CRISPR-Cas9 vectors for genome editing and host engineering in the baculovirus–insect cell system

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The baculovirus–insect cell system (BICS) has been widely used to produce many different recombinant proteins for basic research and is being used to produce several biologics approved for use in human or veterinary medicine. Early BICS were technically complex and constrained by the relatively primordial nature of insect cell protein glycosylation pathways. Since then, recombination has been used to modify baculovirus vectors—which has simplified the system—and transform insect cells, which has enhanced its protein glycosylation capabilities. Now, CRISPR-Cas9 tools for site-specific genome editing are needed to facilitate further improvements in the BICS. Thus, in this study, we used various insect U6 promoters to construct CRISPR-Cas9 vectors and assessed their utility for site-specific genome editing in two insect cell lines commonly used as hosts in the BICS. We demonstrate the use of CRISPR-Cas9 to edit an endogenous insect cell gene and alter protein glycosylation in the BICS.

CRISPR-Cas9 | baculovirus | insect cells | genome editing | glycoengineering

The baculovirus–insect cell system (BICS), first described in 1983 (1), has been used to produce thousands of different recombinant proteins for diverse areas of biomedical research. Since 2009, the BICS has also been used to produce several biologics approved for use in human or veterinary medicine (reviewed in ref. 2). Thus, the BICS is an important recombinant protein production platform that has had and will continue to have a large and broad impact on basic research, biotechnology, and medicine.

Two precedents suggest the BICS would have even more impact if it could be engineered to enhance its capabilities or extend its utility. In the 1980s, the isolation of baculovirus expression vectors was a highly inefficient, time-consuming, and frustrating process. However, by the early 1990s, efforts to engineer the baculoviral genome in various ways had greatly simplified this process (3–5). These refinements effectively converted a complex system created in highly specialized laboratories to a routine tool that could be easily used in many different laboratories. This was followed by efforts to enhance the BICS by engineering host protein N-glycosylation pathways (reviewed in ref. 6). However, host glycoengineering and other host improvement efforts have been limited to the use of nonhomologous recombination to knockin heterologous genes at random sites (reviewed in ref. 7). This is because there have been no tools for site-specific genome manipulation in the insect cell lines most commonly used as hosts in the BICS. These cell lines include Sf9 (8) and High Five (9), which are derived from the lepidopteran insects *Spodoptera frugiperda* (Sf) and *Trichoplusia ni* (Tn), respectively.

Sf9 and High Five cells clearly have the machinery required for protein N-glycosylation, but cannot synthesize the same end products as mammalian cells (reviewed in refs. 10–14). More specifically, both of these lepidopteran insect cell lines can transfer N-glycan precursors to nascent polypeptides and trim them to produce processing intermediates identical to those produced by mammalian cells. However, neither can process those intermediates through the additional steps needed to produce larger, mammalian-like structures with terminal sialic acids. Interestingly, we know insect cells encode the machinery needed to produce sialylated N-glycans and we also know some cells in whole insects naturally produce endogenously sialylated glycoproteins (15–17). Nevertheless, this genetic capacity is not expressed in Sf9 or High Five cells. Insect cells also have a trimming enzyme, absent in mammalian cells, which antagonizes N-glycan elongation (18–21). This enzyme, which is a specific, processing β-N-acetylglucosaminidase called fused lobes (FDL), removes a terminal N-acetylglucosamine residue from trimmed N-glycan–processing intermediates. This antagonizes elongation because it eliminates the N-glycan intermediates used as substrates for N-acetylglucosaminyltransferase II, which initiates the elongation process. The inability of the BICS to produce mammalian-type, elongated N-glycans is a major deficiency of this system because these structures are required for clinical efficacy in glycoprotein biologics (22). Because of its inability to synthesize these structures, it is widely believed that the BICS platform could never be used for glycoprotein biologics manufacturing.

As indicated above, this limitation has been addressed by nonhomologous recombination to engineer insect cell N-glycosylation pathways for mammalian-type N-glycan biosynthesis (reviewed in refs. 12–14 and 23). These efforts have yielded new, transgenic insect cell lines that can be used to produce recombinant glycoproteins with fully elongated, mammalian-type N-glycans. However, further glycoengineering is needed to create host cell lines that can more efficiently process N-glycans in mammalian fashion and produce homogeneously glycosylated proteins. These more refined glycoengineering efforts will require tools for site-specific genome editing in the BICS, and *fdl*, which encodes an antagonistic function, will be a critically important target.

The CRISPR-Cas9 system is a relatively new and exceptionally powerful tool for site-specific genome editing (24–26). CRISPR-Cas9 vectors have been constructed for and used in many different

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Significance

CRISPR-Cas9 is a powerful site-specific genome-editing tool that has been used to genetically engineer many different systems. However, this tool has not yet been adopted for use in the baculovirus–insect cell system, which is an important recombinant protein production platform. Thus, we created new CRISPR-Cas9 vectors that can be used for genome editing in two relevant insect cell lines. This is significant because these tools will enable new efforts to enhance the capabilities and expand the utility of this important protein production platform.
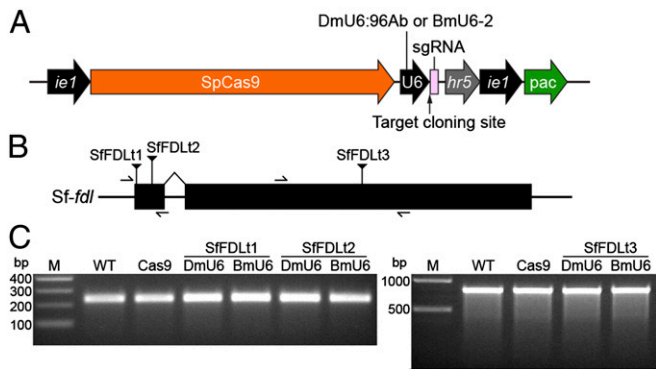
Fig. 1. *D. melanogaster* and *B. mori* U6 promoters do not support CRISPR-Cas9 editing in Sf9 cells. (*A*) Diagram showing generic CRISPR-Cas9 vectors encoding, (*Left* to *Right*) SpCas9 under the control of a baculovirus *ie1* promoter, an sgRNA expression cassette that includes an insect species-specific U6 promoter and a targeting sequence cloning site consisting of two SapI recognition sites, and a puromycin-resistance marker under the control of baculovirus *hr5* enhancer and *ie1* promoter elements. (*B*) Diagram showing Sf-*fdl* gene structure and highlighting specific Cas9 targeting sequences (Table S1) and PCR primer sites. (*C*) CEL-I nuclease assay results obtained using genomic DNA from Sf9 cells edited with CRISPR-Cas9 vectors encoding various Sf-*fdl* targeting sequences (SfFDLt1, SfFDLt2, and SfFDLt3) (Table S1) under the control of either the DmU6:96Ab or the BmU6-2 promoter.

biological systems, including insect cell systems. In fact, it has been shown that endogenous U6 promoters can be used to drive single-guide RNA (sgRNA) expression for CRISPR-Cas9 genome editing in S2R+, a cell line derived from the dipteran insect, *Drosophila melanogaster* (Dm) (27, 28), and BmN, a cell line derived from the lepidopteran insect, *Bombyx mori* (Bm) (29). These findings prompted us to attempt to adopt the CRISPR-Cas9 system for site-specific genome editing in the BICS. The broader purpose of this effort was to provide enabling technology for precise genetic modifications that will further enhance and expand the utility of this important recombinant protein production platform.

Results

Heterologous Insect U6 Promoters Fail to Support CRISPR-Cas9 Editing in Sf9 Cells.
When we undertook this effort, there were no known *S. frugiperda* or *T. ni* RNA polymerase III promoters. However, as noted above, there were DmU6 and BmU6 promoters with the known ability to drive sgRNA expression in *D. melanogaster* and *B. mori* cells, respectively (27–29). Thus, we chose to use the DmU6 and BmU6 promoters as potential surrogates for CRISPR-Cas9 genome editing in Sf9 and High Five cells, based on their ability to drive sgRNA expression in other insect cell systems. *D. melanogaster* is a dipteran and *B. mori* is a lepidopteran, so the former is relatively distantly and the latter more closely related to *S. frugiperda* and *T. ni*, from which Sf9 (8) and High Five (9) were derived.

We initially designed generic CRISPR-Cas9 vectors (Fig. 1*A*) that included an *S. frugiperda* codon-optimized *Streptococcus pyogenes* (Sp) Cas9 coding sequence under the control of a baculovirus *ie1* promoter, which provides constitutive transcription in a wide variety of organisms (30), followed by either the DmU6:96Ab or BmU6-2 promoter for sgRNA expression and a targeting sequence cloning site. These vectors also included a puromycin-resistance marker (puromycin acetyl transferase, *pac*) under the control of baculovirus *hr5* enhancer and *ie1* promoter elements (Fig. 1*A*). After constructing, mapping, and sequencing the generic DmU6:96Ab and BmU6-2 CRISPR-Cas9 vectors, we designed, synthesized, and inserted targeting sequences (Table S1) for the *D. melanogaster* (Fig. S1*A*) or *B. mori* (Fig. S1*C*) *fdl* genes. We then examined the editing capacities of the products by transfecting *D. melanogaster* (S2R+) or *B. mori* (BmN) cell lines, respectively, and performing CEL-I

nuclease assays on puromycin-resistant derivatives. The results of this control experiment showed the Dm-*fdl* gene was efficiently edited in S2R+ cells transfected with the DmU6 vector encoding the Dm-*fdl*–specific sgRNA and in S2R+ cells transfected with AcCas9DmFDLt3, a previously described CRISPR-Cas9 vector encoding a Dm-*fdl*–specific sgRNA (28), but not in S2R+ cells transfected with a vector encoding Cas9 alone (Fig. S1*B*). Similarly, the Bm-*fdl* gene was efficiently edited in BmN cells transfected with each of three BmU6-2 vectors encoding different Bm-*fdl*–specific sgRNAs, but not in BmN cells transfected with a vector encoding Cas9 alone (Fig. S1*D*). These results indicated our CRISPR-Cas9 vectors produced functional Cas9 under *ie1* promoter control, functional sgRNAs under DmU6:96Ab and BmU6-2 promoter control, and also showed they could be used for efficient CRISPR-Cas9 editing of endogenous gene targets in cells from the homologous species.

Therefore, we constructed DmU6:96Ab and BmU6-2 CRISPR-Cas9 vectors encoding sgRNAs with three different Sf-*fdl* targeting sequences (Fig. 1*B* and Table S1) and used them to transfect Sf9 cells in an effort to edit the Sf-*fdl* gene. However, CEL-I nuclease assays revealed no evidence of Sf-*fdl* indels in the resulting puromycin-resistant Sf9 derivatives (Fig. 1*C*). Because the results obtained with *D. melanogaster* and *B. mori* cells indicated these vectors induced adequate Cas9 and *pac* expression, this result suggested the DmU6 and BmU6 promoters were unable to support adequate sgRNA expression in Sf9 cells, which are derived from a heterologous insect species. Therefore, we concluded we needed to identify an endogenous SfU6 promoter to induce sgRNA expression in Sf9 cells.

An Identified SfU6 Promoter Supports CRISPR-Cas9 Editing in Sf9 Cells.
Using the BmU6-2 snRNA sequence (31) as a query to search the *S. frugiperda* draft genome sequence (32), we found only one putative SfU6 snRNA coding sequence. We had no confidence in this hit because insect snRNA sequences are often derived from pseudogenes (31). Thus, we used splinkerette PCR (33) in an attempt to experimentally isolate SfU6 promoter candidates from Sf9 genomic DNA. This approach yielded six unique U6 snRNA upstream sequences (Fig. 2*A*), including the one (SfU6-3) identified using bioinformatics. Additional bioinformatics showed only SfU6-3 included the proximal sequence element A (PSEA) and TATA box required for insect U6 promoter function (Fig. 2*A*).
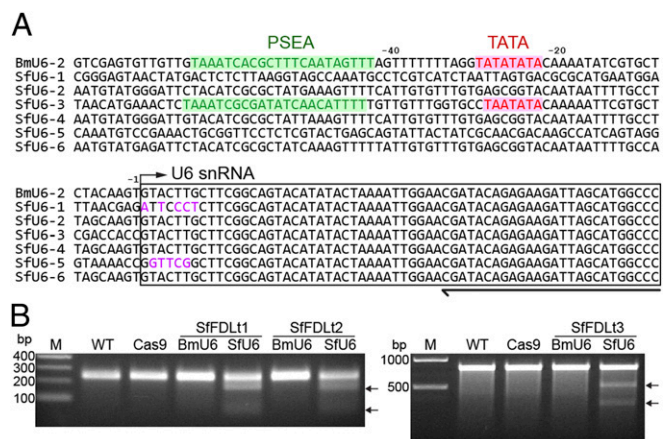

Fig. 2. Identification of putative SfU6 promoters and successful CRISPR-Cas9 editing of Sf-*fdl*. (*A*) Multiple sequence alignment of BmU6-2 promoter and SfU6 promoter candidates. (*B*) CEL-I nuclease assay results obtained using genomic DNA from Sf9 cells edited with CRISPR-Cas9 vectors encoding Sf-*fdl* targeting sequences (Table S1) under the control of the BmU6-2 or SfU6-3 promoters.

Based on these results, we used SfU6-3 to construct a generic CRISPR-Cas9 vector (Fig. 1A) and then constructed three derivatives using the Sf-*fdl* targeting sequences previously inserted into the DmU6 and BmU6 CRISPR-Cas9 vectors (Fig. 1B and Table S1). We used each construct to transfect Sf9 cells, selected puromycin-resistant derivatives, and then performed CEL-I nuclease assays with genomic DNAs from those cells. The results showed all three SfU6–3–based CRISPR-Cas9 vectors produced Sf-*fdl* indels (Fig. 2B) and this was confirmed by PCR and sequencing (Fig. S2). These results demonstrated the SfU6-3 promoter, but not the DmU6:96Ab or BmU6-2 promoters, can be used for CRISPR-Cas9 editing in Sf9 cells.

Identified TnU6 Promoters Support CRISPR-Cas9 Editing in *T. ni* Cells. We extended these results by using splinkerette PCR to identify eight putative TnU6 promoters as potential tools for CRISPR-Cas9 editing of High Five cells (Fig. 3A). We then used TnU6-2, -3, -4, and -5, all of which had PSEA and TATA elements, to construct generic CRISPR-Cas9 vectors. Finally, we inserted a Tn-*fdl*–specific targeting sequence (Fig. 3B and Table S1), transfected High Five cells with the resulting constructs, selected for puromycin resistance, and examined the cellular Tn-*fdl* genes using CEL-I nuclease assays. The results indicated the Tn-*fdl* gene was edited in each case, demonstrating that TnU6-2, -3, -4, and -5 are all effective as promoters for CRISPR-Cas9 editing in High Five cells (Fig. 3C). Interestingly, the CEL-I nuclease assays also indicated the SfU6-3, BmU6-2, and DmU6:96Ab CRISPR-Cas9 vectors encoding the Tn-*fdl*–specific sgRNA produced efficient, inefficient, and no detectable Tn-*fdl* gene editing in High Five cells, respectively (Fig. 3C). These results showed that TnU6-2, -3, -4, -5, and SfU6-3 promoters can all be used for CRISPR-Cas9 editing in High Five cells.

CRISPR-Cas9 Editing Efficiencies Mediated by Various Insect U6 Promoters in Various Insect Cell Lines. Considering the U6 promoters derived from *T. ni* and *S. frugiperda* both mediated Tn-*fdl* gene editing in High Five cells, we chose to more quantitatively document the efficiencies of CRISPR-Cas9 editing provided by various insect U6 promoters in the various insect cell lines used in this study. First, we transformed S2R+, Sf9, High Five, and BmN cells with an EGFP expression plasmid. Then, we transfected each transformed derivative with CRISPR-Cas9 vectors encoding an EGFP-specific sgRNA under the control of *D. melanogaster*, *B. mori*, *S. frugiperda*, or *T. ni* U6 promoters and measured cellular fluorescence (Fig. 4). The results showed only the homologous U6 CRISPR-Cas9 vectors significantly reduced fluorescence in S2R+ and Sf9 cells (Fig. 4 A and B), whereas the U6 promoters from several species reduced fluorescence in *T. ni* and *B. mori* cells (Fig. 4 C and D). Overall, among those tested, the DmU6:96Ab, SfU6-3, and TnU6-4 promoters would be the best choices for CRISPR-Cas9 editing in S2R+, Sf9, and High Five cells, respectively. In contrast, the BmU6-2, SfU6-3, and TnU6-4 promoters all provided about the same efficiencies and the heterologous SfU6-3 promoter would likely be the best choice for CRISPR-Cas9 editing in BmN cells.

Phenotypic Impact of Gene Editing with SfU6-3-SfFDLt1 CRISPR-Cas9 Vector in Sf9 Cells. Finally, we assessed the phenotypic impact of gene editing using one of the CRISPR-Cas9 tools created in this study. Sf9 cells were transfected with the CRISPR-Cas9 vector encoding the Sf-FDLt1 sgRNA under SfU6-3 promoter control, puromycin-selected, and the resulting polyclonal cell population (SfFDLt1) was used to isolate 30 single-cell clones. The Sf-*fdl* sequences in the parental Sf9, polyclonal SfFDLt1, and SfFDLt1 clones were then examined by CEL-I nuclease assays and TIDE analysis, as described in *Materials and Methods*. The
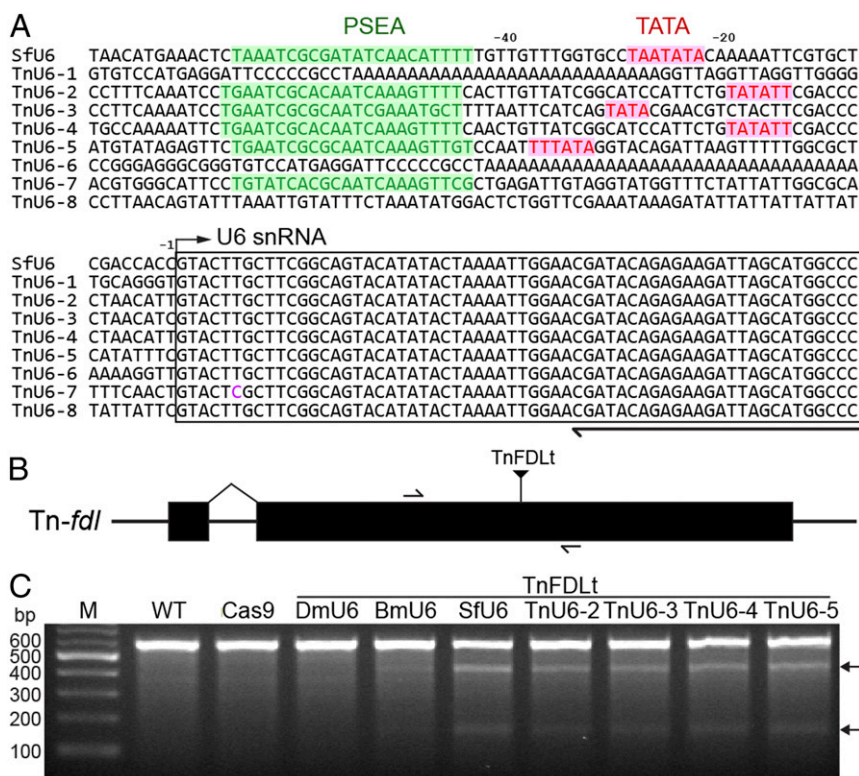

Fig. 3. Identification of putative TnU6 promoters and successful CRISPR-Cas9 editing of Tn-*fdl*. (A) Multiple sequence alignment of SfU6 promoter and TnU6 promoter candidates. (B) Diagram showing Tn-*fdl* gene structure and highlighting specific Cas9 targeting sequences and PCR primer sites. (C) CEL-I nuclease assay results obtained using genomic DNA from High Five cells edited with CRISPR-Cas9 vectors encoding a Tn-*fdl* targeting sequence (Table S1) under the control of the DmU6:96Ab, BmU6-2, SfU6, or TnU6 promoters.
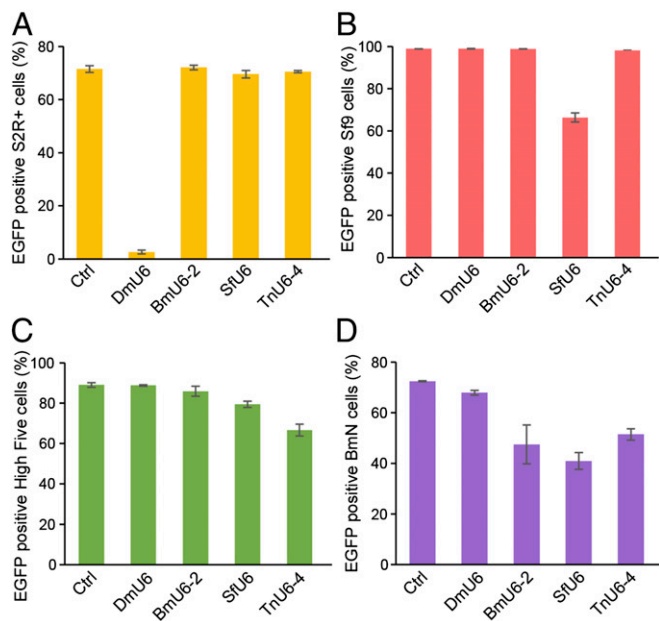
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Fig. 4. CRISPR-Cas9 editing efficiencies by various insect U6 promoters in various insect cell lines. (*A*) S2R-EGFP, (*B*) Sf9-EGFP, (*C*) High Five-EGFP, and (*D*) BmN-EGFP cells were transfected with DmU6:96Ab, SfU6, TnU6-4, and BmU6-2 CRISPR-Cas9 vectors encoding an EGFP-specific sgRNA, selected for puromycin resistance, and EGFP was measured by flow cytometry (the bars show mean fluorescence ± SD, *n* = 3 per group).

CEL-I nuclease assay results indicated all 30 clones had Sf-*fdl* indels (Fig. S3) and TIDE analysis revealed four clones had no wild-type Sf-*fdl* sequences or potentially functional in-frame deletions (Fig. S4 and Table S2).

We subsequently infected one of those clones (#32), as well as Sf9 cells and the polyclonal SfFDLt1 cell population, with a recombinant baculovirus encoding an affinity-tagged version of human erythropoietin (hEPO) and purified the secreted product from each culture, as described in *Materials and Methods*. We then enzymatically released the *N*-glycans from each purified protein preparation and analyzed the permethylated glycan structures by MALDI-TOF-MS, as described in *Materials and Methods*. The spectra showed the major *N*-glycan on hEPO from Sf9 and SfFDLt1 (polyclonal) cells was $Man_3GlcNAc_2$, whereas the major *N*-glycan on hEPO from SfFDLt1 #32 was $GlcNAcMan_3GlcNAc_2$ (Fig. S5). A quantitative analysis showed $Man_3GlcNAc_2$ represented about 90%, 60%, and 8% of the total *N*-glycans on hEPO from Sf9, SfFDLt1 (polyclonal), and SfFDLt1 clone #32, respectively (Fig. 5). Reciprocally, $GlcNAcMan_3GlcNAc_2$ represented about 10%, 30%, and 65% of total *N*-glycans on hEPO from Sf9, SfFDLt1 (polyclonal), and SfFDLt1 clone #32, respectively (Fig. 5). Finally, $GlcNAc_2Man_3GlcNAc_2$ was only detected on hEPO from SfFDLt1 (polyclonal), and SfFDLt1 #32 (Fig. 5).

These results clearly demonstrate the phenotypic impact of genome editing with the SfU6-3-SfFDLt1 CRISPR-Cas9 vector in Sf9 cells. Specifically, the structures of the *N*-glycans observed in the Sf9 cells treated with this vector reveal a partial (polyclonal) and nearly complete (clone #32) loss of FDL function resulting from *fdl* editing with this vector. We conclude that the CRISPR-Cas tools created in this study can be used to engineer host pathways in efforts to enhance and expand the capabilities of the BICS.

Discussion

The major outcome of this study was successful creation of CRISPR-Cas9 tools that can be used for site-specific genome editing in the BICS. These tools will enable far more sophisticated host-cell

engineering efforts, which to date have been limited to using nonhomologous recombination to knockin genes at random sites in the insect cell genome. Thus, these tools will enable new efforts to enhance and expand the utility of the BICS as a recombinant protein production platform.

In our efforts to achieve this goal, we initially tested *D. melanogaster* and *B. mori* U6 promoters that were previously shown to direct effective sgRNA expression and CRISPR-Cas9–mediated genome editing in dipteran and lepidopteran insect cells (27–29). We assumed these promoters might drive these same functions in *S. frugiperda* and *T. ni* cells, which would have allowed us to quickly produce CRISPR-Cas9 vectors for the BICS.

In fact, CRISPR-Cas9 vectors encoding Dm- or Bm-*fdl*–specific targeting sequences under *D. melanogaster* or *B. mori* U6 promoter control produced indels in cell lines from homologous species (Fig. S2). However, CRISPR-Cas9 vectors with these same *D. melanogaster* or *B. mori* U6 promoters encoding sgRNAs with Sf- or Tn-*fdl*–specific targeting sequences failed to produce any detectable indels in *S. frugiperda* (Fig. 1) or *T. ni* cells (Fig. 3), respectively. This forced us to identify putative *S. frugiperda* and *T. ni* U6 promoters, which we then used to produce CRISPR-Cas9 vectors encoding sgRNAs with the same Sf- or Tn-*fdl*–specific targeting sequences. We found CRISPR-Cas9 vectors with the homologous U6 promoters efficiently produced indels in *S. frugiperda* (Fig. 2) and *T. ni* (Fig. 3) cells, respectively.

We subsequently established an EGFP reduction assay, which could be used to more quantitatively measure the relative efficiencies of editing by CRISPR-Cas9 vectors encoding a GFP-specific sgRNA under the control of various insect U6 promoters in different insect cell species, as described in *Materials and Methods*. The results (Fig. 4) indicated only the CRISPR-Cas9 vectors with homologous U6 promoters significantly reduced GFP expression in *D. melanogaster* and *S. frugiperda* cells. In contrast, while the homologous U6 promoter provided the highest CRISPR-Cas9 editing efficiency in High Five cells, SfU6-3 also provided a reasonable efficiency and the *B. mori*, *S. frugiperda*, and *T. ni* promoters all provided about the same efficiencies of CRISPR-Cas9 editing in BmN cells. These results indicate SfU6-3 has the broadest—while DmU6:96Ab has the narrowest—host range among the insect U6 promoters tested in the insect cell lines used in this study. Additional studies will be required to extend these findings to include a broader range of insect U6 promoters and cell lines and,
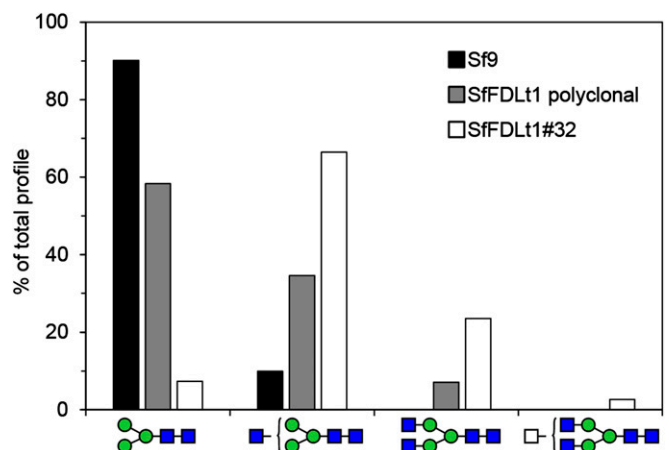

Fig. 5. CRISPR-Cas9–mediated *Sf-fdl* gene editing for host engineering in the BICS. The bar graph shows the relative proportions of different *N*-glycan structures released from hEPO produced by Sf9, SfFDLt1 polyclonal population, and SfFDLt1 clone #32. These data are derived from the MALDI-TOF-MS profiles shown in Fig. S5 and represent the relative percentages of each *N*-glycan shown along the bottom of the figure as a percentage of total.

ultimately, determine their underlying host-range determinants at the molecular level.

In a previous study, a recombinant baculovirus designed to express Cas9 and sgRNAs under the control of mammalian promoters was used as a transducing vector to induce CRISPR-Cas9 genome editing in mammalian cells (34). In contrast, the present study yielded new CRISPR-Cas9 tools designed to express Cas9 and sgRNAs under the control of baculovirus and insect cell promoters and examined their relative utility for genome editing in the BICS. Finally, our study provided proof-of-concept that these tools can be used for host cell engineering in the BICS. In our example, we targeted *fdl*, which encodes a key enzyme that distinguishes insect and mammalian cell protein *N*-glycosylation pathways by antagonizing *N*-glycan elongation. As such, *fdl* has been a high-priority target for knockout, as this would facilitate efforts to glycoengineer the BICS and other insect-based recombinant protein production platforms for high-efficiency mammalian-type protein *N*-glycosylation. Several previous publications demonstrated various RNAi approaches can reduce FDL activity, but with little or no phenotypic impact on *N*-glycan processing (20, 35–37). We previously used existing CRISPR-Cas9 tools to knockout Dm-*fdl* in S2R+ cells and demonstrate this had the expected impact on *N*-glycan processing (28). However, we were unable to knockout Sf-*fdl* or Tn-*fdl* until we created the tools needed for site-specific gene editing in the BICS. We then used a CRISPR-Cas9 vector encoding an Sf-*fdl*–specific sgRNA under the control of the SfU6-3 promoter to produce polyclonal and monoclonal Sf9 cell derivatives. CEL-I nuclease assays and TIDE analysis indicated this CRISPR-Cas9 vector directed efficient editing of the Sf-*fdl* gene (Fig. S4 and Table S2). Finally, we documented the phenotypic impact of these genotypic changes by analyzing the *N*-glycans isolated from recombinant hEPO produced by polyclonal and monoclonal Sf-*fdl* knockout cells described in this study. As expected, we observed reduced proportions of paucimannose ($Man_3GlcNAc_2$) and increased proportions of terminally GlcNAcylated ($GlcNAc_{1–2}Man_3GlcNAc_2$) structures on hEPO produced by SfFDLt1 cells, compared with Sf9 cells (Fig. 5 and Fig. S5). Thus, in summary, this study presents CRISPR-Cas9 vectors for site-specific genome editing and clearly demonstrates they can be used successfully for host cell engineering in the BICS.

Materials and Methods

Cells. S2R+ cells (38) were maintained at 28 °C as adherent cultures in Schneider's *Drosophila* medium (Life Technologies) containing 10% (vol/vol) FBS (Atlanta Biologics). Sf9, High Five, and BmN cells were maintained at 28 °C as adherent cultures in TNM-FH medium containing 10% (vol/vol) FBS. Sf9 cells were transfected using a modified calcium phosphate method (8) and S2R+, High Five, and BmN cells were transfected with polyethyleneimine, as described previously (28). S2R-EGFP, Sf9-EGFP, Tn-EGFP, and BmN-EGFP cells are transgenic derivatives of S2R+, Sf9, High Five, and BmN cells, respectively, produced by transfecting each parental cell line with pIE1-EGFP-Bla and selecting for blasticidin resistance. Blasticidin-resistant cells expressing EGFP in the top quartile were isolated using a MoFlo Legacy Cell Sorter (Beckman Coulter) and enriched cell subpopulations were maintained under the same growth conditions as the parental cell lines.

Plasmid Constructions. All CRISPR-Cas9 constructs were generically designed to include three distinct cassettes for expression of Cas9, an sgRNA, and a puromycin resistance marker. The Cas9 expression cassette consists of a *S. pyogenes* Cas9 sequence codon optimized for *S. frugiperda* and assembled with the AcMNPV *ie1* promoter and *p10* polyadenylation signal using the Golden Gate method. The sgRNA expression cassettes consist of DmU6:96Ab, BmU6-2, SfU6-3, TnU6-2, TnU6-3, TnU6-4, or TnU6-5 promoters assembled with various downstream sgRNA sequences. The targeting sequences incorporated into various sgRNAs are given in Table S1. A targeting sequence cloning site consisting of two SapI recognition sites was inserted between the U6 promoter and sgRNA in each CRISPR-Cas9 plasmid. Finally, the puromycin-resistance marker was codon optimized for *S. frugiperda* and assembled with the AcMNPV *ie1* promoter and *p10* polyadenylation signal. The generic CRISPR-Cas9 plasmid and specific target-sgRNA sequences are given in SI Appendix.

Splinkerette PCR. Splinkerette PCR was performed as described previously (39). Briefly, Sf9 or High Five genomic DNA was digested with BamHI, BglII, BstYI, HindIII, SalI, SpeI, or XbaI, and ligated with splinkerette adaptors complementary to the resulting overhangs. Primary and secondary PCRs were performed with Splink1 and SfU6-Rv1 and Splink2 and SfU6-Rv2 as the primer pairs, respectively (primer sequences are given in Table S3). The resulting amplimers were cloned into pGEM-T (Promega) and three independent clones were sequenced to determine the consensus.

Genomic DNA Isolation and CEL-I Nuclease Assays. Genomic DNA was extracted from Sf9, High Five, BmN, and S2R+ cells using the Wizard genomic DNA extraction kit (Promega) according to the manufacturer's instructions. CEL-I nuclease assays were performed as described previously (28). The sequences of the primers used to amplify various target loci are given in Table S4.

TIDE Analysis. We performed TIDE analysis as described previously (40). Briefly, we directly sequenced the PCR products amplified from Sf9 and SfFDLt1 monoclonals' genomic DNA as described above and used the sequencing results as queries for a TIDE web program (https://tide-calculator.nki.nl/). All analyses were performed with a default setting.

EGFP Reduction Assay. S2R-EGFP, Sf9-EGFP, Tn-EGFP, and BmN-EGFP cells were transfected with various CRISPR-Cas9 vectors targeting EGFP or a control vector encoding no sgRNA and selected for puromycin resistance. Puromycin-resistant survivors were analyzed using a Guava easyCyte HT flow cytometer (Millipore) and EGFP$^+$ cell populations were quantified using FlowJo software.

Expression and Purification of hEPO. We isolated AcRMD2-p6.9-hEPO, a recombinant baculovirus encoding an N-terminally affinity-tagged version of hEPO, in two steps. First, we recombined a gene encoding the *Pseudomonas aeruginosa* GDP-4-dehydro-6-deoxy-D-mannose reductase (*rmd*) cds under the control of the AcMNPV *ie1* promoter into the *chi-cth* locus of a baculovirus vector called BacPAK6-p6.9-GUS (41) to produce AcRMD2. Second, we recombined a honey bee melittin signal peptide, 8XHIS-tag, Strep II-tag, tobacco etch virus recognition site, and mature hEPO cds under the control of the AcMNPV *p6.9* promoter into the *polh* locus of AcRMD2. hEPO was expressed and purified as described previously (42).

Isolation and Characterization of Monoclonal SfFDLKO Cell Lines. Single cell clones were isolated from the polyclonal SfFDLt1 cell population, as described previously (43). Indels were analyzed by CEL-I nuclease assays and the Sf-*fdl* gene sequences in clones 4, 14, 32, and 49 were amplified, sequenced, and the sequences were analyzed by TIDE, as described previously (28, 40).

Mass Spectrometry. *N*-glycans were enzymatically released from purified hEPO and derivatized, as described previously (42), then analyzed by MALDI-TOF-MS using an Applied Biosystems SCIEX TOF/TOF 5800 (SCIEX), with 400 shots accumulated in reflectron positive-ion mode. Structures were manually assigned to peaks based on knowledge of the insect cell *N*-glycan processing pathway. Quantification involved dividing the peak intensities of permethylated *N*-glycan structures by the total intensity of all annotated *N*-glycan peaks having >1% of total intensities.

1. Smith GE, Summers MD, Fraser MJ (1983) Production of human beta interferon in insect cells infected with a baculovirus expression vector. *Mol Cell Biol* 3:2156–2165.
2. Felberbaum RS (2015) The baculovirus expression vector system: A commercial manufacturing platform for viral vaccines and gene therapy vectors. *Biotechnol J* 10:702–714.
3. Kitts PA, Ayres MD, Possee RD (1990) Linearization of baculovirus DNA enhances the recovery of recombinant virus expression vectors. *Nucleic Acids Res* 18:5667–5672.
4. Kitts PA, Possee RD (1993) A method for producing recombinant baculovirus expression vectors at high frequency. *Biotechniques* 14:810–817.

5. Luckow VA, Lee SC, Barry GF, Olins PO (1993) Efficient generation of infectious recombinant baculoviruses by site-specific transposon-mediated insertion of foreign genes into a baculovirus genome propagated in *Escherichia coli*. *J Virol* 67:4566–4579.

6. Geisler C, Mabashi-Asazuma H, Jarvis DL (2015) An overview and history of glycoengineering in insect expression systems. *Methods Mol Biol* 1321:131–152.

7. Harrison RL, Jarvis DL (2016) Transforming lepidopteran insect cells for improved protein processing and expression. *Methods Mol Biol* 1350:359–379.

8. Summers MD, Smith GE (1987) *A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures Tex Agric Exp Stn Bull* 1555:1–57.

9. Wickham TJ, Davis T, Granados RR, Shuler ML, Wood HA (1992) Screening of insect cell lines for the production of recombinant proteins and infectious virus in the baculovirus expression system. *Biotechnol Prog* 8:391–396.

10. Marz L, Altmann F, Staudacher E, Kubelka V (1995) Protein glycosylation in insects. *Glycoproteins*, eds Montreuil J, Vliegenthart JFG, Schachter H (Elsevier, Amsterdam), Vol 29a, pp 543–563.

11. Marchal I, Jarvis DL, Cacan R, Verbert A (2001) Glycoproteins from insect cells: Sialylated or not? *Biol Chem* 382:151–159.

12. Harrison RL, Jarvis DL (2006) Protein *N*-glycosylation in the baculovirus-insect cell expression system and engineering of insect cells to produce "mammalianized" recombinant glycoproteins. *Adv Virus Res* 68:159–191.

13. Geisler C, Jarvis DL (2009) Insect cell glycosylation patterns in the context of biopharmaceuticals. *Post-Translational Modification of Protein Biopharmaceuticals*, ed Walsh B (Wiley-Blackwell, Weinheim, Germany), pp 165–191.

14. Shi X, Jarvis DL (2007) Protein *N*-glycosylation in the baculovirus-insect cell system. *Curr Drug Targets* 8:1116–1125.

15. Koles K, Irvine KD, Panin VM (2004) Functional characterization of *Drosophila* sialyltransferase. *J Biol Chem* 279:4346–4357.

16. Koles K, et al. (2007) Identification of *N*-glycosylated proteins from the central nervous system of *Drosophila melanogaster*. *Glycobiology* 17:1388–1403.

17. Aoki K, et al. (2007) Dynamic developmental elaboration of *N*-linked glycan complexity in the *Drosophila melanogaster* embryo. *J Biol Chem* 282:9127–9142.

18. Altmann F, Schwihla H, Staudacher E, Glössl J, März L (1995) Insect cells contain an unusual, membrane-bound β-*N*-acetylglucosaminidase probably involved in the processing of protein *N*-glycans. *J Biol Chem* 270:17344–17349.

19. Léonard R, et al. (2006) The *Drosophila* fused lobes gene encodes an *N*-acetylglucosaminidase involved in *N*-glycan processing. *J Biol Chem* 281:4867–4875.

20. Geisler C, Aumiller JJ, Jarvis DL (2008) A fused lobes gene encodes the processing beta-*N*-acetylglucosaminidase in Sf9 cells. *J Biol Chem* 283:11330–11339.

21. Geisler C, Jarvis DL (2010) Identification of genes encoding *N*-glycan processing beta-*N*-acetylglucosaminidases in *Trichoplusia ni* and *Bombyx mori*: Implications for glycoengineering of baculovirus expression systems. *Biotechnol Prog* 26:34–44.

22. Solá RJ, Griebenow K (2010) Glycosylation of therapeutic proteins: An effective strategy to optimize efficacy. *BioDrugs* 24:9–21.

23. Jarvis DL (2009) Baculovirus-insect cell expression systems. *Methods Enzymol* 463:191–222.

24. Hsu PD, Lander ES, Zhang F (2014) Development and applications of CRISPR-Cas9 for genome engineering. *Cell* 157:1262–1278.

25. Sander JD, Joung JK (2014) CRISPR-Cas systems for editing, regulating and targeting genomes. *Nat Biotechnol* 32:347–355.

26. Doudna JA, Charpentier E (2014) Genome editing. The new frontier of genome engineering with CRISPR-Cas9. *Science* 346:1258096.

27. Bassett AR, Tibbit C, Ponting CP, Liu JL (2014) Mutagenesis and homologous recombination in *Drosophila* cell lines using CRISPR/Cas9. *Biol Open* 3:42–49.

28. Mabashi-Asazuma H, Kuo CW, Khoo KH, Jarvis DL (2015) Modifying an insect cell *N*-glycan processing pathway using CRISPR-Cas technology. *ACS Chem Biol* 10:2199–2208.

29. Liu Y, et al. (2014) Highly efficient multiplex targeted mutagenesis and genomic structure variation in *Bombyx mori* cells using CRISPR/Cas9. *Insect Biochem Mol Biol* 49:35–42.

30. Fujita R, Ono C, Ono I, Asano S, Bando H (2015) Analysis of the *Bombyx mori* nucleopolyhedrovirus ie-1 promoter in insect, mammalian, plant, and bacterial cells. *Biochem Biophys Res Commun* 464:1297–1301.

31. Hernandez G, Jr, Valafar F, Stumph WE (2007) Insect small nuclear RNA gene promoters evolve rapidly yet retain conserved features involved in determining promoter activity and RNA polymerase specificity. *Nucleic Acids Res* 35:21–34.

32. Kakumani PK, Malhotra P, Mukherjee SK, Bhatnagar RK (2014) A draft genome assembly of the army worm, *Spodoptera frugiperda*. *Genomics* 104:134–143.

33. Devon RS, Porteous DJ, Brookes AJ (1995) Splinkerettes—Improved vectorettes for greater efficiency in PCR walking. *Nucleic Acids Res* 23:1644–1645.

34. Mansouri M, et al. (2016) Highly efficient baculovirus-mediated multigene delivery in primary cells. *Nat Commun* 7:11529.

35. Nomura T, et al. (2015) Improvement of glycosylation structure by suppression of β-*N*-acetylglucosaminidases in silkworm. *J Biosci Bioeng* 119:131–136.

36. Kim NY, et al. (2012) Short-hairpin RNA-mediated gene expression interference in *Trichoplusia ni* cells. *J Microbiol Biotechnol* 22:190–198.

37. Kim YK, et al. (2009) Suppression of beta-*N*-acetylglucosaminidase in the *N*-glycosylation pathway for complex glycoprotein formation in *Drosophila* S2 cells. *Glycobiology* 19:301–308.

38. Yanagawa S, Lee JS, Ishimoto A (1998) Identification and characterization of a novel line of *Drosophila* Schneider S2 cells that respond to wingless signaling. *J Biol Chem* 273:32353–32359.

39. Mabashi-Asazuma H, et al. (2015) Targeted glycoengineering extends the protein N-glycosylation pathway in the silkworm silk gland. *Insect Biochem Mol Biol* 65:20–27.

40. Brinkman EK, Chen T, Amendola M, van Steensel B (2014) Easy quantitative assessment of genome editing by sequence trace decomposition. *Nucleic Acids Res* 42:e168.

41. Maghodia AB, Geisler C, Jarvis DL (2016) Characterization of an Sf-rhabdovirus-negative *Spodoptera frugiperda* cell line as an alternative host for recombinant protein production in the baculovirus-insect cell system. *Protein Expr Purif* 122:45–55.

42. Geisler C, Mabashi-Asazuma H, Kuo CW, Khoo KH, Jarvis DL (2015) Engineering β1,4-galactosyltransferase I to reduce secretion and enhance N-glycan elongation in insect cells. *J Biotechnol* 193:52–65.

43. Harrison RL, Jarvis DL (2016) Transforming lepidopteran insect cells for continuous recombinant protein expression. *Methods Mol Biol* 1350:329–348.