Versatile P(acman) BAC Libraries for Transgenesis Studies in *Drosophila melanogaster*

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Abstract

We constructed *Drosophila melanogaster* BAC libraries with 21-kb and 83-kb inserts in the P(acman) system. Clones representing 12-fold coverage and encompassing more than 95% of annotated genes were mapped onto the reference genome. These clones can be integrated into predetermined *attP* sites in the genome using ΦC31 integrase to rescue mutations. They can be modified through recombineering, for example to incorporate protein tags and assess expression patterns.

Genetic model systems such as *Drosophila melanogaster* are powerful tools for investigating developmental and cell biological processes, properties of inheritance, the molecular underpinnings of behavior, and the molecular bases of disease 1. The approaches

METHODS
Methods and associated references are available as supplementary online material at http://www.nature.com/naturemethods/.

NOTE
Supplementary information is available on the Nature Methods website.

used in model systems rely on the identification of mutations in genes and the characterization of the gene products, often aided by transgenesis techniques 2.

We recently developed a new transgenesis platform for *D. melanogaster*, the P(acman) (P/ ΦC31 artificial chromosome for manipulation) system, that allows modification of cloned fragments by recombineering and germ-line transformation of genomic DNA fragments up to 133 kb in length 3. P(acman) combines a conditionally amplifiable BAC 4, the ability to use recombineering in *E. coli* for retrieval and manipulation of DNA inserts 5, and bacteriophage ΦC31 integrase-mediated germ-line transformation into the *D. melanogaster* genome 6,7. Clones are maintained at low-copy number to improve plasmid stability and facilitate recombineering, but can be induced to high-copy number for plasmid isolation to facilitate microinjection of embryos. Recombineering can be used to insert protein tags for *in vivo* protein localization or acute protein inactivation 8, and to create deletions 9 and point mutations 5 for structure/function analysis. ΦC31-mediated transgenesis integrates DNA constructs at specific pre-determined *attP* sites dispersed throughout the genome 3,6,7,10, eliminating the need to map integration events and reducing variability in expression due to position effects 10. The technique allows rescue of mutations in large genes 3 and facilitates comparative expression analysis of engineered DNA constructs 7,10–12. Previously, genomic regions of interest were cloned into P(acman) by gap-repair from available mapped BAC clones 3. Here, we describe a more efficient approach: we constructed two genomic BAC libraries in the P(acman) system and mapped the cloned inserts by alignment of paired end sequences to the reference genome sequence.

We engineered a novel P(acman) BAC vector for construction of genomic libraries (Fig. 1a). In addition to the published features 3, we included a polylinker embedded within a mutant α-*lacZ* fragment. It became apparent that in the low-copy-number condition necessary to ensure stability of large genomic inserts, standard α-*lacZ* fragments are expressed at insufficient levels to permit reliable blue-white colony screening. We isolated a mutant with significantly enhanced β-galactosidase activity resulting from a premature stop codon in the α-*lacZ* fragment (Supplementary Fig. 1) that permits blue-white selection for cloned inserts at low-copy number using an automated colony picking device.

To create a resource for manipulation and analysis of *D. melanogaster* genes, we constructed two P(acman) libraries with different insert sizes (Supplementary Fig. 2). For analysis of most genes, we used the library with an insert size of 20 kb. Ninety percent of protein-coding gene annotations in *D. melanogaster* are less than 12.1 kb in length, and a 20 kb insert size should provide sufficient flanking genomic sequence to contain most genes, including regulatory sequences required for normal expression. For analysis of large genes and gene complexes, we constructed a library with an insert size of 80 kb. High molecular weight genomic DNA was prepared from the *D. melanogaster* strain used to produce the reference genome sequence. The DNA was fragmented by partial restriction digestion, and size fractions in the 20 kb and 80 kb ranges were recovered and cloned separately to produce two genomic BAC libraries. The libraries produced from the 20 kb and 80 kb fractions were designated CHORI-322 and CHORI-321, respectively. We stocked 73,728 CHORI-322 clones and 36,864 CHORI-321 clones.

To map P(acman) BACs on the genome, paired end sequences were determined and aligned to the reference genome sequence. We mapped consistent paired ends of 33,314 CHORI-322 clones representing 4.3-fold coverage of the X chromosome and 5.9-fold coverage of the autosomes, and 12,328 CHORI-321 clones representing 8.2-fold coverage of the X chromosome and 9.3-fold coverage of the autosomes. The mapped paired end sequences show that the average insert sizes of the CHORI-322 and CHORI-321 libraries are 21.0 kb (+/− 4.0 kb) and 83.3 kb (+/− 21.5 kb), respectively. An additional 18,767 CHORI-322 clones and 11,571 CHORI-321 clones were partially mapped to the genome sequence by alignment of one end sequence only. The two libraries together represent deep coverage of the genome and span most annotated genes (Supplementary Table 1). The mapped CHORI-322 and CHORI-321 clones span 88.9% and 99.3% of annotated genes, respectively. P(acman) clones containing genes and genomic regions of interest can be identified through a web-accessible genome browser (http://pacmanfly.org/) (Fig. 1b) and are available for distribution from the BACPAC Resources Center (http://bacpac.chori.org/).

We tested the P(acman) library resource for transformation efficiency using clones encompassing several genes. For each gene, we identified a clone containing substantial flanking sequences biased toward the 5' end of the gene annotation. These clones are likely to include the regulatory sequences necessary for normal expression of the gene. For small genes (12 kb), a CHORI-322 clone was preferred over a CHORI-321 clone, as smaller clones tend to have higher transformation efficiencies [3]. When a mapped CHORI-322 clone was not available for a small gene (e.g. *hh*, *vas* and *shi*) or sufficient 5' regulatory sequence did not appear to be present in a mapped CHORI-322 clone (e.g. *jar*, *lt* and *cta*), we chose a CHORI-321 clone instead. In total, we selected 38 clones from the CHORI-322 library (Table 1) and 24 clones from the CHORI-321 library (Table 2). The largest clone, encompassing *Hnf4*, has an insert size of 105 kb. Each clone was isolated and tested for integration into a genomic *attP* docking site, either VK37 on chromosome arm 2L or VK33 on chromosome arm 3L [3], using ΦC31 integrase [6,7]. The transformation efficiency of each clone was defined as the percentage of G0 fertile crosses that yielded at least one transgenic animal. We were successful in obtaining at least one transformant for all CHORI-322 clones (Table 1) and 13 of the 24 CHORI-321 clones (Table 2). In addition, 16 of 17 CHORI-322 clones used for recombineering-mediated tagging (see below) were successfully integrated (Supplementary Table 2). Moreover, 53 of 72 CHORI-321 clones have been integrated successfully in an independent experiment to generate a set of duplication lines, each carrying a clone from a tiling path of overlapping CHORI-321 clones spanning the entire X chromosome (Ellen Popodi and Thom Kaufman, personal communication). These data show that more than 98% (54/55) of CHORI-322 clones and at least 68% (66/96) of CHORI-321 clones can be successfully integrated. For all transformants, the presence of the expected DNA fragment sizes at the integration junctions - indicative of site-specific integration at the respective docking site - was confirmed by multiplex PCR that tests simultaneously for the presence of *attP*, *attB*, *attR* and *attL* sites (Supplementary Fig. 3).

The range of integration efficiencies observed is surprisingly broad. Efficiencies ranged from 0% to 28.1 % for CHORI-322 clones and from 0% to 11.6 % for CHORI-321 clones. The insert sizes of CHORI-322 clones are very similar to each other, so the observed range

suggests that some fragments are less efficiently transformed than others due to sequence content or specific interference between certain fragments and docking sites (e.g. *Csp* and *wg*). Notably, the high efficiency observed for some CHORI-321 clones (e.g. CH321-16H04, CH321-64G01 and CH321-79N05) suggests that further optimization of the integration efficiency of large clones is possible.

We tested transgenic insertions of ten CHORI-322 and six CHORI-321 clones for their ability to complement lethal mutations in genes. All CHORI-322 clones tested, encompassing the genes *CG6017*, *chc*, *dap160*, *drp1*, *endo*, *Eps15*, *n-syb*, *sqh*, *synj* and *vha100-1*, rescue lethal mutations in the corresponding genes. To our knowledge, rescue of mutations in *endo*, *n-syb* and *vha100-1* using genomic fragments has not been reported previously. Similarly, CHORI-321 clones encompassing the genes *cac*, *Dscam*, *lt* and *shakB* complement lethal mutations in the corresponding genes. Rescue of *cac*, *lt* and *shakB* using genomic fragments has also not been reported previously. Rescue of a lethal mutation in *lt* with a 92 kb genomic fragment inserted in euchromatin is surprising, because full expression of *lt* and several other heterochromatic genes has been shown to be dependent on their heterochromatic context 13. Only one of three clones tested complemented *lt* lethality, suggesting that essential regulatory elements or sufficient genomic context were absent in the other two clones.

To test the utility of recombineering in P(acman) BACs, we introduced EGFP reporter tags into 17 genes encoding transcription factors with well-documented embryonic expression patterns. We inserted the coding region of EGFP in-frame at the 3' end of the open reading frame, replacing the stop codon and creating C-terminal protein fusions 14 (Supplementary Fig. 4). Both the untagged and tagged constructs were tested for integration using ΦC31 integrase (Supplementary Table 2). Eleven tagged constructs were tested for expression of the fusion protein. Since this EGFP does not fold efficiently in embryos prior to stage 15, we performed immunohistochemistry on embryos with an anti-GFP antibody (Fig. 2 and Supplementary Fig. 5a,b). EGFP fluorescence could be used to visualize fusion protein expression in live embryos only in the late stages of embryonic development (Supplementary Fig. 5c). The expression patterns of *eve*, *D*, *cad*, *Dfd*, *tll*, *slp2* , and *exd* are reproduced by the transgenic fusion constructs (**Supplementary Discussion**). The *en* and *h* gene expression patterns appeared to be exceptions (Fig. 2k–l). For *h*, only two stripes (1 and 5) of expression in the embryo were observed, instead of eight 15. Interestingly, enhancers for stripes 1 and 5 are located in the 7 kb region proximal to the transcription start site, whereas the regulatory elements for the other stripes are located more distally 16. The latter regulatory elements are lacking in CH322-135D17 used to tag *h*. Hence, the tagged construct is expressed in the expected pattern. Similarly, *en* expression was only observed in 13 stripes and not the head region 17. This may be due to the absence of regulatory regions in the *en* clone CH322-92I14 (Judith Kassis, personal communication). These experiments show that recombineering-mediated deletion of genomic sequences in P(acman) constructs can be used to dissect the control of transcription by *cis*-regulatory elements.

In conclusion, we have described a versatile P(acman) BAC library resource for functional analysis of transgenes in *D. melanogaster* (**Supplementary Discussion**). We conservatively estimate that the new resource enables *in vivo* analysis of more than 95% of *D*.

melanogaster genes including large genes, gene complexes and heterochromatic genes (Supplementary Fig. 6). Moreover, protein tagging should prove a valuable alternative to antibody production, particularly when proteins are poorly immunogenic. Finally, the flexibility of recombineering 5 permits the integration of a variety of protein tags for numerous applications 18. The few genes and gene complexes that are too large to be contained within clones in the P(acman) libraries or are otherwise not represented in them can be obtained using the previously described gap-repair procedure 3 and previously mapped and end-sequenced BAC libraries constructed from the same isogenized strain 19,20.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

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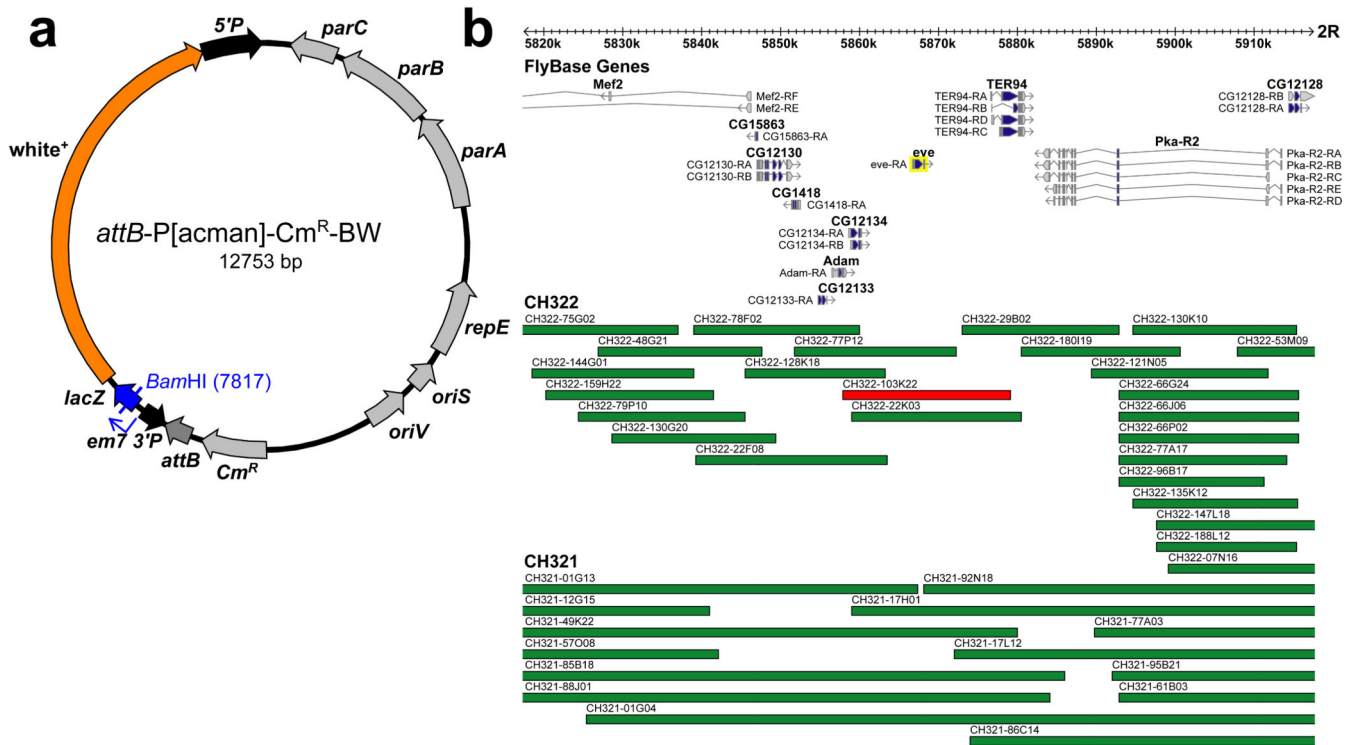
Figure 1. The P(acman) BAC Vector and Mapped Clones in the *eve* Region

(**a**) Map of *attB*-P(acman)-Cm^R-BW with partitioning functions (*parA parB* and *parC*), low-copy replication functions (*repE* and *oriS*), selectable marker (chloramphenicol acetyl transferase, *Cm^R*), conditionally inducible origin of replication (*oriV*) and dominant eye color marker *white* $^+$. Genomic DNA was cloned into the *Bam*HI site (Supplementary Fig. 2). (**b**) A 100-kb region surrounding the *eve* gene (yellow) on chromosome arm 2R. Mapped CHORI-321 and CHORI-322 clones are indicated below the FlyBase R5.9 gene annotation. CH322-103K22, selected for transformation (Supplementary Table 2) and protein tagging (Fig. 2), is indicated in red.
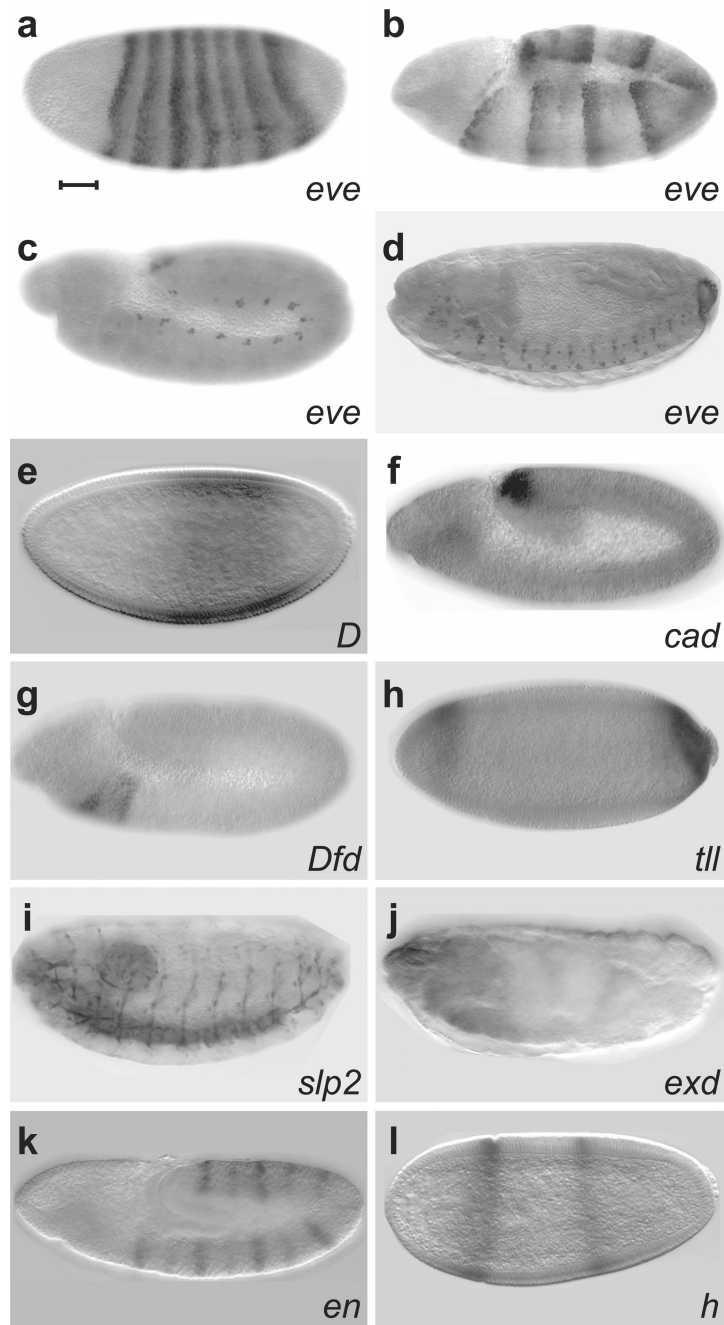
Figure 2. Expression of EGFP Fusion Proteins in Transgenic Embryos
Fusion proteins were detected using an anti-GFP antibody and peroxidase staining. (**a-d**)
Expression of an *even skipped* fusion construct recapitulates the native pattern of Eve
expression. (**a**) embryonic stage 5, (**b**) embryonic stage 9, (**c**) embryonic stage 11, (**d**)
embryonic stage 15. (**e**) *Dichaete*, embryonic stage 5. (**f**) *caudal*, embryonic stage 9. (**g**)
Deformed, embryonic stage 11. (**h**) *tailless*, embryonic stage 5. (**i**) *sloppy paired 2*,

embryonic stage 17. (**j**) *extradenticle*, embryonic stage 15. (**k**) *engrailed*, embryonic stage 9. (**l**) *hairy*, embryonic stage 6. Scale bar (**a**) indicates 50 μm.

Table 1
Characterization of CHORI-322 Clones

Genes contained in 38 CHORI-322 clones are indicated. For each clone, the deduced genomic insert length in bp (insert), *attP* VK docking site used (VK#), number of fertile G0 crosses (G0), number of vials resulting in at least one transgenic animal (Tr) and integration efficiency (%) are indicated. Note that only the gene of interest is shown; most clones contain more than one gene.

Gene(s)	Clone	Insert	VK#	G0	Tr	%
Act42A	*12M14*	19,321	33	84	2	2.4%
Act87E	*158M20*	19,317	37	65	5	7.7%
alphaTub84B	*158D05*	20,091	37	51	1	2.0%
bcd	*100D18*	18,452	37	59	4	6.8%
CG14438	*191E24*	20,115	33	103	4	3.9%
CG6017	*55J22*	19,815	37	24	3	12.5%
Chc	*123J21*	17,647	33	57	8	14.0%
Clc	*92D22*	20,882	37	32	2	6.3%
Csp	*06D09*	23,790	16	44	5	11.4%
Csp	*06D09*	23,790	22	11	2	18.2%
Csp	*06D09*	23,790	37	115	0	0.0%
Dap160	*154I22*	22,027	33	73	1	1.4%
DIP1	*146O15*	20,342	33	69	2	2.9%
Drp1	*83H15*	19,373	33	115	2	1.7%
endoA	*19L12*	20,365	37	25	1	4.0%
Eps-15	*150F15*	21,222	33	61	1	1.6%
ERR	*54A09*	20,644	37	70	1	1.4%
His2Av	*97I07*	20,846	37	51	1	2.0%
Hr4	*137G06*	22,025	33	56	3	5.4%
Hr96	*155C21*	22,752	37	38	2	5.3%
Khc	*162G07*	21,662	33	63	1	1.6%
ncd	*118A01*	22,196	37	104	4	3.8%
Nmnat	*175G15*	24,376	37	87	2	2.3%
Nrx-IV	*154P15*	21,688	37	45	3	6.7%
n-syb	*83G13*	21,536	37	48	1	2.1%
ogre	*155A19*	22,329	33	109	1	0.9%
Pak	*05M18*	21,251	37	76	4	5.3%
pen	*08M17*	22,049	33	71	1	1.4%
piwi	*103C03*	24,728	33	43	2	4.7%
polo	*104P12*	23,954	37	28	1	3.6%
Rab5	*97N16*	20,416	33	59	4	6.8%
sec15/Rab11	*152E24*	21,979	37	58	2	3.4%
sens	*01N16*	19,175	37	49	9	18.4%

Gene(s)	Clone	Insert	VK#	G0	Tr	%
spn-E	*93D04*	23,226	37	39	2	5.1%
sqh	*130G10*	20,074	33	65	8	12.3%
stau	*15P05*	20,978	33	57	3	5.3%
synj	*188H18*	18,311	33	36	1	2.8%
Syx1A	*142K16*	18,384	37	45	1	2.2%
Vha100-1	*119J05*	19,299	37	53	3	5.7%
wg	*192I14*	22,791	13	21	0	0.0%
wg *wg*	*192I14* *192I14*	22,791 22,791	31	35	5	14.3%
			33	78	0	0.0%

Table 2

Characterization of CHORI-321 Clones

Genes contained in 24 CHORI-321 clones are indicated Colum headings are identical to those in Table 1.

Gene(s)	Clone	Insert	VK#	G0	Tr	%
cac	60D21	77,150	33	62	2	3.2%
cta	03L03	83,080	33	65	1	1.5%
cta	04I17	91,022	33	81	1	1.2%
dpp	23O18	86,898	33	41	1	2.4%
Dscam	22M14	87,314	33	99	2	2.0%
eag	77E01	97,072	33	56	1	1.8%
ftz-f1	47I12	92,406	37	51	0	0.0%
gfa	57O14	77,006	33	76	0	0.0%
hh	61H05	101,201	37	61	0	0.0%
Hnf4	12P12	104,925	33	63	1	1.6%
Hr38	25N09	85,274	33	107	0	0.0%
Hr46	23L02	86,475	33	62	1	1.6%
jar	76B03	92,518	37	46	0	0.0%
lt	16H04	92,084	33	66	6	9.1%
lt	64G01	92,368	33	60	4	6.7%
lt/cta	05E14	78,102	33	64	2	3.1%
para	18K02	98,254	33	68	0	0.0%
rl	36L01	53,704	33	63	0	0.0%
rl	81D16	102,491	33	79	0	0.0%
shakB	27E22	88,712	33	76	2	2.6%
shi	71G22	70,514	33	53	0	0.0%
syt	08F02	82,244	33	59	0	0.0%
tweek	79N05	76,842	33	69	8	11.6%
vas	69O09	80,238	33	62	0	0.0%