length attB sites flanking a GFP gene, piB-GFP (BATEMAN *et al.* 2006) (Table 2), indicating that our strategy for PCR-generated donor cassettes has no discernible adverse effect on RMCE integration.

To verify RMCE events produced by our PCR-generated attB40 donor, we arbitrarily selected 30 candidate males from nine independent vials (six targeted to 37B, three targeted to 53F) and backcrossed them to establish stocks. We then subjected genomic DNA prepared from these stocks to PCR analysis using primers that would flank the predicted recombination junctions at both ends of a successfully integrated donor and a primer pair internal to the GFP gene (see Table 2 legend). We observed PCR products of the expected size for both junctions and the internal fragment in 30/30 cases (data not

shown), indicating that all flies tested had resulted from a clean exchange of the target for the donor.

Our strategy using attB40 oligonucleotide tails for the production of PCR-generated donor cassettes will greatly simplify the construction of donor vectors that are compatible with our RMCE targets. We anticipate that it will be possible to combine the use of attB40 tails with other approaches for PCR-based mutagenesis, such as splicing by overlap extension PCR (HORTON *et al.* 1990), to easily create a series of deletion mutants, genes of interest carrying various useful "tags," or other sets of constructs for *in vivo* analysis. Furthermore, it should be possible to incorporate attB40 tails into primers used to create flanking homology arms in recombineering plasmids such as P[acman] (VENKEN *et al.* 2006), thus adapting this and other vectors for cloning large genomic fragments into RMCE-compatible donors. Finally, parallel strategies may be useful for generating suitable constructs for transgenesis via other integration systems in Drosophila and in other organisms, including those using single attP/attB sites (GROTH *et al.* 2000, 2004) and RMCE using the Cre and FLP recombinases (reviewed by BRANDA and DYMECKI 2004).

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Communicating editor: K. G. Golic