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A toolkit for transformation and mutagenesis in *Drosophila* using piggyBac.

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We have generated a high efficiency germline transformation system using the piggyBac transposable element and a series of vectors for gene misexpression under the control of the GAL4/UAS system. We have adapted Gateway recombination technology to the rapid and simple production of inverted repeat constructs which produce an RNAi effect when expressed. Finally, we have created a stable source of piggyBac transposase in the genome that is capable of mobilizing piggyBac elements in somatic and germline cells.

High efficiency transformation using ubiquitin-driven transposase

Using the known sequence of the piggyBac transposable element as a reference, we amplified the intact, full-length transposon from genomic DNA of the lepidopteran *Trichoplusia ni*. Our clone contains one open reading frame with 99.8% identity to that previously described (Handler and Harrell II, 1999). We subcloned this open reading frame via PCR downstream of the poly-ubiquitin promoter (Lee *et al.*, 1988) in pBluescript KS to create **pBlu-uTp** (Figure 1A). This helper plasmid was then used as the transposase source for the germline transformation of *Drosophila*. Using 20 different piggyBac vector constructs at a concentration of 600 mg/ml, and the helper plasmid at 400 mg/ml, we achieved transformation rates ranging from 4% to 36% of fertile G0s. This compares favorably to efficiencies obtained by Li *et al.* (2001) and Handler and Harrell II (1999). Table 1 details the rates of transformation using this helper plasmid.

Table 1.

20 w+ pB constructs	eggs	larvae	pupae	adults	fertile	transformed	percent
total	2297	N.D.	N.D.	801	608	80	13%
per construct average	115	79	49	40	30	4	13%

Useful vectors for germline transformation

The utility of the piggyBac transformation system is currently limited by the available plasmid constructs. To remedy this, we generated four vectors that may prove useful for misexpression. All of the vectors contain a mini-white transgene at the 5' end of the modified piggyBac as the selectable marker. Mini-white was amplified from flies containing a pUAS P element transgene, and the integrity of the amplified segment was verified by sequencing. The 5' and 3' piggyBac ends were amplified from

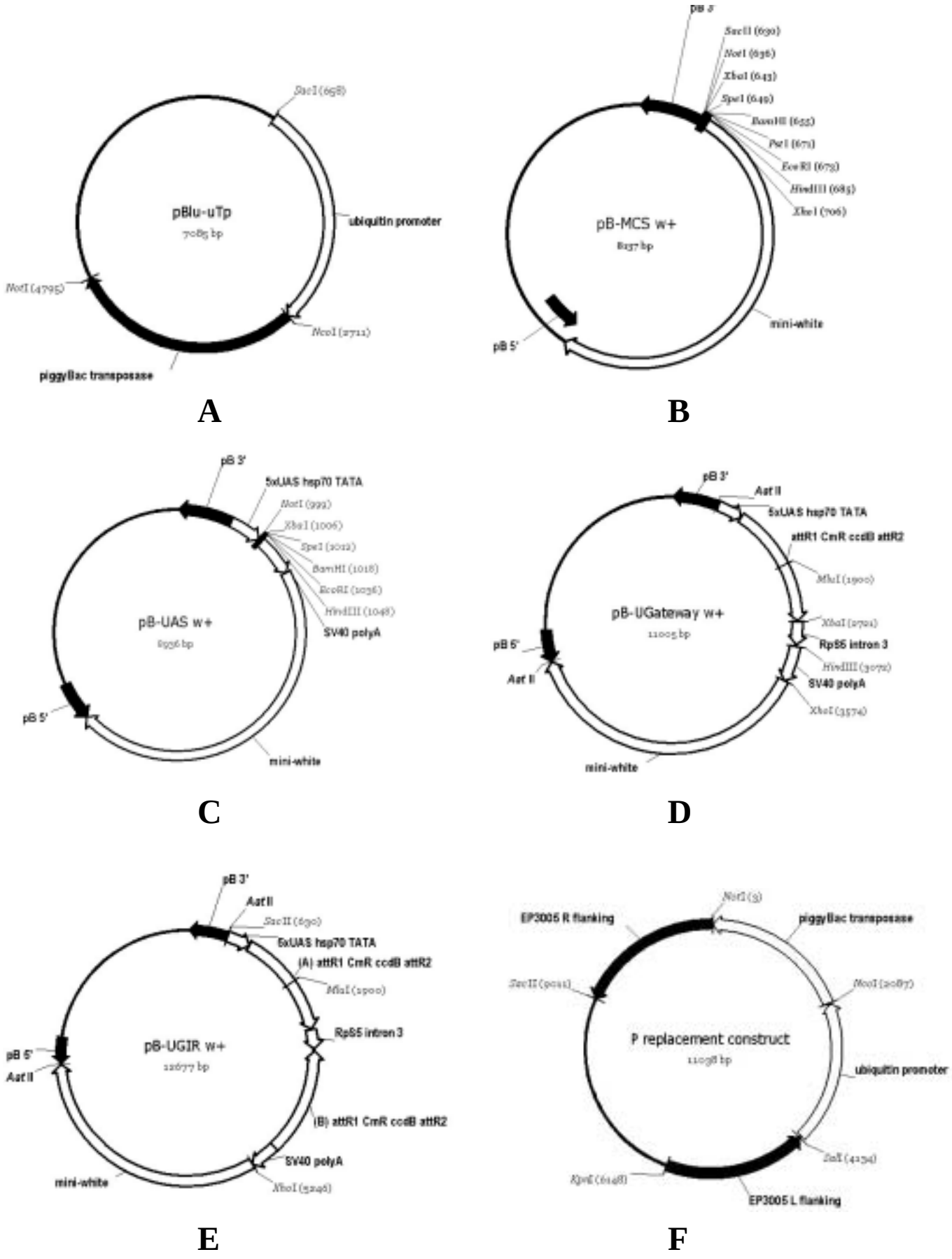
pBlu-uTp
7085 bp
SacI (658)
ubiquitin promoter
NotI (4795)
NcoI (2712)
piggyBac transposase
A
pB-MCS w+
8137 bp
pB 3'
SacII (630)
NotI (636)
XbaI (643)
SpeI (649)
BamHI (655)
PstI (671)
EcoRI (679)
HindIII (685)
XhoI (706)
mini-white
pB 5'
B
pB-UAS w+
6956 bp
pB 3'
5xUAS hsp70 TATA
NotI (999)
XbaI (1006)
SpeI (1012)
BamHI (1018)
EcoRI (1036)
HindIII (1048)
SV40 polyA
pB 5'
mini-white
C
pB-UGateway w+
11005 bp
pB 3'
AatII
5xUAS hsp70 TATA
attR1 CmR ccdB attR2
MluI (1900)
XhoI (2701)
RpS5 intron 3
HindIII (3078)
SV40 polyA
XhoI (3574)
pB 5'
AatII
mini-white
D
pB-UGIR w+
12677 bp
pB 3'
AatII
SacII (630)
5xUAS hsp70 TATA
(A) attR1 CmR ccdB attR2
MluI (1900)
RpS5 intron 3
pB 5'
AatII
(B) attR1 CmR ccdB attR2
SV40 polyA
mini-white
XhoI (9246)
E
P replacement construct
11038 bp
NotI (3)
EP3005 R flanking
piggyBac transposase
SacII (9041)
NcoI (10087)
ubiquitin promoter
ZraI (4254)
KpnI (6148)
EP3005 L flanking
F

Figure 1.

the original *Trichoplusia* fragment, and cloned into opposite ends of the pBluescript KS multiple cloning site. The mini-white gene was cloned next to the 5' piggyBac end to generate the plasmid **pB-MCS w+** (Figure 1B). This plasmid contains nine unique restriction sites within the transposable element.

Using pB-MCS w+ as a starting point, we generated a vector capable of misexpressing genes using the GAL4/UAS system, similar to Brand and Perrimon (1993). A fragment containing UAS binding sites and the Hsp70 transcriptional start site was amplified from flies harboring a transgene derived from the pUAS P element. The fragment was verified by sequencing and was placed at one end of the pB-MCS w+ cloning site. A fragment containing the SV40 polyadenylation signal was inserted downstream of the pB-MCS w+ multiple cloning site to generate the plasmid **pB-UAS w+** (Figure 1C). This arrangement allows genes to be cloned into any of six unique restriction sites under the control of the UAS enhancer. We have demonstrated that genes cloned into pB-UAS w+ and transformed into flies are controlled as expected by the expression of GAL4.

The Gateway cloning technology (Invitrogen), based on lambda phage site-specific recombination, is a rapid and simple method for transferring fragments of DNA between vectors in a directional fashion. We have adapted this technology to two piggyBac vectors to enable cloning DNA fragments into a transformation vector without the use of restriction enzymes. The first vector is based on the pB-UAS w+ plasmid and has a Gateway cassette in a "forward" orientation. Genes transferred from Invitrogen pENTR- or pDONR-derived plasmids will be under the control of the UAS enhancer. A map of this plasmid, called **pB-UGateway w+**, is shown in Figure 1D.

The second vector taking advantage of Gateway cloning technology is called **pB-UGIR w+** (Figure 1E). This vector contains a Gateway cloning cassette in the forward orientation just downstream of the UAS binding sites, a 303 bp intron from the RpS5 locus, followed by another Gateway cassette in the reverse orientation. The recombination reaction forces the fragment of interest into both Gateway cassettes and maintains the forward and reverse orientations. Plasmids containing an intact Gateway cassette must be propagated in *E. coli* containing the gyrA462 allele, such as DB3.1 (Invitrogen). After the recombination reaction (carried out as directed by Invitrogen), the plasmids are transformed into the *E. coli* strain Stbl2. Only plasmids that have undergone recombination to remove both Gateway cassettes are propagated. We found that all constructs tested were stable in Stbl2 cells (n = 16). Once transformed into *Drosophila,* transcription initiated by the UAS produces a hairpin RNA (after the intron is spliced out) that serves as a source of dsRNA used in the degradation of endogenous mRNA via RNAi. This vector allows production of an inducible dsRNA by a simple recombinase reaction followed by germline transformation.

Stably integrated Transposase

The availability of a stable source of P element transposase in a fly stock has made a wide variety of techniques available for *Drosophila* research. While such a stock might exist for piggyBac, it is not available from public stock centers. The high transformation frequency of the ubiquitin-transposase plasmid led us to initiate integration of that construct into the *Drosophila* genome. We used the P element replacement method (Keeler *et al*., 1996) so that the final product would be stable in any genetic background. We replaced the P element insertion EP3005, located at 100D2. We chose this P element because it was approximately 5 kb from the nearest predicted gene, and showed a high degree of mosaicism when crossed to Delta2-3 stocks. We injected 5292 embryos from a cross between w; EP3005 / TM3, Sb males and y^1, w^1; Delta2-3, Ki virgins with a replacement construct that carried 2 kb fragments of genomic DNA flanking the EP3005 insert on both sides of the ubiquitin-transposase (Figure 1F). From these injections we collected 336 males of the genotype y^1, w^1; EP3005 / Delta2-3,

Ki. We crossed these males to w; TM3 Sb / TM6B and isolated 375 lines where the w+ of the EP3005 insert had been lost. We established stable stocks from three individuals of each of these lines and screened these 1125 lines by PCR for insertion of the transposase. We isolated one positive, line 36C. Further analysis showed that the ubiquitin-transposase construct was functional and caused the mobilization of w+ piggyBac inserts in the eye. To enhance the genetic usefulness of this insert, we recombined dominant markers onto the 36C insert chromosome. Two stocks are now available: w^{1118}; Tb^{1}, 36C / TM3, Sb and w^{1118}; Sb^{1}, Gl^{1}, Tb^{1}, 36C/ TM3, Ser.

We tested the ability of the 36C transposase transgene to mediate the transposition of piggyBac inserts in the germline by examining the mobilization frequency of two independent inserts. The first contained only the w^{+} marker inside piggyBac ends and was located on a second chromosome marked with Tft^{1}. We could not detect any mobilization of this piggyBac insertion onto a different chromosome in 55 crosses. The second insertion, a pB-UAS w+ transgene on the first chromosome, was more amenable. We identified two autosomal jumps from 32 crosses. The percentages shown with these two attempts indicate that the 36C insert does not catalyze movement of piggyBac elements frequently enough for mutagenesis screens. It is sufficient, however, to generate new inserts of piggyBac transgenes already in the genome.

GenBank accession numbers:

pBlu-uTp	AY196821
pB-MCS w+	AY196822
pB-UAS w+	AY196823
pB-UGateway w+	AY196824
pB-UGIR w+	AY196825
P replacement construct	AY196826

References: Brand, A.H., and N. Perrimon 1993, Development 118: 401-415; Handler, A.M., and R.A. Harrell II 1999, Insect Molecular Biology 8: 449-457; Keeler, K.J., T. Dray, J.E. Penney, and G.B. Gloor 1996, Molecular and Cellular Biology 16: 522-528; Lee, H.S., J.A. Simon, and J.T. Lis 1988, Molecular and Cellular Biology 8: 4727-4735; Li, X., J.C. Heinrich, and M.J. Scott 2001, Insect Molecular Biology 10: 447-455.