Fly 5:4, 1-8; October/November/December 2011; © 2011 Landes Bioscience

An efficient method for recombineering GAL4 and QF drivers

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Key words: GAL4, QF, recombineering, tyramine beta-hydroxylase, tyramine receptor, bacterial artificial chromosome

Neural circuit mapping and manipulation are facilitated by independent control of gene expression in pre- and post-synaptic neurons. The GAL4/UAS and Q binary transcription systems have the potential to provide this capability. Of particular use in neural circuit mapping would be GAL4 and QF drivers specific for neurotransmitters and neurotransmitter receptors. Recently available Drosophila genomic BAC libraries make recombineering large genes including those specific for neurotransmitters and neurotransmitter receptors feasible. Here the functionality of cassettes that allow efficient recombineering of GAL4 and QF drivers based on kanamycin selection is demonstrated in Drosophila. The cassettes should, however, be generalizable for recombineering in other species.

Introduction

A current challenge in neuroscience is to develop connectomes, maps of the functional connections between neurons in a nervous system. A set of GAL4 and QF drivers specific for neurotransmitters and neurotransmitter receptors would facilitate the development of a Drosophila connectome since knowledge of the neurotransmitter usage of a presynaptic neuron entails knowledge of the neurotransmitter receptor usage of its post-synaptic neuron and vice versa. In Drosophila, many neurotransmitter-specific genes, such as neurotransmitter synthesis enzymes, and most neurotransmitter receptors have extensive conserved noncoding regions (presumably regulatory) that are too large to be contained in traditional plasmid vectors suitable for generating GAL4 and QF drivers.[1-3] Therefore, generation of GAL4 and QF drivers that faithfully recapitulate the entire endogenous spatial and temporal expression patterns of neurotransmitter and neurotransmitter receptor-specific genes will require a different type of vector capable of maintaining larger DNA fragments. The recently developed Drosophila genomic CH321 bacterial artificial chromosome (BAC) library has an average insert size of 83 kb[4] and therefore has much greater potential for including in a single clone the complete regulatory region of large Drosophila genes including neurotransmitter synthesis enzymes and neurotransmitter receptors. Since BACs of this size are too large for traditional restriction enzyme cloning, modifying them into GAL4 and QF drivers requires recombineering. In this report an efficient, generally applicable method for recombineering GAL4 and QF cassettes into BACs using kanamycin selection is functionally demonstrated.

Results

One method for recombineering genes of interest into BACs utilizes a two-step process that involves positive selection for *galK* in the first step followed by replacement of *galK* with the gene of interest using negative selection in the second step.[5] Twenty-two BACs from the recently generated Drosophila genomic BAC libraries[4] containing either neurotransmitter synthesis enzymes or neurotransmitter receptors were chosen for recombineering using the *galK* method. *GalK* was successfully recombineered into these BACs in only 10/22 attempts. For most of the failures, recombineering was attempted at least twice and ≥20 colonies were screened in each attempt by colony PCR for site-specific integration of the targeting construct. In these recombineering attempts 50–60 bp of DNA sequence homology was included at each end of the *galK* targeting constructs as this length of targeting homology was previously shown to be sufficient for successful recombineering of *galK*.[5] Other groups have also been successful using this length of sequence homology for BAC recombineering.[6-8] Nevertheless, after consideration of the various factors for the high failure rate, increasing the length of targeting homology at the termini of the recombineering constructs seemed the most likely modification to improve the success rate.

However, before attempting recombineering with increased lengths of targeting homology using the *galK* method, consideration was given to alternative recombineering strategies because of several disadvantages of the *galK* method. These disadvantages include that both *galK* selection steps require 3–4 days incubation time to obtain bacterial colonies, *galK* media preparation is cumbersome, and the 2-deoxy-galactose used in the negative *galK* selection step is somewhat expensive. An alternative strategy

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Submitted: 06/21/11; Revised: 07/27/11; Accepted: 07/27/11
DOI:

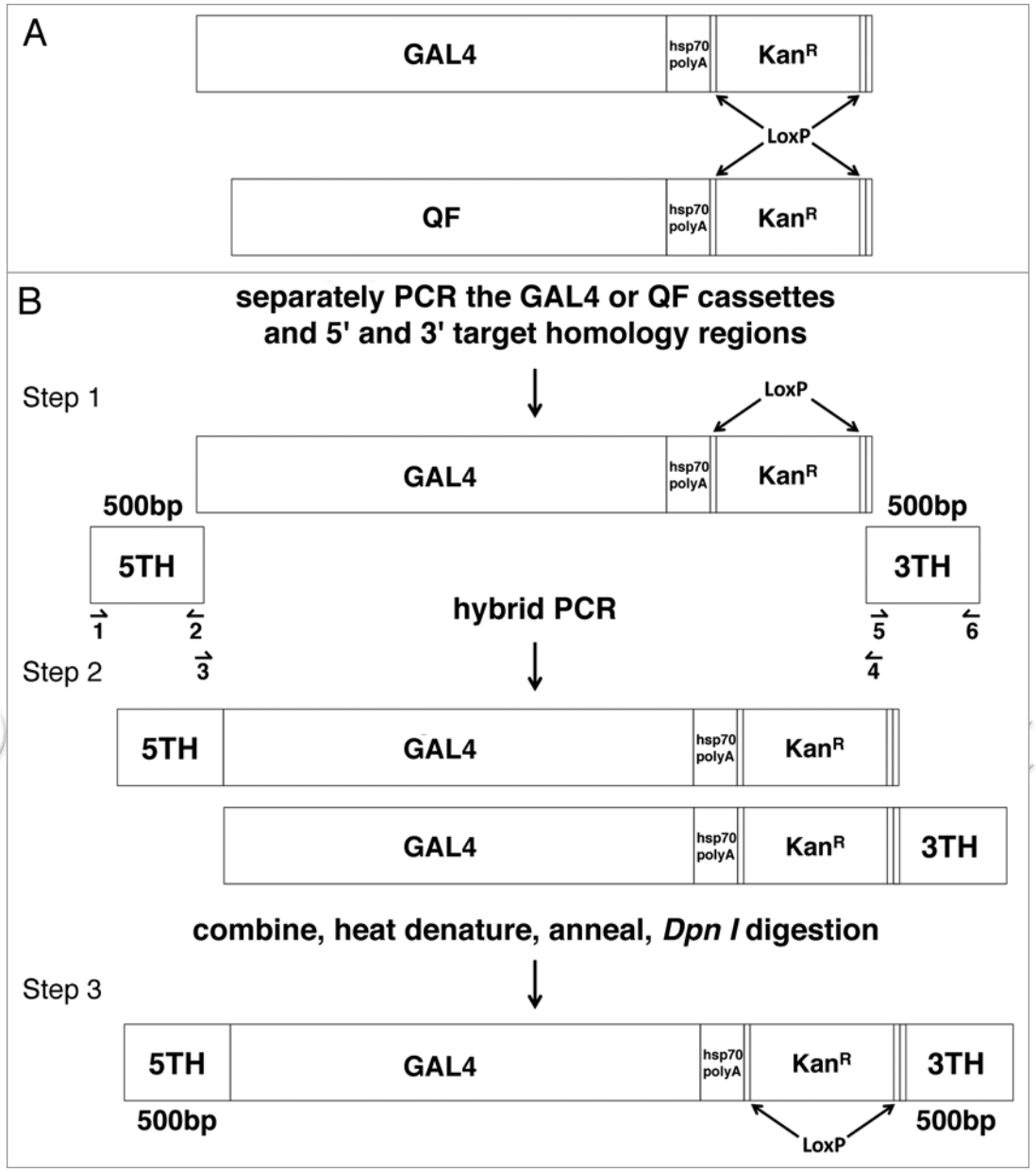

Figure 1. The pGAL4Kan and pQFKan recombineering cassettes and hybrid PCR method for adding flanking targeting homology. (A) Schematic diagrams of the pGAL4Kan and pQFKan recombineering cassettes. These cassettes contain the coding sequences of GAL4 or QF followed by hsp70 polyadenylation sites and LoxP-flanked kanamycin resistance sequences. (B) Hybrid PCR method for adding 5' and 3' targeting homology sequences to the GAL4 or QF cassettes (only GAL4 is shown but the method for QF is identical). In step 1, the 5' target homology (5TH), GAL4 cassette and 3' target homology (3TH) fragments are separately amplified by PCR using primers 1 and 2 for 5TH, primers 3 and 4 for the GAL4 cassette, and primers 5 and 6 for 3TH. In step 2, 5TH is added to the 5' end of the GAL4 cassette by hybrid PCR using primers 1 and 4 and the products of the 5TH and GAL4 cassette PCR reactions from step1 as templates. Similarly, 3TH is added to the 3' end of the GAL4 cassette by hybrid PCR using primers 3 and 6 and the products of the GAL4 cassette and 3TH PCR reactions from step 1 as templates. In step 3, the 5TH-GAL4 and GAL4-3TH products from the hybrid PCR reactions of step 2 are combined, heat denatured, annealed and subjected to *Dpn I* digestion (to remove residual template DNA from the step 1 PCR reactions). The end result is a recombineering-ready GAL4 targeting cassette with ~500 bp of targeting homology on each end.

that was appealing because it has none of these disadvantages was to use kanamycin selection to identify clones that have integrated recombineering targeting constructs.[9] Before a kanamycin-based recombineering strategy could be implemented, however, GAL4 and QF cassettes first had to be constructed that incorporated kanamycin resistance. As shown in **Figure 1A**, pGAL4Kan and pQFKan contain cassettes with the coding sequence of GAL4 or QF adjacent to a polyadenylation sequence from the hsp70 gene followed by sequences encoding kanamycin resistance flanked by LoxP sites. The LoxP sites were included so that the kanamycin resistance sequences could be removed in a second step using Cre recombinase,[5] if desired.

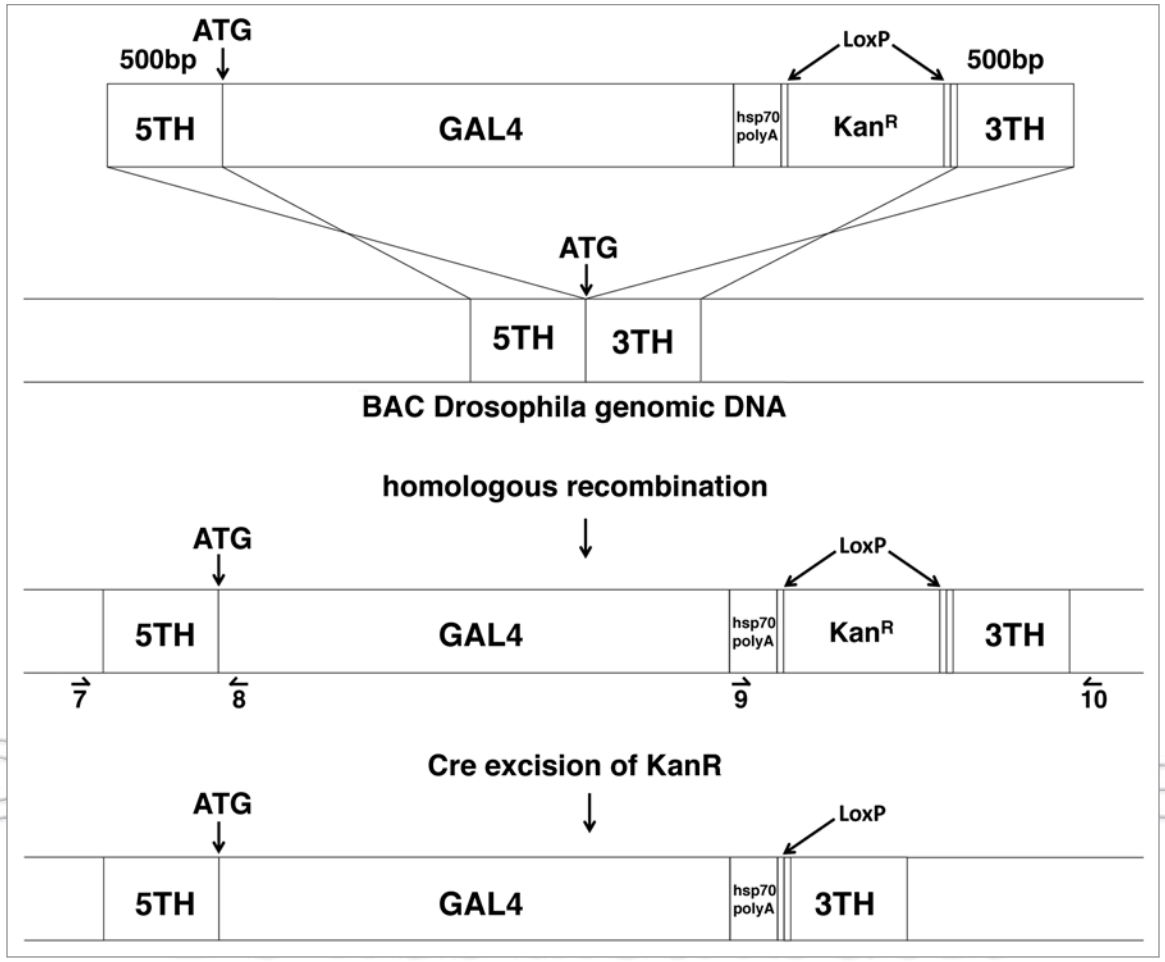

Figure 2. Integration of the GAL4 cassette by recombineering and Cre excision of KanR. To integrate the GAL4 cassette containing flanking ~500 bp 5' and 3' targeting homology regions into the desired location of a Bacterial Artificial Chromosome (BAC), the 5TH-GAL4-3TH cassette is transformed into the recombineering bacterial strain SW106 that already contains the appropriate Drosophila genomic BAC. The recombineering enzymes recombine the GAL4 cassette specifically into the desired location based on sequence homology in the 5TH and 3TH regions between the targeting construct and the genomic BAC. The crossed lines indicate potential cleaveage and religation sites for homologous recombination. Integration of the 5TH-GAL4-3TH cassette converts the bacteria from kanamycin-sensitive to kanamycin-resistant. Bacteria containing integrations at the desired location are identified by colony PCR using primer pairs 7 and 8 or 9 and 10. In a subsequent step, arabinose-inducible Cre recombinase present in the SW106 bacterial strain excises the KanR sequences using the flanking LoxP sites. The end result is a GAL4 driver BAC ready for Drosophila germline transformation.

To potentially improve the recombineering success rate the length of targeting homology on the termini of the targeting constructs was increased 10-fold to ~500 bp. The strategy chosen to do this was to use hybrid PCR as outlined in **Figure 1B**. The first step involves separate PCR amplification of the 5' targeting homology (5TH) region (using primers 1 and 2), the GAL4 or QF cassettes (using primers 3 and 4), and the 3' targeting homology (3TH) region (using primers 5 and 6). Primer locations are indicated below the top panel of **Figure 1B**. The 5TH and 3TH fragments were designed to overlap with the GAL4 and QF cassettes by 22–26 bp to make the hybrid PCR possible. In the second step, two separate hybrid PCR reactions were used to fuse either 5TH to the 5' end of the GAL4 or QF cassettes (using primers 1 and 4 and the products of the 5TH and GAL4 or QF cassette PCR reactions as templates) or 3TH to the 3' end of the GAL4 or QF cassettes (using primers 3 and 6 and the products of the 3TH and GAL4 or QF cassette PCR reactions as templates). In step 3, the products of the two hybrid PCR reactions from step 2 are combined, heat denatured, reannealed and digested with *Dpn I* (to remove residual template DNA). The result is that one-third of the products of the reannealing are predicted to be a hybrid between 5TH-GAL4 or QF and GAL4 or QF-3TH that have ~500 bp of targeting homology on each end as shown at the bottom of **Figure 1B**. Although this hybrid could be purified by agarose gel electrophoresesis from the 5TH-GAL4 or QF and GAL4 or QF-3TH products due to its larger size, this was determined unnecessary as electroporation of the entire mixture was sufficient for a high rate of recombineering success as described below.

The targeting constructs containing the GAL4 or QF cassettes with ~500 bp of targeting homology on each end were used for recombineering as outlined in **Figure 2**. Targeting constructs were electroporated into the bacterial strain SW106 that had already been transformed with the BACs containing

the genomic DNA being targeted. Heat shock induction of the recombineering functions present in SW106 results in integration of the GAL4 or QF cassettes by homologous recombination at the location specified by the 5TH and 3TH target homology regions (**Fig. 2**-middle). Since the goal was to make GAL4 and QF drivers that recapitulate the complete endogenous temporal and spatial expression pattern of genes of interest, the strategy was to place the ATG start codons of GAL4 or QF at the same location as the ATG start codons of the genes of interest. Only the endogenous ATG start codons of the genes of interest were deleted with the remaining sequences downstream of the start codons shifted 3' by 4.2 kb (GAL4) or 3.8 kb (QF). After electroporation of the targeting cassettes, the bacteria were selected on chloramphenicol (the resistance marker of the BAC)/kanamycin plates to identify colonies that have integrated the GAL4 or QF cassettes into the BAC. Colonies that exhibit both chloramphenicol and kanamycin resistance are, however, not guaranteed to have integrated the GAL4 or QF cassettes at the desired location as nonhomologous integrations do occur. To distinguish homologous from nonhomologous integrations, colonies were assessed by colony PCR using primer pairs 7 and 8 and primer pairs 9 and 10 (primer locations are shown below the middle diagram of **Fig. 2**). Note that primers 7 and 10 lie just outside the 5TH and 3TH regions, respectively, and will therefore not yield a PCR product from the GAL4 or QF cassettes containing 5TH and 3TH when used in combination with primers 8 and 9 unless targeted integration of the GAL4 or QF cassettes has occurred. Positive colonies that produced PCR fragments of the sizes predicted if homologous integration has occurred were next subjected to colony PCR using primers 7 and 10. If the product of this PCR reaction yielded a fragment of the predicted size, indicating integration of the entire cassette, the GAL4 or QF region of this PCR fragment was directly sequenced to assess whether mutations were introduced by PCR. If no PCR errors were found, the kanamycin resistance sequences were excised in a second step using an arabinose-inducible Cre present in the SW106 strain.[5] A single 34 bp LoxP "scar" remains in this final construct that is immediately ready for Drosophila germline transformation.

Using this method GAL4 and QF were successfully recombineered in 11/12 attempts with BACs containing genomic DNA for neurotransmitter synthesis enzymes or neurotransmitter receptors. Most of these successful attempts were into BACs in which recombineering had previously failed using 50–60 bp of targeting homology with the *galK* method as described above. Six of these constructs were selected for Drosophila transgenesis and germline transformants were obtained for two of them. One of these contained a GAL4 insertion in the tyramine receptor gene CG7485 (hereafter TyrR-GAL4) that is included in the 83,965 bp insert of BAC CH321-64J06. This BAC contains the entire 30 kb transcription unit of TyrR including 42 kb of genomic DNA upstream and downstream of the TyrR transcription start site that extends into one or more adjacent genes on each side. TyrR-GAL4 is therefore likely to contain most, if not all, of the TyrR regulatory region. TyrR-GAL4 exhibits robust expression in a large subset of neurons in the larval ventral nerve cord as revealed with the 20XUAS-CD8-GFP reporter as shown in **Figure 3A**. Many of these neurons were determined to be motor neurons based on the abundant neuromuscular junction expression observed in TyrR-GAL4 larva as shown in **Figure 3B**. It was not possible to assess to what extent the expression of TyrR-GAL4 recapitulates the expression pattern of endogenous TyrR since the expression pattern of TyrR has not been described and no antibody is available. However, a β-Gal enhancer trap insertion in TyrR has been previously reported to exhibit broad expression in the larval ventral nerve cord[10] that is highly reminiscent of the observed pattern of TyrR-GAL4.

The second construct for which a germline transformant was obtained contained a QF insertion in the Tyramine β hydroxylase gene CG1543 (hereafter TβH-QF). TβH converts the neurotransmitter tyramine into the neurotransmitter octopamine. TβH-QF is contained in the 85,268 bp insert of BAC CH321-34K02. This BAC contains the entire 31 kb transcription unit of TβH including 14 kb of genomic DNA upstream and 71 kb downstream of the TβH transcription start site that extends into one or more adjacent genes on each side. TβH-QF is therefore likely to contain most, if not all, of the TβH regulatory region. To assess the degree to which TBH-QF expression recapitulates the endogenous expression of TβH, the expression of TβH-QF was revealed using the QUAS-mtdTOM-3XHA reporter and double-labeled with anti-TβH antiserum. As shown in **Figure 3D**, TβH-QF exhibits sparse expression in the larval ventral nerve cord. This expression pattern mirrors TβH expression as shown in **Figure 3C and E**. The TβH-QF larval expression pattern is also highly reminiscent of the larval distribution of octopamine.[11] These observations thus establish that the cellular expression of TβH-QF closely approximates the cellular expression of endogenous TβH.

Discussion

In this report the functionality of cassettes for efficient recombineering of GAL4 and QF using kanamycin selection has been demonstrated. In addition, a hybrid-PCR method has been introduced for incorporation of targeting homology onto the termini of recombineering targeting constructs. Given the success of numerous other groups using ~50 bp of targeting homology for recombineering, the 45% success rate observed with the same length of targeting homology was surprising. However, since negative results are not often published it is possible these groups or others have experienced similar recombineering failures using 50–60 bp of homology that have simply gone unreported. Increasing the targeting homology region on the termini of recombineering targeting constructs by hybrid PCR ~10-fold to ~500 bp improved the success rate to 92%. Since intermediate lengths of targeting homology were not tested, it is possible the point of diminishing returns for efficient homologous recombination is reached at less than 500 bp and essentially the same success rate could have been achieved with targeting homology lengths less than 500 bp. Although somewhat labor intensive, the entire process of preparing a recombineering targeting construct using the hybrid PCR method of adding targeting homology to the termini can

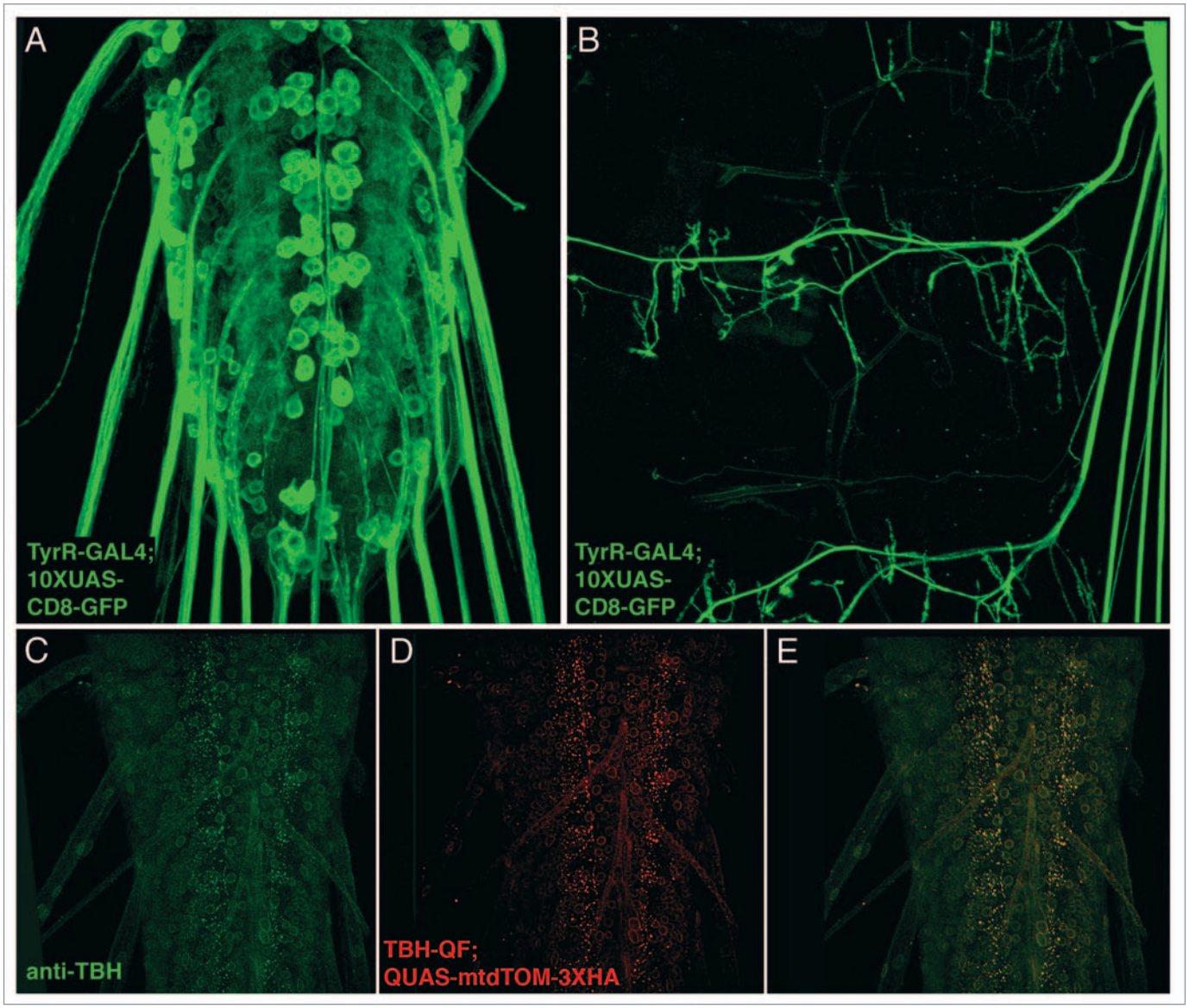

Figure 3. The TyrR-GAL4 and TβH-QF drivers express in specific neuronal subsets of the larval nervous system. (A and B) Representative confocal images of *yw*; *10XUAS-mCD8-GFP/+*; *TyrR-GAL4/+* in the larval ventral nerve cord (A) and larval neuromuscular junctions (B) labeled with anti-GFP. TyrR-GAL4 expresses in a broad subset of neurons in the larval nervous system many of which are motor neurons. (C–E) Representative confocal images of a *yw*; *QUAS-mtdTOM-3XHA/+*; *TβH-QF/TβH-QF* larval ventral nerve cord double-labeled with anti-TβH (C) and anti-HA (D). TβH-QF exhibits expression in the same subset of larval neurons as TβH.

be completed in a single day and numerous targeting constructs can be prepared in parallel. It is, however, not essential to use the hybrid PCR method to add targeting homology to the termini of the GAL4 and QF cassettes. If recombineering success can be achieved using targeting homology lengths of ~50 bp that can be incorporated into the oligonucleotides used for PCR, targeting constructs using these cassettes can be prepared from a single PCR reaction.

Using a kanamycin-based selection strategy for recombineering is advantageous over the *galK* method for at least three reasons. First, it saves time. Although similar to the *galK* method in that it is still a two-step process, both steps of the *galK* method require 3–4 days incubation for colony growth while the kanamycin selection and excision steps only require overnight incubations. This cuts the time of the entire recombineering process in half, from around two weeks to one. Second, the media preparation for using kanamycin selection is less labor intensive. The *galK* method requires complex media for auxotrophic selection of *galK* while the kanamycin method uses standard LB kanamycin media. Third, the kanamycin selection media is less expensive. The *galK* method utilizes complex media for both steps that is more expensive (especially the 2-deoxy-galactose used in the second step) than the standard LB media used by the kanamycin selection method.

The described method for increasing recombineering efficiency, or variations of it, can potentially be used for

numerous other applications besides generating GAL4 or QF drivers that recapitulate the complete endogenous expression patterns of genes. For instance, for large genes with multiple isoforms, isoform-specific GAL4 or QF drivers could be generated that reveal isoform-specific expression patterns that would not be distinguished with an antibody that recognizes all isoforms. The method could also be used to remove sequences from a clone that might confound the analysis in some situations. As an example, for a mutant rescue experiment it might be undesirable to have more than one gene in the clone else it would not be possible to distinguish which gene is responsible for rescue. If all available BACs containing the candidate rescue gene also contain other genes then the method could be used for deleting the unwanted genes by recombineering. Another potential application of the method is for dissecting the regulatory elements of GAL4 or QF drivers of large genes such as the ones presented in this report. Assessment of the potential importance of regulatory regions of interest of GAL4 or QF drivers of large genes could be made by serial recombineering. After generation of the initial GAL4 or QF driver, additional recombineering steps could be performed that result in mutations ranging from large deletions to the mutation of single base pairs. Alternatively, dissection of regulatory regions of GAL4 or QF drivers could be accomplished by combining with other methods such as MSSMR that have the capacity for generating deletion or point mutations in large clones.[12]

In conclusion, in this report GAL4 and QF cassettes have been functionally demonstrated for use in Drosophila BAC recombineering. A QF driver for the octopamine neurotransmitter synthesis enzyme tyramine beta-hydroxylase and a GAL4 driver for the tyramine neurotransmitter receptor were efficiently recombineered using these cassettes, germline transformed, and shown to express in vivo. These results demonstrate the feasibility of generating GAL4 and QF drivers for any other gene using the same approach, especially those with regulatory regions too large to be contained in plasmid vectors. Other such large Drosophila genes include several neurotransmitter synthesis enzymes and most neurotransmitter receptors. It is thus now possible to efficiently generate a complete set of neurotransmitter and neurotransmitter receptor-specific GAL4 and QF drivers that would significantly enhance the mapping of Drosophila neural circuits. Lastly, the GAL4 and QF cassettes are not Drosophila-specific and should be compatible with recombineering in other species and model systems.

Materials and Methods

Fly stocks. Fly stocks were maintained on standard cornmeal/molasses media. The 10XUAS-CD8-GFP[13] (BDSC#-32186) and QUAS-tdTOM-3XHA[3] (BDSC#-30004) fly stocks have been previously described.

Construction of the pGAL4Kan and pQFKan recombineering cassettes. pGAL4Kan was constructed in two steps. In the first step a 3.3 kb *BamHI*/*Not I* fragment from pBPGUw[14] encoding GAL4 and the polyadenylation sequence from the hsp70 gene was cloned into *BamHI*/*Not I* sites of pBSC5.[15] In the second step, a 1.1 kb *Not I*/*EcoRI* PCR fragment containing LoxP flanked KanR derived from template pDONR P5-P4 (Invitrogen) was cloned into the *Not I*/*EcoRI* sites of the resulting plasmid from step 1. pQFKan was constructed in three steps. In the first step, a 2.5 kb *Asc I*/*Nhe I* PCR fragment encoding QF derived from pattBQF[3] template was cloned into the *Asc I*/*Nhe I* sites of pBSC5. The QF open reading frame was sequenced and determined to be free of mutations introduced by PCR. In the second step, a 258 bp *Nhe I*/*BamHI* PCR fragment derived from template pBPGUw containing a polyadenylation sequence from the hsp70 gene was cloned into the *Nhe I*/*BamHI* sites of the resulting plasmid from step 1. In the third step, a 1.1 kb *BamHI*/*EcoRI* PCR fragment containing LoxP flanked KanR derived from template pDONR P5-P4 was cloned into the *BamHI*/*EcoRI* sites of the resulting plasmid from step 2. Oligos used for the construction of these vectors are shown in **Table 1**.

Hybrid PCR. For the first round PCR reactions the 5TH, GAL4 or QF cassettes, and 3TH fragments were separately amplified for 10 cycles (to minimize PCR errors) in 20 μl reactions using 1 ng of pGAL4Kan or pQFKan plasmid or CH321-64J06 or CH321-34K02 BAC template DNA, AccuPrime *Pfx* SuperMix (Invitrogen-Cat. # 12344-040), and 5 pmols of primers 1 and 2 (5TH), primers 3 and 4 (GAL4 or QF), or primers 5 and 6 (3TH) (primer locations are indicated on **Fig. 1B**). For the second round 5TH-GAL4 or 5TH-QF hybrid PCR reactions, 1 μl each of the 5TH and either GAL4 or QF first round PCR reactions were used as templates with 5 pmols of primers 1 and 4, AccuPrime *Pfx* SuperMix, and amplified for 30 cycles in 20 μl reactions. For the second round GAL4-3TH or QF-3TH hybrid PCR reactions, 1 μl each of the GAL4 or QF and 3TH first round PCR reactions were used as templates with 5 pmoles of primers 3 and 6, AccuPrime *Pfx* SuperMix, and amplified for 30 cycles in 20 μl reactions. After second round PCR amplification, fragments were gel purified, combined, heat denatured at 95°C for five minutes in a heat block, annealed in the same heat block for ≥1 hr at room temp, and digested with *Dpn I* (to remove plasmid and BAC template DNA). Annealed targeting cassettes were subsequently run through a spin column (Zymo Research Cat # D4104) and eluted in 6 μl.

Theoretically, it should have been possible to do a single three-way hybrid PCR amplification using primers 1 and 6 and the products of all three PCR reactions from the first step as templates to generate the GAL4 or QF targeting constructs with ~500 bp on each end, but in practice attempts to do this resulted in a ~1 kb PCR product due to the presence of residual BAC templates from the first step 5TH and 3TH PCR reactions. This could not be overcome even with *Dpn I* digestion after the first round PCR reactions.

Recombineering. *GalK* recombineering was performed according to previously described methods using bacterial strain SW102.[5] Recombineering of GAL4 and QF as well as Cre excision of kanR was performed essentially as described in reference 16. Typically, half of the GAL4 or QF targeting cassettes preparations (3 μl/~250 ng) were electroporated into SW106,[5] bacteria containing the appropriate BAC before plating on chloramphenicol (12.5 μg/ml)/kanamycin (25 μg/ml) plates.

Table 1. Recombineering oligos

TyrRF1	5'-CCA AGT TCA GAT AAT TCG AGA AG-3'
TyrRGAL4R2	5'-GAT AGA AGA CAG TAG CTT CAT GTT GCC CTG CCC TGC CAC TTG CCT TC-3'
TyrRQFR2	5'-GTG TCT TGC GTT TAG GCG GCA TGT TGC CCT GCC CTG CCA CTT GCC TTC-3'
TyrRF5	5'-GCA GTC ATG CGT CTG AGA ATT CCA TCG GCA GAT CAG ATC CTG-3'
TyrRR6	5'-CTC AAC TTC CTG TTG TAC ATT C-3'
TyrRF7	5'-GAT ATC CCT GAA CAT CTT AGA AC-3'
TyrRR10	5'-GTG AAA TTT GTG GCA GGC GAA C-3'
TβHF1	5'-CCG CAC ATA ATT TAC CCA TC-3'
TβHGAL4R2	5'-GAT AGA AGA CAG TAG CTT CAT GTT GAG ATT TGA GAT TGC ACG GCC GGC AGA T-3'
TβHQFR2	5'-GTG TCT TGC GTT TAG GCG GCA TGT TGA GAT TTG AGA TTG CAC GGC CGG CAG AT-3'
TβHF5	5'-GCA GTC ATG CGT CTG AGA ATT GCT TAA AAT TCC GCT GCA GCT GAG CAG T-3'
TβHR6	5'-CGT AAT TGA GAT GAC GGC GTG-3'
TβHF7	5'-GAG GCG TGG CTG CAA AGT AC-3'
TβHR10	5'-AGC CAT CCA TCC GGA CGG AC-3'
GAL4F3	5'-CAA CAT GAA GCT ACT GTC TTC TAT C-3'
QFF3	5'-CAA CAT GCC GCC TAA ACG CAA GAC AC-3'
R4	5'-AAT TCT CAG ACG CAT GAC TGC-3'
GAL4R8	5'-GAG AGT AGC GAC ACT CCC AGT TG-3'
QFR8	5'-GTG TCT TGC GTT TAG GCG GCA T-3'
Hsp70F9	5'-ACT ACG GCT AGC GTC GAC TAA AGC CAA ATA GAA AAT-3'
LoxPKanNotBamF	5'-ACT ACG GGA TCC GCG GCC GCA TAA CTT CGT ATA GCA TAC ATT ATA CGA AGT TAT GGC AGC TCT GGC CCG TGT CTC A-3'
LoxPKanRIR	5'-ACT ACG GAA TTC TCA GAC GCA TGA CTG CAT AAC TTC GTA TAA TGT ATG CTA TAC GAA GTT ATG GTC ATG AGC TTG CGC CGT C-3'
QFAscIF	5'-ACT ACG GGC GCG CCC AAC ATG CCG CCT AAA CGC AAG ACA C-3'
QFNheIR	5'-ACT ACG GCT AGC CTA TTG CTC ATA CGT GTT GAT ATC-3'
Hsp70NheF	5'-ACT ACG GCT AGC GTC GAC TAA AGC CAA ATA GAA AAT-3'
Hsp70BamHIR	5'-ACT ACG GGA TCC GAT CTA AAC GAG TTT TTA AGC A-3'

A list of the oligo names and sequences used for recombineering and for the construction of pGAL4Kan and pQFKan. Numbers at the ends of the oligo names correspond to the locations of oligos of the same number on **Figures 1 and 2**.

After kanR excision, the SW106 bacteria were plated on chloramphenicol (12.5 μg/ml) plates. Drosophila BACs CH321-64J06 (TyrR) and CH321-34K02 (TβH) were obtained from the CHORI BACPAC Resources Center.

Colony PCR. For the colony PCR reactions, 5 pmoles each of primers 7, 8, 9 and 10 (primer locations are indicated on **Fig. 2**) were used with Taq 2X master mix (New England Biolabs-Cat. # M0270L) in 20 μl reactions and amplified for 30 cycles. Templates were derived from bacterial colonies touched with a pipet tip that had been spotted onto a separate bacterial plate for subsequent recovery of positives.

Immunohistochemistry. Larva were dissected on Sylgard coated slides using minuten pins. After dissection, larvae were fixed in either 4% paraformaldehyde (TyrR-GAL4) or Bouin's fixative (TBH-QF) for 30 minutes at room temperature and washed 3x two minutes in PBS. Larvae were then blocked in PBSTNGS (PBS + 2% Triton X-100 + 5% normal goat serum) for ≥1 hr at room temperature, incubated with primary antibody diluted in PBSTNGS overnight at 4°C, washed 5x two minutes in PBS, incubated with secondary antibody diluted in PBSTNGS overnight at 4°C, washed 5x two minutes in PBS, before imaging on a Leica TCS-SP confocal microscope. Primary antibodies were rabbit anti-GFP Abfinity mAb (Invitrogen-Cat # G10362; 1:200), Rat anti-HA mAb 3F10 (Roche-Cat # 11 867 423 001; 1:200), and Rabbit anti-TβH.[17] Secondary antibodies were goat anti-Rabbit Alexa Fluor 488 (Invitrogen-Cat # A-11034; 1:500), goat anti-rabbit Alexa Fluor 633 (Invitrogen-Cat # A-21071; 1:500), and goat anti-rat Alexa Fluor 568 (Invitrogen-Cat # A-11077; 1:500).

Drosophila transformation. After recombineering and kanamycin excision, GAL4 and QF BACs were transformed into bacterial strain EPI300 (Epicentre) before shipment to Bestgene, Inc., for DNA isolation and embryo injections. The insertion site for both TyrR-GAL4 and TβH-QF was VK00013.[18]

Acknowledgments

I would like to thank Emily Morrison for technical assistance in the early stages of this work.

References

1. Sharma Y, Cheung U, Larsen EW, Eberl DF. pPTGAL, a convenient GAL4 P-element vector for testing expression of enhancer fragments in Drosophila. Genesis 2002; 34:115-8.
2. Apitz H. pChs-GAL4, a vector for the generation of Drosophila Gal4 lines driven by identified enhancer elements. Dros Inf Serv 2002; 85:118-20.
3. Potter CJ, Tasic B, Russler EV, Liang L, Luo L. The Q system: a repressible binary system for transgene expression, lineage tracing and mosaic analysis. Cell 2010; 141:536-48.
4. Venken KJ, Carlson JW, Schulze KL, Pan H, He Y, Spokony R, et al. Versatile P[acman] BAC libraries for transgenesis studies in *Drosophila melanogaster*. Nat Methods 2009; 6:431-4.
5. Warming S, Costantino N, Court DL, Jenkins NA, Copeland NG. Simple and highly efficient BAC recombineering using galK selection. Nucleic Acids Res 2005; 33:36.
6. Venken KJ, Kasprowicz J, Kuenen S, Yan J, Hassan BA, Verstreken P. Recombineering-mediated tagging of Drosophila genomic constructs for in vivo localization and acute protein inactivation. Nucleic Acids Res 2008; 36:114.
7. Tran KD, Miller MR, Doe CQ. Recombineering Hunchback identifies two conserved domains required to maintain neuroblast competence and specific early-born neuronal identity. Development 2010; 137:1421-30.
8. Perry MW, Boettiger AN, Bothma JP, Levine M. Shadow enhancers foster robustness of Drosophila gastrulation. Curr Biol 2010; 20:1562-7.
9. Liu P, Jenkins NA, Copeland NG. A highly efficient recombineering-based method for generating conditional knockout mutations. Genome Res 2003; 13:476-84.
10. Kutsukake M, Komatsu A, Yamamoto D, Ishiwa-Chigusa S. A tyramine receptor gene mutation causes a defective olfactory behavior in *Drosophila melanogaster*. Gene 2000; 245:31-42.
11. Monastirioti M, Gorczyca M, Rapus J, Eckert M, White K, Budnik V. Octopamine immunoreactivity in the fruit fly *Drosophila melanogaster*. J Comp Neurol 1995; 356:275-87.
12. Jacobs JS, Hong X, Eberl DF. A 'mesmer'izing new approach to site-directed mutagenesis in large transformation-ready constructs: Mutagenesis via Serial Mismatch Recombineering. Fly 2011; 5:162-9.
13. Pfeiffer BD, Ngo TT, Hibbard KL, Murphy C, Jenett A, Truman JW, Rubin GM. Refinement of tools for targeted gene expression in Drosophila Genetics 2010; 186:735-55.
14. Pfeiffer BD, Jenett A, Hammonds AS, Ngo TT, Misra S, Murphy C, et al. Tools for neuroanatomy and neurogenetics in Drosophila. Proc Natl Acad Sci 2008; 105:9715-20.
15. Le T, Yu M, Williams B, Goel S, Paul SM, Beitel GJ. CaSpeR5, a family of Drosophila transgenesis and shuttle vectors with improved cloning sites. Biotechniques 2007; 42:164-6.
16. Sharan SK, Thomason LC, Kuznetsov SG, Court DL. Recombineering: a homologous recombination-based method of genetic engineering. Nat Protoc 2990; 4:206-23.
17. Koon AC, Ashley J, Barria R, DasGupta S, Brain R, Waddell S, et al. Autoregulatory and paracrine control of synaptic and behavioral plasticity by octopaminergic signaling. Nat Neurosci 2011; 14:190-9.
18. Venken KJ, He Y, Hoskins RA, Bellen HJ. P[acman]: a BAC transgenic platform for targeted insertion of large DNA fragments in *D. melanogaster*. Science 2006; 314:1747-51.