P[acman]: A BAC Transgenic Platform for Targeted Insertion of Large DNA Fragments in *D. melanogaster*

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We describe a transgenesis platform for *Drosophila melanogaster* that integrates three recently developed technologies: a conditionally amplifiable bacterial artificial chromosome (BAC), recombineering, and bacteriophage ϕC31–mediated transgenesis. The BAC is maintained at low copy number, facilitating plasmid maintenance and recombineering, but is induced to high copy number for plasmid isolation. Recombineering allows gap repair and mutagenesis in bacteria. Gap repair efficiently retrieves DNA fragments up to 133 kilobases long from P1 or BAC clones. ϕC31-mediated transgenesis integrates these large DNA fragments at specific sites in the genome, allowing the rescue of lethal mutations in the corresponding genes. This transgenesis platform should greatly facilitate structure/function analyses of most *Drosophila* genes.

Drosophila is an important model organism for studying biology and disease, and new tools are continually being developed to facilitate this research (*1*, *2*). A major advance was the development of *P*-element–mediated transformation after the injection of plasmids into *Drosophila* embryos (*3*). Hence, *P*-element vectors have been engineered for numerous applications (*4*). However, *P*-element–mediated transformation has a number of limitations: inability to clone large DNA fragments in available *P*-element vectors, difficulties in manipulating large DNA fragments, inability to transfer large DNA fragments into the fly genome, and failure to target DNA to specific sites in the genome.

Cloning large DNA fragments in high–copy-number plasmids, such as typical *P*-element vectors, is inefficient because large fragments are unstable at high copy number in bacteria. Hence, low–copy-number vectors, including P1 (*5*) and bacterial artificial chromosome (BAC) (*6*) vectors, were developed to stably maintain large cloned DNA fragments. Unfortunately, low–copy-number vectors hamper sequencing, embryo injection, and other manipulations requiring large amounts of plasmid DNA. An elegant solution is a conditionally amplifiable plasmid that has two origins of replication (*ori*'s): *oriS* for low-copy propagation, typical for P1 and BAC vectors; and *oriV*, which can be experimentally induced to high copy number (*7*). Hence, the introduction of conditionally amplifiable BAC features into fly transformation vectors is a first key step to manipulating large DNA fragments in *Drosophila*.

Cloning of large DNA fragments is limited by conventional methods that rely on restriction enzymes and DNA ligases, hampering analyses of large genes and gene complexes. Recently, efficient in vivo cloning technologies using enhanced and regulated recombination systems, commonly known as recombineering, have been developed (*8*). Recombineering greatly facilitates the retrieval of DNA fragments through gap repair and their subsequent site-directed mutagenesis. Because recombineering is based on homologous recombination, restriction enzymes and DNA ligases are not required. Recombineering is widely used by mouse geneticists to generate transgenic and knockout constructs. Recombineering-mediated mutagenesis is much more efficient with low-copy plasmids (*8*). Hence, using recombineering in a conditionally amplifiable BAC should greatly facilitate the gap repair of large DNA fragments and subsequent mutagenesis at low copy number.

P-element–mediated transformation is limited by DNA size, precluding the study of large genes (>40 kb) and gene complexes. In addition, more than 75% of *P* elements insert in regulatory elements of genes (*9*), often disrupting genes in subtle ways (*10*). Moreover, *P* elements are subject to position effects: the effect of a local chromosomal environment on the levels or patterns of transgene expression. This necessitates the generation and characterization of several transgenes for each DNA construct studied. Hence, site-specific integration would greatly facilitate structure-function analysis of transgenes, permitting direct comparison of differently mutagenized DNA fragments integrated at the same site in the genome. Recently, site-specific integration using the integrase of bacteriophage ϕC31 has been demonstrated in *Drosophila* (*11*). ϕC31-integrase mediates recombination between an engineered "docking" site, containing a phage attachment (*attP*) site, in the fly genome, and a bacterial attachment (*attB*) site in the injected plasmid. Three pseudo-*attP* docking sites were identified within the *Drosophila* genome, potentially bypassing desired integration events in engineered *attP* sites. Fortunately, they did not seem receptive to *attB* plasmids, because all integration events were at the desired *attP* sites (*11*). Thus, recovery of large DNA fragments by gap repair into a low-copy plasmid containing an *attB* site, followed by ϕC31-mediated transformation, might allow the integration of any DNA fragment into any engineered *attP* docking site dispersed throughout the fly genome.

Here we describe new vectors that overcome the limitations associated with *P*-element–mediated transgenesis. We developed P/ϕC31 artificial chromosome for manipulation (P[acman]), a conditionally amplifiable BAC vector that contains recognition sites for both *P*-transposase– (*3*) and ϕC31-mediated integration (*11*). P[acman] permits recombineering-mediated cloning of any genomic DNA fragment from *Drosophila* P1 or BAC clones (*12*–*15*) and enables the transfer of large DNA fragments into the fly genome. The ability to easily manipulate these DNA fragments through recombineering and to introduce them into specific sites in the fly genome will greatly facilitate and accelerate in vivo genetic manipulations of *Drosophila*.

Results

Construction of a BAC transgenesis vector for Drosophila. The motivation to construct P[acman] grew from our inability to clone 29- and 39-kb DNA fragments in existing *P*-element vectors for the transformation rescue of mutations in the gene *senseless2* (*16*). We were unable to identify restriction sites for cloning the entire gene. Moreover, recombineering-mediated gap repair in existing high-copy *P*-element vectors was unsuccessful. This was consistent with published data demonstrating that gap repair in high-copy or medium-copy plasmids has an upper size limit of 25 and 80 kb, respectively (*17*). Because high-copy *P* elements have a substantial size, gap repair is limited to fragments of about 20 kb. We hypothesized that a low-copy plasmid might alleviate size limitations and improve the stability of large DNA fragments. Hence, we added *P*-element components to a chloramphenicol-resistant conditionally amplifiable BAC (*7*), resulting in P[acman]-Cm^R (Fig. 1A). The *P*-element components include the 5′*P* and 3′*P* termini required for *P*-transposase–mediated integration, a multiple cloning site (MCS), and the *white*$^+$ marker. The conditionally amplifiable BAC contains two origins of replication: *oriS* and *oriV* (*7*). *oriS* keeps P[acman] at low

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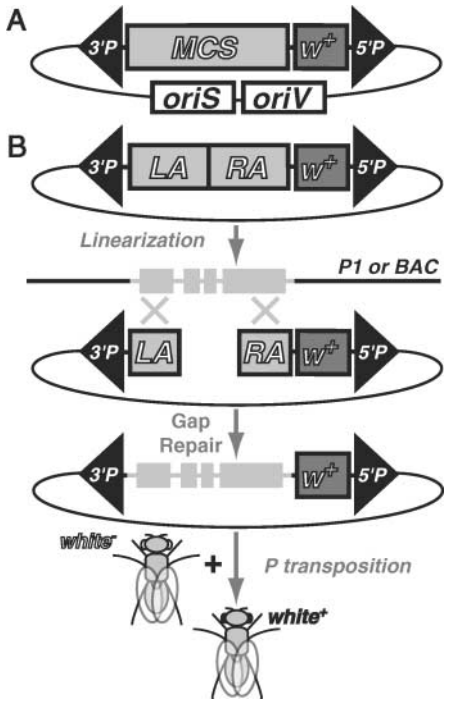

Fig. 1. P[acman]: BAC transgenesis for *Drosophila*. **(A)** P[acman] contains *P*-element transposase sites (3′*P* and 5′*P*), *white*$^{+}$, and an MCS. This *P* element is inserted in the conditionally amplifiable BAC, containing a low-copy origin of replication (*oriS*) and a copy-inducible origin of replication (*oriV*). **(B)** P[acman] is linearized between both homology arms (LA and RA) and transformed into recombineering bacteria containing P1 or BAC clones. Integration into the germ line of *white*$^{-}$ flies is mediated by *P*-element–mediated transformation.

copy number for the stability of large cloned inserts and efficient recombineering, whereas *oriV* permits copy-number induction for high-yield DNA preparation for sequencing and embryo injections. We created transgenic flies containing P[acman]-Cm^{R} using *P*-transposase–mediated integration. To facilitate cloning by gap repair from a variety of sources, we also replaced the ampicillin-resistance marker into P[acman]-Cm^{R}, resulting in P[acman]-Ap^{R}. Both plasmids can be used to clone any DNA fragment from a variety of donor vectors.

Recombineering-mediated gap repair into P[acman]. P[acman] was used to retrieve fragments by gap repair. For each gap repair, we designed four primer sets (fig. S1). Two homology arms, located at either end of the DNA fragment, were cloned into P[acman] (fig. 1B). Linearization between both homology arms and the subsequent transformation of the linearized construct into recombineering-competent bacteria containing the necessary genomic clone allow retrieval of the DNA fragment by gap repair. Gap repair was performed with the use of two similar strategies, relying on *Red* recombination functions (fig. S2) (*17*, *18*). Both strategies were used to retrieve DNA fragments ranging from 9.4 to 39 kb, from different donor plasmids (Table 1). Colony polymerase chain reaction (PCR) screening identified correct recombination events at both junctions after gap repair. After plasmid copy-number induction, DNA fingerprinting and sequencing demonstrated that the desired fragment was obtained. Hence, the methodology is reliable and large DNA fragments can easily be retrieved.

Table 1. Retrieval of genes in P[acman]. Genomic fragments containing genes of interest retrieved by gap repair into P[acman]-Cm^{R} or -Ap^{R} are shown. Donor plasmids, with clone coordinates, are P1, BACs, or ampicillin-modified BACs (BAC-Ap^{R}). Mutations in the corresponding genes were lethal or showed a phenotype. Rescue was obtained for most genes using *P*-element–mediated transformation. NA, not applicable.

Gene	Construct	Size	Donor	Clone	P[acman]	Mutation	Rescue
sens2	sens2-22	22.3 kb	P1	DS05421	Cm^{R}	NA	NA
sens2	sens2-29	28.9 kb	P1	DS05421	Cm^{R}	NA	NA
sens2	sens2-39	38.9 kb	P1	DS05421	Cm^{R}	NA	NA
CG10805	CG10805-S	9.7 kb	P1	DS05421	Cm^{R}	Lethal	Yes
CG10805	CG10805-L	14.7 kb	P1	DS05421	Cm^{R}	Lethal	Yes
dap160	dap160	10.9 kb	P1	DS02919	Cm^{R}	Lethal	Yes
sens	sens-S	12.1 kb	BAC-Ap^{R}	BACR17E13	Cm^{R}	Lethal	Yes
sens	sens-L	18.1 kb	BAC-Ap^{R}	BACR17E13	Cm^{R}	Lethal	Yes
Dalpha7	Da7	29.4 kb	BAC-Ap^{R}	BACR02B03	Cm^{R}	Phenotype	Yes
Drp	Drp	9.4 kb	BAC	BACR30P05	Ap^{R}	Lethal	Yes*
Sec8	Sec8-L	12 kb	BAC	BACR02L23	Ap^{R}	Lethal	No
Eps15	Eps15-L	11.9 kb	BAC	BACR27P17	Ap^{R}	Lethal	Yes

*From (*25*).

To test the functionality of the gap-repaired constructs, we used *P*-mediated transposition. Although germline transformation of the constructs was efficient with small inserts, the efficiency dropped for fragments over 20 kb. Transgenic flies containing the gap-repaired fragments in P[acman] were crossed to flies carrying mutations in the corresponding genes. Eight out of 9 fragments tested fully rescued the lethality or visible phenotype associated with the mutations, in the homozygous or transheterozygous condition (Table 1). One fragment (Sec8-L) did not rescue, although a smaller fragment encompassing the same gene did.

ϕC31-mediated integration of P[acman]. Because *P*-transposase–mediated transformation has size limitations, we explored the possibility of using ϕC31-mediated integration to integrate larger constructs at specific sites within the genome. To combine the power of recombineering with ϕC31, we equipped P[acman] with an *attB* site, resulting in *attB*-P[acman]. To create genomic *attP* docking sites, we introduced an *attP* site and *yellow*$^{+}$ into a minimal *piggyBac* transposon (*19*) (*piggyBac-yellow*$^{+}$*-attP*) and integrated and remobilized this *piggyBac* in the *Drosophila* genome using *piggyBac* transposase. We isolated 34 homozygous viable insertions and determined their exact genomic location (Fig. 2A and table S1). Because *piggyBac* has a more random distribution than *P*-transposase (*9*), *attB*-P[acman] should integrate into both gene-poor and gene-rich regions.

To integrate *attB*-P[acman], we co-injected circular plasmid DNA and mRNA encoding ϕC31-integrase (*11*) into embryos carrying *piggyBac-yellow*$^{+}$*-attP* (Fig. 2B). Integration results in flies with a "yellow^{+}" body color and "white^{+}" eye color phenotype. Because the *attB* site is upstream of the MCS and *white*$^{+}$ is downstream of the MCS, only transgenic flies that are "yellow^{+} white^{+}" should have undergone an integration event containing most of the injected DNA, including the cloned insert (Fig. 2B). Hence, successful integration events can be genetically traced. Moreover, because both transposons (*P*-element and *piggyBac*) are maintained after integration, the inserted DNA can be remobilized using the respective transposases.

We tested whether different *attP* docking sites are equally receptive to *attB*-P[acman]-Ap^{R}. We focused on seven docking sites: two each on chromosomes X, 2, and 3, and one on chromosome 4. As shown in table S2, all but one of these docking sites were receptive, with similar integration efficiencies of 20 to 30%. We occasionally obtained efficiencies as high as 60%, similar to those in a previous report (*11*). However, in general we observed higher survival rates than Groth *et al*. (*11*). In our hands, such efficiencies were higher than the *P*-transposase–mediated integration of similar-sized constructs. Because ϕC31-mediated–integration is site-specific, only a single insertion is needed, and the injection procedure can therefore be scaled down to 50 embryos or less. Integration of *attB*-P[acman]-Ap^{R} in the same docking site led to the same *white*$^{+}$ expression level (fig. S3). This is convenient, because rare events that were not integrated in the proper site exhibited a different eye color and were therefore distinguished from true integration events. Integration of *attB*-P[acman]-Ap^{R} in a single docking site on chromosome 2 consistently caused a patchy red eye phenotype (fig. S3D), indicating position-effect variegation of *white*$^{+}$

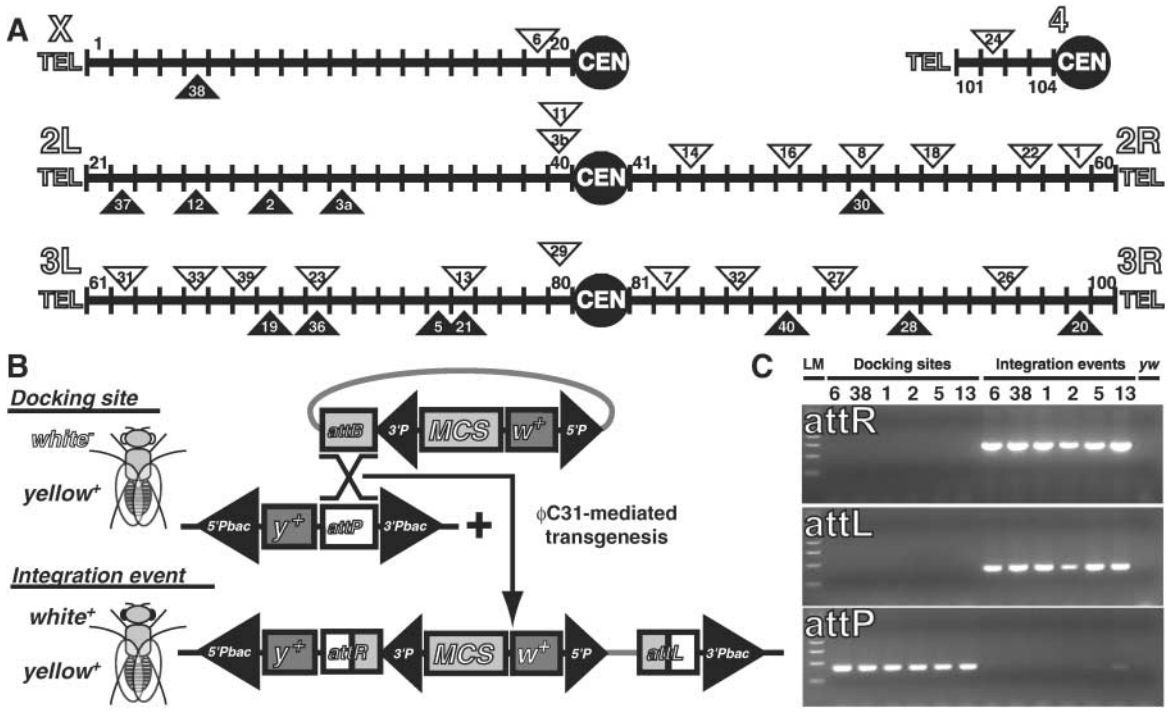

Fig. 2. P[acman] transgenesis in *Drosophila* using the ϕC31 system. (**A**) The *piggyBac-y*$^+$*-attP* docking element was transformed or remobilized in the *Drosophila* genome to obtain multiple *attP* docking lines (VK lines). Locations are indicated on a schematic representation of the polytene chromosomes. White triangles represent insertions between, and black triangles within, annotated genes. VK line numbers (table S1) are indicated within the triangles. (**B**) *attB*-P[acman] can integrate at an *attP* docking site in the fly genome. (**C**) Correct integration events in docking sites are PCR-positive for the *attR* and *attL* assays, whereas original docking sites are PCR-positive for the *attP* assay. *yw* served as a negative control. Two docking sites on chromosomes X (VK6 and VK38), 2 (VK1 and VK2), and 3 (VK5 and VK13) were used. LM, length marker.

expression. This was not observed for the *yellow*$^+$ marker present at the same docking site. Moreover, the insertion of *attB*-P[acman]-Ap^R into different docking sites resulted in different eye color phenotypes, indicating position effects on the expression level of the *white*$^+$ marker (fig. S3). The main difference between ϕC31- and *P*-element–mediated integration is that position effects with the ϕC31 system are predictable, allowing for the selection of different but defined expression levels.

To identify correct integration events, we developed PCR assays specific for the *attP*, *attL* (left attachment), and *attR* (right attachment) sites (Fig. 2C). Correct integration events were identified by the loss of the *attP* PCR product (specific for the original docking site) and the appearance of *attL* and *attR* PCR products (specific for the integration event). PCR analysis indicated that all but one of the insertions was correctly integrated. Moreover, correct integration events in homozygous viable docking sites maintained homozygous viability, demonstrating that the insertion does not detrimentally affect the local chromosomal environment. In numerous transformation experiments, in addition to proper integration events, we recovered only one event that converted a homozygous viable docking site into a recessive lethal locus. PCR analysis demonstrated that this event did not result in correct integration. Therefore, ϕC31-mediated integration is very efficient.

Cloning of large DNA fragments into attB-*P[acman].* To clone large DNA fragments into *attB*-P[acman]-Ap^R, we performed recombineering-mediated gap repair, as described above. We retrieved fragments up to 102 kb in length from single P1 or BAC clones (Table 2). Multiple genes and gene complexes, including *bancal*, *Dscam*, *teashirt*, and the *Bearded* and *Enhancer of Spl* complexes, were cloned into *attB*-P[acman]-Ap^R (Table 2). Unfortunately, some large genes were not contained within a single BAC (*15*). We therefore decided to reconstitute large genes through serial gap repair from two overlapping BAC clones into *attB*-P[acman]-Ap^R: In step 1, the smaller part is retrieved from one BAC, which is followed by step 2, retrieving the remainder of the gene (Fig. 3 and fig. S4). This two-step procedure was successful in retrieving a 133-kb fragment encompassing the *Tenascin-major* gene (Table 2).

Table 2. Retrieval of genes in *attB*-P[acman]. Genome fragments containing genes of interest retrieved by gap repair into *attB*-P[acman]-Ap^R are shown. Donor fragments are from P1 or BACs, or from PCR. Clone coordinates of the donor clones are indicated. Mutations in all corresponding genes are lethal. Rescue is indicated for *P*-element– or ϕC31-mediated transformation. ND, not determined; NA, not applicable.

Gene	Construct	Size	Donor	Clone	Transgenics			
					P	Rescue	phiC31	Rescue
Sec6	Sec6-S	2.8 kb	PCR	BACR27L09	Yes	No	Yes	ND
miR-4	miR-4	4.5 kb	BAC	BACR02J10	ND	NA	ND	NA
Sec8	Sec8-S	4.9 kb	BAC	BACR02L23	Yes	Yes	ND	NA
Eps15	Eps15-S	10.8 kb	BAC	BACR3B7	ND	NA	Yes	ND
Sec6	Sec6-L	11.5 kb	BAC	BACR27L09	Yes	ND	Yes	ND
sens	sens-L	18.1 kb	BAC	BACR17E13	ND	NA	No	NA
miR-9a	miR-9a	20.1 kb	BAC	BACR01D04	ND	NA	Yes	ND
Tsh	Tsh-1	28.4 kb	BAC	BACR03L08	ND	NA	Yes	Yes
grp	grp	29.8 kb	P1	DS00592	ND	NA	Yes	ND
Brd-C	Brd-C	37.2 kb	BAC	BACR01H12	ND	NA	ND	NA
bancal	bancal	39.5 kb	BAC	BACR33D17	ND	NA	ND	NA
Dscam	Dscam-1	73.3 kb	BAC	BACR26B18	ND	NA	Yes	Yes
E(Spl)-C	E(Spl)-C	77.7 kb	BAC	BACR13F13	ND	NA	Yes	Yes
Tsh	Tsh-2	86.4 kb	BAC	BACR03L08	ND	NA	Yes	Yes
Dscam	Dscam-2	102.3 kb	BAC	BACR26B18	ND	NA	No	NA
ten-m	ten-m	20 kb	BAC	BACR02D04	NA	NA	NA	NA
ten-m	ten-m	133 kb	BAC	BACR22C11	ND	NA	Yes	Yes

ϕC31-mediated integration of large gap-repaired fragments. To obtain transgenic flies with *attB*-P[acman]-Ap^R constructs, we can use either *P*-transposase or ϕC31-integrase. We first tested *P*-transposition for three small fragments. Transgenic animals were obtained for all three (Table 2). Because *P*-transposase–mediated integration is inefficient for fragments larger than 20 kb, we switched to ϕC31-mediated integration. Several injection rounds suggested that the molar DNA concentration was critical. We empirically established that 75 ng of DNA per microliter of injection buffer (ng/μl) is the lower limit for *attB*-P[acman]-Ap^R (~13 kb). Therefore, large (75- to 135-kb) supercoiled plasmid DNA (300 to 750 ng/μl) was co-injected with ϕC31 mRNA (250 to 500 ng/μl) (*11*). As shown in Table 2, we obtained transgenic animals for most constructs, including 73-, 76-, 86-, and 133-kb fragments. In these cases, the transformation efficiency was about 10% for medium (15 to 50 kb) and 2 to 4% for large plasmids (>50 kb). PCR analysis confirmed that all constructs integrated correctly. For two transgenes, 18 and 102 kb in length, we did not obtain transformants, illustrating that optimization for certain constructs might be required. Different gap-repaired fragments inserted in the same docking site differentially affected *white*$^+$ expression, suggesting that DNA context is important. Finally, multiple fragments fully rescued two independent lethal mutations, in the homozygous or transheterozygous condition, in each of the four corresponding genes or gene complexes tested (Table 2). These data show that gap repair permits the cloning of BAC-sized fragments, large transgenes can be integrated site-specifically in the *Drosophila* genome using ϕC31, and mutations in essential genes and entire gene complexes can be rescued by large transgenic fragments.

Discussion

P[acman] provides numerous improvements when compared to current strategies for *Drosophila* transgenesis. First, DNA constructs larger than 100 kb can be retrieved from genomic P1 and BAC clones using recombineering-mediated gap repair. Fragments are retrieved into a plasmid fitted with an inducible *oriV* replication origin that allows easy preparation of large quantities of DNA for sequencing and *Drosophila* transgenesis. The retrieved fragments do not need to be resequenced because they are directly retrieved from the genomic clone without PCR amplification. Second, unlike *P*-transposase, ϕC31-integrase enables the integration of large fragments into the *Drosophila* genome. Because ϕC31-integrase catalyzes recombination between two ectopic attachment sites (*attB* and *attP*), transgenes are integrated at specific docking sites in the fly genome. This largely eliminates the problem of position effects, a highly desirable feature when comparing different mutagenized constructs derived from the same transgene for structure/function analysis. Finally, site-directed mutagenesis via recombineering is much more efficient in low-copy plasmids such as P[acman].

We were able to clone fragments as large as 102 kb from single BACs. Only one report documents the gap repair of a similarly sized fragment from one BAC (*20*). In both cases, gap repair was successful because of the use of a low-copy vector. Indeed, gap repair into high-copy or medium-copy plasmids has an upper size limit of 25 and 80 kb, respectively (*17*). We also reconstituted one of the largest *Drosophila* genes, using serial gap repair, resulting in reconstitution of a 133-kb fragment. One recent report demonstrated a variation of seamless recombination of two large DNA fragments (*20*). The methodology described here may facilitate the cloning of even larger genomic fragments into P[acman], which may not be contained within single BAC clones in the genome tiling path set (*15*).

The numerous docking sites (Fig. 2A and table S1) created in this work will have to be characterized in more detail to determine the expression levels of different genes that are inserted in the same site. It will also be important to determine whether adjacent enhancers or regulatory elements influence gene expression in each of the docking sites in order to identify sites that are as "neutral" as possible, a requirement important for the study of regulatory elements. The neighboring genome environment may also become important when overexpression or RNA interference transgenes are inserted. We feel that *piggyBac-y+-attP* insertions in intergenic regions may be the best candidates for these applications, although this will have to be determined experimentally.

The circular ϕC31 bacteriophage genome is integrated into the linear genome of its host, *Streptomyces lividans*, by ϕC31-integrase (*21*). The genome of ϕC31 is about 41.5 kb, which is larger than standard high-copy plasmids. This fact suggested that ϕC31-integrase would be useful to integrate circular DNA molecules up to 41.5 kb and potentially larger into the *Drosophila* genome. Indeed, we integrated fragments up to ~146 kb at defined sites in the genome, which was previously impossible. Large gap-repaired fragments should complement many molecularly defined deficiencies (*22*), and subsequent mutagenesis should permit the analysis of genes within the deletion, obviating the need to recover mutations using conventional genetic screens or imprecise excision of mapped *P* elements. This approach also opens the road for clonal analysis of any gene that maps close to centromeric heterochromatin, because transgenic constructs for these genes can be recombined onto an *FRT*-containing chromosome. Finally, recombineering will allow the integration of any peptide or protein tag into the genomic rescue constructs to study protein localization and function in vivo.

The methodology proposed here opens up a wide variety of experimental manipulations that were previously difficult or impossible to perform in *Drosophila*. Moreover, it is expected that it should be possible to adapt this methodology to other model organisms, because *piggyBac* transposes in many species, including mammals (*23*), and ϕC31 is operational in many species, including mammalian cells (*24*).

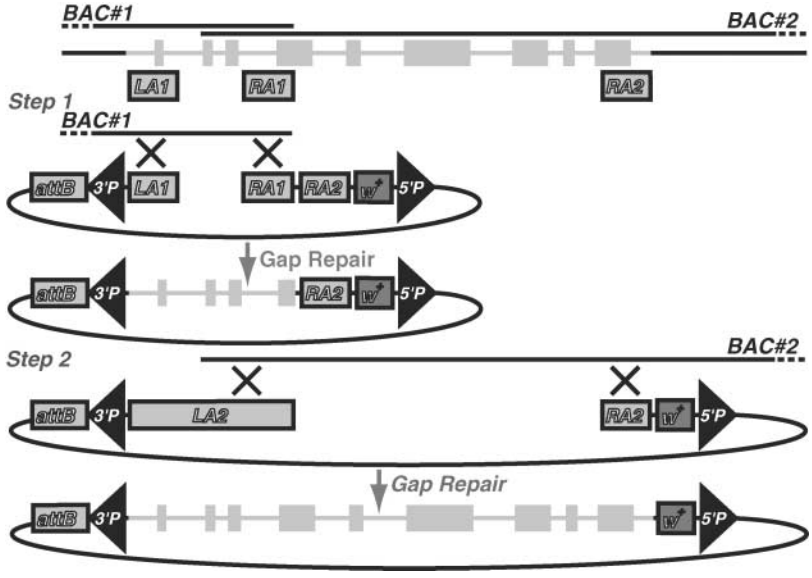

Fig. 3. Reconstitution of large fragments from two overlapping BACs by serial gap repair. Three homology arms are designed: LA1, RA1, and RA2, located at the left end, the region of overlap of the two BACs, and the right end of the desired DNA fragment, respectively. During step 1, the construct is linearized between LA1 and RA1, and the left segment of the gene is obtained from BAC 1, resulting in LA2. In step 2, the construct is linearized between LA2 and RA2, and the remaining segment of the gene is obtained from BAC 2 to reconstitute the entire gene.

References and Notes

1. K. J. Venken, H. J. Bellen, *Nat. Rev. Genet.* **6**, 167 (2005).
2. K. A. Matthews, T. C. Kaufman, W. M. Gelbart, *Nat. Rev. Genet.* **6**, 179 (2005).
3. G. M. Rubin, A. C. Spradling, *Science* **218**, 348 (1982).
4. E. Ryder, S. Russell, *Brief. Function. Genom. Proteom.* **2**, 57 (2003).
5. N. Sternberg, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 103 (1990).
6. H. Shizuya *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 8794 (1992).
7. J. Wild, Z. Hradecna, W. Szybalski, *Genome Res.* **12**, 1434 (2002).
8. N. G. Copeland, N. A. Jenkins, D. L. Court, *Nat. Rev. Genet.* **2**, 769 (2001).
9. H. J. Bellen *et al.*, *Genetics* **167**, 761 (2004).
10. K. K. Norga *et al.*, *Curr. Biol.* **13**, 1388 (2003).
11. A. C. Groth, M. Fish, R. Nusse, M. P. Calos, *Genetics* **166**, 1775 (2004).
12. W. Kimmerly *et al.*, *Genome Res.* **6**, 414 (1996).
13. R. A. Hoskins *et al.*, *Science* **287**, 2271 (2000).
14. M. D. Adams *et al.*, *Science* **287**, 2185 (2000).
15. S. E. Celniker *et al.*, *Genome Biol.* **3**, RESEARCH0079 (2002).
16. H. Jafar-Nejad, H. J. Bellen, *Mol. Cell. Biol.* **24**, 8803 (2004).
17. E. C. Lee *et al.*, *Genomics* **73**, 56 (2001).
18. D. L. Court *et al.*, *Gene* **315**, 63 (2003).
19. X. Li, N. Lobo, C. A. Bauser, M. J. Fraser Jr., *Mol. Genet. Genomics* **266**, 190 (2001).
20. G. Kotzamanis, C. Huxley, *BMC Biotechnol.* **4**, 1 (2004).
21. H. M. Thorpe, M. C. Smith, *Proc. Natl. Acad. Sci. U.S.A.* **95**, 5505 (1998).
22. A. L. Parks *et al.*, *Nat. Genet.* **36**, 288 (2004).
23. H. F. Xu *et al.*, *Mol. Genet. Genomics* **276**, 31 (2006).
24. D. A. Sorrell, A. F. Kolb, *Biotechnol. Adv.* **23**, 431 (2005).
25. P. Verstreken *et al.*, *Neuron* **47**, 365 (2005).
26. GenBank accession numbers for the P[acman] vectors are as follows: P[acman]-Cm^R-F-2, EF106977; P[acman]-Ap^R-F-2-5, EF106978; *attB*-P[acman]-Cm^R-F-2, EF106979; and *attB*-P[acman]-Ap^R-F-2, EF106980. We thank the Bloomington Stock Center and Drosophila Genome Resource Center for fly stocks and plasmids, respectively; and M. Calos, J. Carlson, A. Celik, H.-T.Chao, K.-W. Choi, J. Clemens, N. Copeland, D. Court, C. Delidakis, M. Evans-Holm, D. Featherstone, M. Fraser, A. Handler, N. Jenkins, H. Kim, E. Lai, P. Liu, P. Ponsaerts, R. Mann, D. Schmucker, S. Speese, W. Szybalski, U. Tepass, L. Zipursky, and past and present members of the Bellen lab for flies, bacteria, plasmids, help, and/or stimulating discussions. H.J.B is an investigator with the Howard Hughes Medical Institute. This work was supported by NIH (grant GM067858-05) and the Howard Hughes Medical Institute. Plasmids are available through the Drosophila Genome Resource Center. Fly strains are available through the Bloomington Stock Center.

Supporting Online Material

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Materials and Methods
Figs. S1 to S4
Tables S1 to S4
References

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Rubidium-Rich Asymptotic Giant Branch Stars

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A long-debated issue concerning the nucleosynthesis of neutron-rich elements in asymptotic giant branch (AGB) stars is the identification of the neutron source. We report intermediate-mass (4 to 8 solar masses) AGB stars in our Galaxy that are rubidium-rich as a result of overproduction of the long-lived radioactive isotope ^{87}Rb, as predicted theoretically 40 years ago. This finding represents direct observational evidence that the ^{22}Ne(α,n)^{25}Mg reaction must be the dominant neutron source in these stars. These stars challenge our understanding of the late stages of the evolution of intermediate-mass stars and would have promoted a highly variable Rb/Sr environment in the early solar nebula.

Low- and intermediate-mass stars (1 to 8 solar masses $M_\odot$) evolve toward the asymptotic giant branch (AGB) phase (*1*) after the completion of hydrogen and helium burning in their cores, before they form planetary nebulae, ending their lives as white dwarfs. Basically, an AGB star is composed of an inert carbon-oxygen (C-O) core surrounded by a He-rich intershell and an extended H-rich convective envelope. Nuclear energy release is dominated by the H shell and interrupted periodically by thermonuclear runaway He-shell "thermal pulses" that initiate a series of convective and other mixing events. Strong mass loss enriches the interstellar medium (ISM) with the products of the resulting complex nucleosynthesis (*2*). During this thermally pulsing AGB (TP-AGB) phase, stars originally born O-rich (reflecting the ISM composition) can turn C-rich (C/O > 1) as a consequence of the "dredge-up" of processed material from the bottom of the convective envelope to the stellar surface. In AGB stars at the higher end of this range (4 to 8 $M_\odot$), the convective envelope penetrates the H-burning shell, activating the so-called "hot bottom burning" (HBB) process (*3*, *4*). HBB takes place when the temperature at the base of the convective envelope is hot enough ($T \geq 2 \times 10^7$ K) that ^{12}C can be converted into ^{13}C and ^{14}N through the CN cycle, so these AGBs are no longer C-rich and become again or remain O-rich despite the dredge-up. HBB models (*3*, *4*) also predict the production of the short-lived ^{7}Li isotope through the "^{7}Be transport mechanism" (*5*), which should be detectable at the stellar surface. The HBB activation in massive AGB stars is supported by lithium overabundances in luminous O-rich AGB stars of the Magellanic Clouds (*6*, *7*). In our own Galaxy, a small group of stars showing OH maser emission at 1612 MHz (sometimes without an optical counterpart but very bright in the infrared, the OH/IR stars) has recently been found to show strong Li abundances (*8*).

Mixing of protons into the He-rich intershell during the TP-AGB phase leads to reaction chains producing free neutrons, which allow production of neutron-rich elements such as Rb, Sr, Y, Zr, Ba, La, Nd, and Tc by slow neutron capture on iron nuclei and other heavy elements (the s process) (*9*–*11*). There are two possible chains for the neutron production: ^{13}C(α,n)^{16}O and ^{22}Ne(α,n)^{25}Mg. The ^{13}C neutron source operates at relatively low neutron densities ($N_n < 10^7$ cm^{-3}) and temperatures $T < 0.9 \times 10^8$ K (*11*, *12*) in TP-AGB stars during the interpulse period. The ^{22}Ne neutron source operates at much higher neutron densities ($N_n > 10^{10}$ cm^{-3}) and requires higher temperatures ($T > 3.0 \times 10^8$ K), which are achieved only while the convective thermal pulse is ongoing. In the more massive AGB stars (> 4 to 5 $M_\odot$), where these high temperatures are more easily achieved, the s-process elements are expected to form mainly through the ^{22}Ne(α,n)^{25}Mg reaction (*11*, *13*). The ^{22}Ne neutron source also strongly favors the production of the stable isotope ^{87}Rb because of the operation of a branching in the s-process path at ^{85}Kr (*14*) that modifies the isotopic mix between ^{85}Rb and ^{87}Rb (*14*–*17*). Unfortunately, the Rb isotope ratio cannot be measured in stellar sources (*17*) even with the help of very-

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