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Technical Note

Expanding the CRISPR Toolbox in Culicine Mosquitoes: *In Vitro* Validation of Pol III Promoters

Michelle A. E. Anderson,[‖] Jessica Purcell,[‖] Sebald A. N. Verkuijl, Victoria C. Norman, Philip T. Leftwich, Tim Harvey-Samuel, and Luke S. Alphey*

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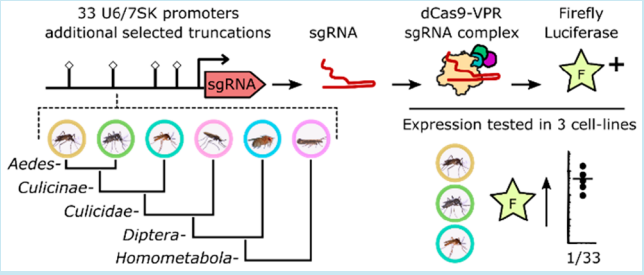
ABSTRACT: CRISPR–Cas9-based "gene drive" technologies have been proposed as a novel and effective means of controlling human diseases vectored by mosquitoes. However, more complex designs than those demonstrated to date—and an expanded molecular toolbox with which to build them—will be required to overcome the issues of resistance formation/evolution and drive spatial/temporal limitation. Foreseeing this need, we assessed the sgRNA transcriptional activities of 33 phylogenetically diverse insect Polymerase III promoters using three disease-relevant Culicine mosquito cell lines (*Aedes aegypti*, *Aedes albopictus*, and *Culex quinquefasciatus*). We show that U6 promoters work across species with a range of transcriptional activity levels and find 7SK promoters to be especially promising because of their broad phylogenetic activity. We further show that U6 promoters can be substantially truncated without affecting transcriptional levels. These results will be of great utility to researchers involved in developing the next generation of gene drives.



KEYWORDS: *Polymerase III, Cas9, U6 promoter, 7SK promoter, gene drive, mosquito*

A limited set of RNA Polymerase III (Pol III) promoters, mostly from U6 and H1 genes, have been used in eukaryotic synthetic biology systems to express short noncoding RNAs without the 5′ and 3′ mRNA modification associated with Polymerase II expression. The U6 small nuclear RNA (snRNA) has a highly conserved 106–108 nt sequence and an external 5′ promoter structure that is remarkably similar to that of an RNA Pol II promoter, namely, a TATA-like box and proximal sequence element (PSE). 7SK is another RNA Pol III-transcribed abundant snRNA, whose function in transcriptional regulation is conserved from invertebrates to humans. Like U6, 7SK has an external 5′ promoter structure, similar conserved domains (a TATA-like box and PSE), and in mammals distal elements consisting of an SPH domain and OCT motif.[1] 7SK RNAs have been identified in multiple arthropod species, including Dipterans.[2,3] In this work, we have explored their use for expression of sgRNAs.

In mosquitoes, Pol III promoters have been utilized for genetic control strategies that depend on CRISPR guide RNA (sgRNA) or RNAi expression.[4−8] The ability to express multiple noncoding RNAs while minimizing repetitive sequences is a significant advantage to these systems and may be necessary to create robust technologies.[9,10] More broadly, as the use of mosquitoes as insect synthetic biology chassis develops, it will be highly advantageous for researchers to have access to a diverse range of validated noncoding RNA promoters with varied expression levels; such a toolbox does not yet exist in the Culicines, and we address this need here. Alternative methods for multiplexing sgRNAs from a single transcript, *e.g.*, using tRNAs or ribozyme-based processing, have been demonstrated in other species[11−13] with varying efficiencies but have not yet been applied to mosquitoes.

We hypothesized that adapting existing promoters from related species would be a rapid and cost-effective way of expanding the available Pol III expression toolbox in Culicines, as cross-species activity of U6 promoters in mosquitoes has previously been demonstrated by Konet et al.[6] We systematically tested the activities of a range of previously reported insect U6 promoters in *Aedes aegypti*, *Aedes albopictus*, and *Culex quinquefasciatus* cell lines. This was supplemented by the identification and testing of additional new U6 promoters and the testing of U6[14] and 7SK[2] promoters that had previously been identified but not experimentally tested in cell lines. We used a standardized cell- CRISPR/dCas9–VPR binding assay to systematically quantify the promoter activity across cell lines. Our results represent a large advance in the available

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expression tools and provide a general guide for efficiently identifying additional expression modalities.

RESULTS AND DISCUSSION

The transcriptional activities of 33 phylogenetically diverse insect Pol III promoters were tested in three cell lines from disease-relevant Culicine mosquito species (*A. aegypti*, *A. albopictus* and *C. quinquefasciatus*). Potential U6 promoters were identified by BLAST using a previously published *A. aegypti* U6 RNA sequence, AAEL017774.[6] The presence of highly conserved sequence elements (a TATA-like box, PSE, and poly-T terminator) were verified for those sequences taken forward experimentally (sequence alignments are provided in the Supporting Information). Each promoter was used to express the same sgRNA, targeting a tetracycline response element (TRE) upstream of the coding sequence of the firefly luciferase gene. Expression of functional sgRNA by the putative promoters, in conjunction with dCas9−VPR, binds the TRE and activates expression of firefly luciferase. Firefly luciferase activity was normalized to the levels of *Renilla* luciferase expressed independently of the sgRNAs (Figure 1).

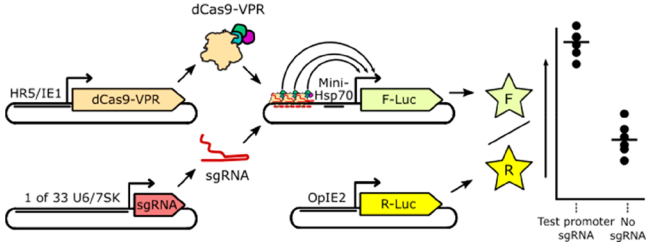

Figure 1. dCas9−VPR assay components. Our assay consists of four plasmids, each expressing a single component. HR5/IE1, a constitutive promoter in insect cells of baculoviral origin, is used to express a dCas9−VPR fusion protein. A second plasmid containing seven tetO repeats upstream of the *D. melanogaster* minimal hsp70 promoter expresses firefly luciferase upon activation. Test promoters all express the same sgRNA targeting the tetO repeat region. Finally, a plasmid expressing *Renilla* luciferase from the OpIE2 promoter was used as a control to normalize for transfection efficiency.

In Aag2 (*A. aegypti*) and Hsu (*C. quinquefasciatus*) cells, the levels of promoter activity were broadly in line with the species of origin of the promoter, decreased with phylogenetic distance, and accounted for most of our observed variance (R^2_m) (R^2_m = 0.73, R^2_c = 0.89 for Aag2, R^2_m = 0.46, R^2_c = 0.84 for Hsu; Tables S1 and S2 and Figure 2A,B), while random variance introduced by technical replicates (replicate wells transfected with the same mix on the same day) and experimental blocking were low.

In *A. albopictus*-derived U4.4 cells we found no significant effect of the species of origin of the promoter sequence on the relative luciferase expression and larger random variance than in our other experiments (R^2_m = 0.22, R^2_c = 0.89; Table S3 and Figure 2C). We speculate that there may be less overall activity from one or more of our promoter sequences in these cells, which with fewer replicate experiments (still at least three performed on different days) likely explains the lack of an observable pattern here.

Within those species where we tested U6 and 7SK promoter sequences, there was a trend toward 7SK promoter sequences having stronger activity levels than U6 promoter sequences regardless of their species of origin (Tables S1 and S2 and

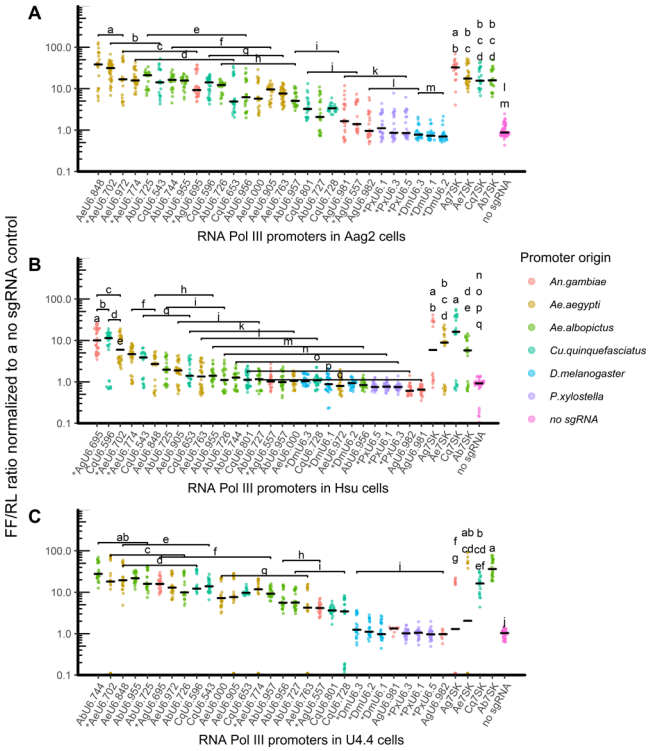

Figure 2. dCas9−VPR assay *in vitro*. Ratios of FF/RL luciferase normalized to a no-sgRNA control are shown. Promoters are organized by median relative activation within U6 and 7SK promoter categories, and the colors denote the promoter origin by species. Lowercase letter groupings denote significant differences at $P < 0.05$ following post hoc analysis. Each point represents one well of a 96-well plate, with at least eight replicate wells transfected in at least three replicate experiments.

Figure 2). None of the U6 promoters from *Drosophila melanogaster* or *Plutella xylostella* showed any activity above background in our mosquito cell lines (Figure 2). Promoters are denoted by the last three digits of their accession numbers.

Shorter versions of several U6 promoters were tested in Aag2 and Hsu cells (Figure 3) in order to determine the minimum possible promoter fragment without compromising the activity. For all seven promoters, the PSE and TATA-like box were present within 100 nt upstream of the transcriptional start and are likely the principal requirements for expression. We did not identify any distal sequence elements with a strong effect on the promoter activity, except in CuU6.801, where deletion from 200 bp to 100 bp essentially eliminated the activity. These results indicate that most of the U6 promoters identified can be used in a very compact form.

Furthering the work of Mount et al.[15] and Konet et al.,[6] we have demonstrated a pipeline for cell culture verification of Pol III promoter sequences in Culicine mosquitoes. In these experiments, we showed that Pol III promoter sequences from closely related species can be used to drive high levels of noncoding RNA expression in mosquito species of interest. Regulatory elements from more distantly related species may be applicable for complex applications where a range of expression levels is desirable. We anticipate that these findings will provide a valuable resource for those involved in the rapidly developing field of mosquito genome editing and synthetic biology.

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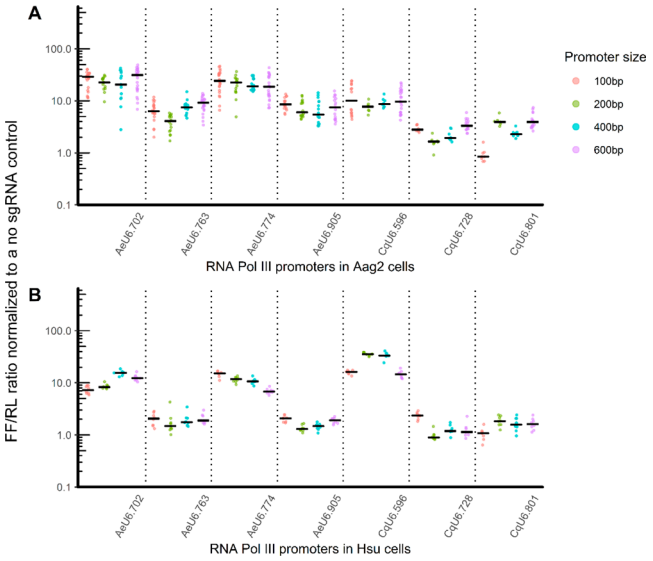

Figure 3. Mosquito U6 promoters maintain dCas9−VPR activity down to 100 bp. Seven promoters were deleted incrementally from a −600 bp fragment down to −100 bp upstream of the transcriptional start. Four promoter lengths were tested for each Pol III promoter, shown in each group left to right as 100 bp (orange), 200 bp (green), 400 bp (blue), and 600 bp (purple). Activity was assessed by the dCas9−VPR assay in Aag2 cells and Hsu cells. FF/RL luciferase ratios have been normalized to a no-sgRNA control. Each point represents one well of a 96-well plate, with at least eight replicate wells transfected at least three replicate experiments.

METHODS

Plasmids and Constructs. Cloning details and complete plasmid sequences are available in the Supporting Information.

Cells, Transfections, and Luciferase Assay. All of the cell lines were maintained at 28 °C without CO_2 or humidity control. Aag2 and U4.4 were maintained in L-15 (Thermo Fisher Scientific, Waltham, MA, U.S.) supplemented with 10% fetal bovine serum (FBS) (Labtech, Lewes, U.K.), 1% penicillin/streptomycin (Pen/Strep) (Thermo Fisher Scientific), and 10% tryptose phosphate broth (Thermo Fisher Scientific). Hsu cells were maintained in Schneider's Drosophila Medium (Lonza, Basel, Switzerland) supplemented with 10% FBS and 1% Pen/Strep. Cell lines were a kind gift of Rennos Fragkoudis.

Cells were seeded in 96-well plates 1 day prior to transfection with the TransIT-PRO transfection kit (Mirus Bio, Madison, WI, U.S.) according to the manufacturer's recommendations. Master mixes were prepared for 8.8 wells of a 96-well plate, and eight replicate wells per experimental construct were transfected in three to eight replicate experiments. In each well, 25 ng of dCas9−VPR plasmid, 25 ng of TRE-firefly reporter plasmid, 0.3 ng of Pol III-sgRNA expressing plasmid, and 50 ng (Aag2, U4.4) or 30 ng (Hsu) of pRL-OpIE2 were used.

Two days after transfection, the cells were washed twice with phosphate-buffered saline, lysed with 1× Passive Lysis Buffer, and then analyzed using the Dual Luciferase Assay Kit on a GloMax Multi+ plate reader (Promega, Southampton, U.K.).

Data Analysis. Luciferase readings were normalized for transfection by dividing the firefly activity by the *Renilla* activity and then normalized to the average of background readings (no-sgRNA control). Data were analyzed by generalized linear mixed models using a Γ distribution with a log link with the glmer function within lme4.[16] Models that encountered convergence errors were fitted with the boyqa optimizer. Each transformed data reading for a promoter was analyzed together with the species of origin and promoter type (U6 or 7SK), and experimental replicates and blocking were nested as a random effect within promoter identity. After each model was fitted, marginal and conditional R^2 values (R^2_m and R^2_c, respectively) were calculated to express the variance explained by the fixed and random factors using the package piecewiseSEM.[17] Pairwise comparisons of different promoter strengths were calculated using Tukey HSD multiple comparison tests using the lsmeans package.[18] All analyses were conducted in R ver. 3.5.3.[19] Scripts and raw data can be found at doi: 10.6084/m9.figshare.11407752.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssynbio.9b00436.

Additional information on all constructs, including complete sequences; sequence alignment of mosquito U6 and 7SK promoters analyzed; and summary output tables of full generalized linear mixed models (Tables S1−S3) (PDF)

AUTHOR INFORMATION

Corresponding Author

Luke S. Alphey − *Arthropod Genetics, The Pirbright Institute, Pirbright GU24 0NF, U.K.*; Email: luke.alphey@pirbright.ac.uk

Authors

Michelle A. E. Anderson − *Arthropod Genetics, The Pirbright Institute, Pirbright GU24 0NF, U.K.*; orcid.org/0000-0003-1510-2942

Jessica Purcell − *Arthropod Genetics, The Pirbright Institute, Pirbright GU24 0NF, U.K.*

Sebald A. N. Verkuijl − *Arthropod Genetics, The Pirbright Institute, Pirbright GU24 0NF, U.K.; Department of Zoology, University of Oxford, Oxford OX1 3SZ, U.K.*

Victoria C. Norman − *Arthropod Genetics, The Pirbright Institute, Pirbright GU24 0NF, U.K.*

Philip T. Leftwich − *Arthropod Genetics, The Pirbright Institute, Pirbright GU24 0NF, U.K.; School of Biological Sciences, University of East Anglia, Norwich, Norfolk NR4 7TJ, U.K.*; orcid.org/0000-0001-9500-6592

Tim Harvey-Samuel − *Arthropod Genetics, The Pirbright Institute, Pirbright GU24 0NF, U.K.*

Complete contact information is available at: https://pubs.acs.org/10.1021/acssynbio.9b00436

Author Contributions

‖M.A.E.A. and J.P. contributed equally to this work. M.A.E.A., J.P., T.H.-S., and L.S.A. conceived and designed the experiments. M.A.E.A., J.P., S.A.N.V., T.H.-S., and V.C.N. designed and generated constructs or components. M.A.E.A. and J.P. performed the experiments. P.T.L. analyzed the data. M.A.E.A., S.A.N.V., P.T.L., and T.H.-S. wrote the manuscript, and all of the authors read and approved the final draft.

Notes

The authors declare no competing financial interest.

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