Benchmarks

Streamlined scanning for enhancer elements in *Drosophila melanogaster*

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Enhancer elements in most eukaryotic organisms are often positioned at a great distance away from the transcription start site of the gene they regulate. Complex three-dimensional chromatin organization and insulators usually guide and limit the range of an enhancer's regulatory activity to a specific genetic locus. Rigorous testing of an entire genomic locus is often required in order to uncover the complete set of *cis*-regulatory modules (CRMs) regulating a gene, especially those with complex and dynamic expression patterns. Here we report a fast and efficient method for enhancer element identification by scanning large genomic regions using transgenic reporter genes.

The fruit fly *Drosophila melanogaster* has long been a key model organism for investigation of enhancer element structure and function. Genome-scale chromatin studies assisted by bioinformatic approaches have described many of the *cis*-regulatory modules (CRMs) and their architecture throughout the fruit fly genome (reviewed in Reference 1). However, enhancer elements that are active at particular developmental stages might easily be missed using standard approaches. In addition, in many cases multiple shadow enhancers function in a semi-redundant manner, and their combined analysis is required to fully describe a gene's transcriptional regulation. Because enhancers can be far apart from one another and from the transcription start sites of the genes they regulate, we have developed a novel technique to rapidly and efficiently scan large genomic regions.

Venken and colleagues generated valuable genomic libraries, CHORI-322 and CHORI-321, which cover the *Drosophila* genome and can be used for gene rescue

experiments and gene tagging since the backbone contains a selectable marker (*mini-white+*) and ϕ C31 attB site for transgenesis (2). While CHORI-321 consists of clones carrying large genomic fragments (average size ~80 kb), CHORI-322 was designed to carry only ~20 kb fragments (available at BACPAC Resource Center, Oakland, CA). Because of their relatively small size, CHORI-322 clones can be efficiently inserted into the genome using the ϕ C31 system (2). Since the CHORI-322 library virtually tiles the entire genome, we reasoned that even a large gene locus of ~200 kb (from one insulator to another) could be scanned with ~10 CHORI-322 clones.

To create reporter constructs from the CHORI-322 clones, all that is required is insertion at a feasible location along the DNA fragment of a reporter gene containing its own minimal promoter and sequences for transcriptional termination. This is quickly achievable by using a unique restriction site in each clone (there are many available in any 20 kb fragment) to open the vector and

insert a promoter-reporter-3'-terminator PCR product through Gibson Assembly (3). Each set of primers in this case would contain ~25 bp overhangs that are complementary to the corresponding clone sequence flanking the chosen restriction enzyme site (Figure 1A). To avoid the possibility that a putative enhancer element would be destroyed at the chosen restriction site location, an overlapping BAC clone could be used to cover the site of the reporter insertion in another construct. In addition, two different reporter cassettes could be inserted in opposite ends of a BAC in order to be able to compare expression of both in cases where secondary DNA structure or repressive elements are predicted or suspected in a particular genomic region.

To test this strategy, we chose the 3' region of the gene *teashirt* (*tsh*), which spans ~60 kb to the next downstream gene (Figure 1B) and contains numerous non-coding RNAs. There are no predicted strong insulators (as defined by modENCODE; www.modencode.org) in this region that would prevent a putative enhancer from activating the inserted reporter gene and generating an expression pattern. This 60 kb region could be covered with only 3 CHORI-322 clones: 176G09, 161P06, and 74E24 (Figure 1B). These clones were digested with enzymes *San*DI, *Bs*WI, and *As*cl, respectively, and purified with the QIAquick PCR Purification Kit (#28106; Qiagen, Valencia, CA). PCR was used to amplify a cassette carrying the *Drosophila* synthetic minimal promoter (4) driving *tdTomato* (5) followed by the SV40 late polyA 3' terminator (6) (*synthP-tdTomato-SV40*), and to insert each reporter cassette, flanked by clone-specific overhangs, into the corresponding clones using Gibson Assembly (Figure 1A). The resulting mix was transformed by electroporation into TransforMax EPI300 *E. coli* cells (#EC300110; Epicenter Biotechnologies, Madison, WI). Each transformation generated ~30 colony-forming units (CFUs). We grew three colonies per construct in standard LB media and induced each of them to high-copy number using CopyControl solution (#CCIS125; Epicenter Biotechnologies). We extracted DNA corresponding to each colony via the QIAprep

METHOD SUMMARY

We developed a method for quick discovery of novel *cis*-regulatory modules (CRMs) by generating enhancer-reporter constructs that span vast genomic regions in the fruit fly *Drosophila melanogaster*. Our method introduces a small promoter-reporter-3'-terminator cassette into ϕ C31 system-compatible CHORI-322 clones using Gibson Assembly.

Figure 1. Efficient generation of enhancer-reporter constructs using *Drosophila melanogaster* BAC clones. (A) Schematic representation of the conversion of any CHORI-322 clone into a reporter construct through Gibson Assembly. The following oligonucleotides were used for PCR amplification of the *synthP-tdTomato-SV40* cassette: 5'-cgactgtgaaaggggtgagcggtacagtATCGAGCGCAGC-GGTATAAAAGGGCGCGGG-3' and 5'-aatatttataaattggtgcccccgccaactAAAAACCTCCCA-CACCTCCCCCTGAACCT-3' in the case of clone 176G09; 5'-gtttattatataagtgagaatccgtacgATCGAGCGCAGCGGTATAAAAGGGCGCGGG-3' and 5'-atcataatcggaattcaataattaagatcAAAAACCTCCCAACCTCCCCCTGAACCT-3' in the case of clone 161P06; 5'-caagccaaaataggaatagattgggaaATCGAGCGCAGCGGTATA-AAAGGGCGCGGG-3' and 5'-gattgctgctcttgtttt-gtgtgctggcAAAAACCTCCCAACCTCCCCCTGAACCT-3' in the case of clone 74E24; lower case letters represent complementary overhangs. (B) Schematic representation of the *tsh* 3' genomic region on chromosome 2L and the CHORI-322 clones used in this study; black arrows represent the sites of reporter cassette insertion; blue arrow represents the relative location of a previously described *tsh* embryonic *cis*-regulatory module (CRM) (9).

Figure 2. Expression pattern of the generated reporter constructs. Comparison between the expression of *tdTomato* (red) with *tsh* (green) in a 176G09-*tdTomato* stage 11 embryo (A), a 176G09-*tdTomato* stage 14 embryo (B), a 176G09-*tdTomato* larval brain (C), a 161P06-*tdTomato* stage 14 embryo (D), a 161P06-*tdTomato* L3 larval female genital imaginal disc (E), a 161P06-*tdTomato* L3 larval male genital imaginal disc (F), a 161P06-*tdTomato* L3 larval leg imaginal disc (G), a 161P06-*tdTomato* L3 larval wing imaginal disc (H), a 74E24-*tdTomato* stage 14 embryo (I), a 74E24-*tdTomato* L3 larval male genital imaginal disc (J), and a 74E24-*tdTomato* L3 larval ventral nerve cord (VNC) (K). Note that *tdTomato* is non-nuclear and *Teashirt* is nuclear. Immunostaining was performed with goat anti-*tdTomato* antibody (#LS-C340696; LifeSpan BioSciences, Seattle, WA) and guinea pig anti-*Teashirt* antibody (gift from Gary Struhl). Corresponding donkey Alexa-Fluor-conjugated secondary antibodies [donkey anti-goat Alexa Fluor 555 (ThermoFisher Scientific, Waltham, MA) and donkey anti-guinea pig Alexa Fluor 488 (Jackson ImmunoResearch Laboratories, West Grove, PA)] were used to visualize the primary antibody staining. Anterior is left; posterior is right.

Spin Miniprep Kit (#27106; Qiagen) and verified each of the constructs by sequencing the *synthP-tdTomato-SV40* minigene and insertion site. Next, we extracted sufficient amounts of DNA for injection (250 ng/ μ l) with the Qiagen Plasmid Midi Kit (#12143; Qiagen) and generated transgenic flies carrying each of the constructs using the phiC31 system (7,8) and the attP2 insertion site (7).

We analyzed the expression pattern of the generated reporter constructs in fly imaginal discs, embryos, and larval nervous system. Given the size of each construct, multiple CRMs may contribute to the reporter expression in each case. Clone 176G09 contains a previously described Hox-dependent *tsh* embryonic enhancer (9), and the transgene made with this clone recapitulated *tsh*'s embryonic expression pattern, verifying that the reporter cassette is capable of recruiting, at the very least, this *tsh* embryonic CRM (Figure 2, A and B), which is located ~10 kb downstream of the reporter cassette insertion site (Figure 1B). In addition, clone 176G09 drove expression in the larval brain that co-localizes with the *tsh* expression in the ventral nerve cord (VNC) but diverges from the *tsh* expression in the

lobes of the brain (Figure 2C). This suggests that the region covered by 176G09 contains at least one *tsh* VNC enhancer element. Clone 176G09 did not drive expression in third instar larval imaginal discs.

Clone 161P06 drove expression in a *tsh*-like pattern in stage 14 embryos (Figure 2D), which argues that this region contains additional embryonic *tsh* CRMs. This clone also drove expression in multiple imaginal discs. Some patterns coincided nicely with the *tsh* expression, while in other cases, the 161P06-*tdTomato* expression deviated from that of *tsh*. For example, in female genital discs 161P06-*tdTomato* matches almost completely the *tsh* expression pattern (Figure 2E), while in male genital discs the expression driven by 161P06 is adjacent to that of *tsh* (Figure 2F). Most likely this region contains one or more legitimate female genital disc CRMs, while the male genital CRM is either interrupted or several CRMs are required for the correct expression pattern of *tsh* in male genital imaginal discs and some of them are positioned in different genomic regions (e.g., 5' of *tsh*). The reporter expression driven by clone 161P06 also overlapped well with the *tsh* expression pattern in leg imaginal discs,

suggesting the presence of one or more *tsh* leg CRMs in this region (Figure 2G). In contrast to the leg disc, 161P06-*tdTomato* drove expression in the proximal wing disc that overlapped with *tsh* expression only in a few regions (Figure 2H). The expression pattern in the haltere disc was analogous (data not shown), suggesting that there are other *tsh* wing and haltere CRMs outside of 161P06.

Clone 74E24 generated some reporter expression in stage 14 embryos (Figure 2I), in male genital imaginal discs (Figure 2J), and in the larval nervous system (Figure 2K), none of which co-localized with the *tsh* expression, suggesting that this region contains regulatory elements unrelated to *tsh*. None of the generated reporter constructs drove expression in the mouth and eye-antennal imaginal discs (although *tsh* is expressed in the anterior eye disc).

The method described here allows for rapid and efficient scanning of any *Drosophila* genomic region as a first step toward identifying enhancer elements of a gene of interest at any developmental stage and tissue. It will complement other genome-wide tiling projects (10–12) and computa-

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tional approaches (reviewed in References 13 and 14), thus significantly improving the identification and verification of putative CRMs.

Author contributions

R.V. conceived this work, performed the experiments, and wrote the article. R.S.M. edited the article text and supported this study.

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Competing interests

The authors declare no competing interests.

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