Germline transformation in *Drosophila melanogaster* expressing the mammalian sialic acid synthase and CMP-sialic acid synthetase

(Received: 05.04.2009; Accepted: 30.04.2009)

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ABSTRACT

The insect-dependent baculovirus expression vectors are frequently used to express glycoproteins. Such glycoproteins include many products with biomedical value. The N-glycosylation pathway of insect cells differs than the mammalian pathway. In turn, it limits expression systems to produce glycoproteins with full activities. The current study is a part of transgenic experiment series to produce drosophila capable of processing the glycoprteins as mammalian glycosylation pathways. The w^{1118} eye mutant strain of Drosophila melanogaster was transformed with piggyBac-derived construct carrying DNA fragment encoding both mammalian sialic acid synthase and CMP-sialic acid synthetase under the "ie1-hr5-ie1" dual back to back constitutive promoter. The transformation experiments yielded six positive flies showing eye-yellow fluorescence. The putative transgenic flies were further analyzed. Subsequent southern analyses, RT-PCR and sequencing of piggyBac-flanking region within the drosophila genome proved at least two transgenic drosophila lines which are piggyBac-mediated transformants and express mammalian enzymes.

***Key words**: Transformation, D. melanogaster, mammalian, sialic acid.*

INTRODUCTION

Typically, there are three distinct types of protein modifications with glycan in eukaryotic cells: N-linked glycosylation of asparagine, 0-linked glycosylation of serine or threonine and glycosylphosphatidyl inositol derivatization of the terminal carboxyl groups (Orlean. 1996, Hounsell *et al.*, 1996 and Udenfriend and Kodukula, 1995). N-linked carbohydrates play roles in different biological processes such as protein folding and conformation, stability, and targeting to subcellular and extracellular sites as well as cell-matrix and cell-cell interactions (Varki 1993; Fiedler and Simons, 1996).

In all eukaryotes, the initial steps are identical involving the synthesis of lipid-linked oligosaccharide precursor followed by the subsequent events in the ER and Golgi (Kukuruzinska and Lennon, 1998). The intermediate complex is a common structure between mammalian and insect N-glycosylation pathway (For review see: Jarvis 2003, and Rendie *et al.*, 2008). Three glucose residues at the high mannose structure "Glu3Man9GlcNac2-N-Asn" are removed by α1,2 glucosidase I and α1,3 glucosidase II.

Further trimming of five mannose residues is catalyzed by α-mannosidase I. One residue of N-acetylglocusamine is added to the structure by the N-acetylglocusaminyltransferase I. The common intermediate structure is achieved by trimming two mannose residues by α-mannosidase II and adding one fucose residue by fucosyltransferase I. Further modifications of the intermediate structure are different in mammalian than those in insect cells. In mammalian cells, the pathway tends to elongate this common intermediate by the action of glycosyl-transferases to produce hybrid and complex N-glycans with terminal sialic acids. In contrast, insect cells have N-acetylglucosaminidase that can remove the terminal N-acetylglucosamine residue from "GlcNAcMan3GlcNAc2-N-Asn" forming the major N-glycan product in insect cells, the paucimannose, "Man3GlcNAc2-N-Asn". However, in some cases, only extremely low levels terminal glycosyltransferase activities have been detected in insect cells.

Due to the inability of insect cells to produce biantennary complex, baculovirus-insect cell expression systems produce glycoproteins complex with N-linked oligosaccharide lacking the terminal sialic acid residues. Modifying the insect cell N-glycosylation pathway is critical for the insect cell-dependant expression systems. The *Spodoptera frugiperda* cells (Sf9) were engineered to express the mammalian β1,4-galactosyltransferase (GalT) and α2,6-sialyltransferase (ST6). The cells, designated as Sf4GalT/ST6, produced a recombinant glycoprotein with mono-antennary structure with only α1,3 arm elongated (Hollister and Jarvis 2001; Hollister *et al.*, 2002). The Sf4GalT/ST6 cells were further transformed with N-acetylglucoseaminyltransferase II gene creating the transgenic cells SfSWT-1. These cells produced a recombinant glycoprotein with bi-antennary sialylated N-glycans (Hollister *et al.* 2002). Aumiller *et al.* (2003) transformed SfSWT-1 cells with two additional mammalian genes sialic acid synthase and CMP-sialic acid synthetase. The resulted cells (SfSWT-3), with a total of seven N-glycosylation-related mammalian genes, produced CMP-sialic acid and sialylated the recombinant glycoprotein in serum free-media. The growth media was only supplement with N-acetylmannosamine as a sugar precursor for sialic acid salvage.

The current study is one of transformation experimental series to generate transgenic *Drosophila melanogaster* that are capable to process the N-glycan as mammalian pathways. The drosophila flies were transformed with *piggyBac*-construct encoding both the mouse sialic acid synthase and CMP-sialic acid synthetase. The transgenic lines were analyzed for the expression of both genes at molecular level.

MATERIALS AND METHODS

Plasmids

The pXLBacII-SAS/CMP.SAS-EYFP clone#42-3 plasmid (Fig. 1) was provided by Dr. Donald Jarvis, Wyoming State University (Shi *et al.*, 2007). The *piggyBac* vector system pXLBacII (Li *et al.*, 2005) was utilized as template construct. The plasmid was designed to encode a mouse sialic acid synthase (SAS) (Nataka *et al.*, 2000) and a mouse CMP-sialic acid synthetase (CMP-SAS) (Munster *et al.*, 1998) under the "*ie1-hr5-ie1*" dual constitutive transcriptional control element. The dual element consisted of back-to back baculovirus *ie1* promoters separated by a baculovirus *hr5* enhancer. Both genes were terminated with a DNA fragment containing the bovine growth hormone polyadenylation signal, BGHpolyA. The yellow fluorescent marker gene (EYFP), under the transcriptional control of the eye-specific promoter, 3xP3, was also incorporated into the plasmid. The phspBac plasmid

(Handler and Harrell, 1999) was used for microinjection as a source of *piggyBac* transposase.

Drosophila culture

Drosophila strains were reared under standard laboratory conditions (Roberts, 1998). The preblastodermal embryos were microinjected as described in (Rubin and Spradling, 1982). The *D. melanogaster* w^{1118} white eye strain was used for microinjection with a concentration of 0.4µg/µl of the donor plasmid, pXLBacII-SAS/CMP.SAS-EYFP clone#42-3, and 0.4µg/µl of the helper plasmid, phspBac. All injected embryos were subjected to one hour heat shock period at 37°C after 24 hours from injection to induce expression of the *piggyBac* transposase. The injected embryos were allowed to grow to adulthood at 28°C. Emerging adults were individually mated with w^{1118} flies, and their progeny were screened as adults for fluorescent eye color using an Olympus SZX12 fluorescent microscope equipped with YFP filter set. Positive adults were individually crossed with the w^{1118} flies, and subsequent generations were homozygosed to establish the separate transformed lines.

Southern hybridization

The genomic DNA was prepared from adult flies using DNAzol (MBL) essentially as described in the manufacture manual. Southern hybridization was performed using standard procedures. Twenty five micrograms of genomic DNA were digested overnight with *EcoR*V restriction enzyme. The digested genomic DNA was transferred overnight onto Nylon membrane. The DNA was cross-linked to the membrane using a UV crosslinker (Stratgene). The plasmid DNA that initially used for mircoinjection, was used as a positive control as well as a probe for each hybridization. The probe was radio-labeled with ^{32}P dCTP (Amersham) using Prime-a-Gene Labeling System (Promega). Hybridizations were performed overnight at 65°C in 6x standard saline citrate (SSC), 0.5% SDS, 5x Denhardt's solution and 100µg/ml salmon sperm DNA. After hybridization, the membrane was initially washed in 2x SSC; 0.5% SDS at room temperature and two washes in 0.2x SSC; 0.5% SDS at 55°C for 30 min. Autoradiography was performed by exposure on Kodak X-ray film at -80°C.

IPCR

Inverse PCR was performed by completely digesting 5µg of the genomic DNA from the transformed strain with *Sau*3AI for both ends followed by heat inactivation of the restriction enzyme and DNA precipitation and finally self ligated in a 100µl volume overnight. The self-ligated DNAs were precipitated and resuspended in 30µl ddH$_2$O. A 5µl portion of each ligation was used for first round PCR (94°C for 1 min, 40°C for 1 min, 72°C for 2 min, thirty-five cycles) with the primer set (RightArm42-3FWD: 5'-ATCAAGCTTATCGATACCGTCGACC-3'/ RightArm42-3RVS: 5'-CCTAAATGCACAGCGACGGA-3') for the 5' end, and the primer set (LeftArm42-3FWD: 5'-AACCTCGATATACAGACCGAT-3' / LeftArm42-3RVS: 5'-CTTTTATCGAATTCCTGCAGCC-3') for the 3'end. Two microliters of the first-round PCR products were used as templates for the second-round PCR (94°C for 1 min, 50°C for 1 min, 72°C for 2 min, thirty-five cycles) using the primer set (RightArmIIFWD-42-3: 5'-CATGTTCTACTTACGTGATAAC-3' and RightArmIIRVS-42-3: 5'-CGACTGAGATGTCCTAAATC-3') for the 5'end, and the primer set (LeftArmIIFWD-42-3: 5'-CACATGCGTCAATTTTACGCA-3' and LeftArmIIRVS-42-3: 5'-GTAACAAAACTTTTATCGAATTCCTGC-

3') for the 3' end. All PCR products were cloned into the pCR2.1 (Invitrogen). DNA sequence was determined on ABI Prism 310 DNA sequencer using BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems). Amplified products were determined on an ABI Prism 373 DNA Sequencer using BigDye termination DNA sequencing kit containing AmpliTaq FS polymerase (Perkin Elmer Cetus, Foster City, CA).and sequences were subjected to the Blast search of the NCBI database to identify the location of the insertions.

RT-PCR

The total RNA was extracted from the homogenized tissue using the Trizol reagent (Inovitrogen). First strand cDNA was prepared from the total RNA to be used as a template for RT-PCR analysis. The total RNA (1-5µg) was treated with RNase-free DNase, to get ride of any DAN contaminants, using 1 U of DNasI amplification grade (Invitrogen) /1µg RNA and incubated for 15 minutes at room temperature. The reaction was stopped by adding 1µl of 25 mM EDTA and 10 minutes at 65°C. The DNase treated RNA was mixed with 2 pmoles of the Oligo dT primers and heated to 70°C for 10 minutes and immediately chilled on ice. The reaction was performed by adding 1X 1st strand buffer, 10 mMDTT and 0.5 mM dNTP and warmed at 42°C for 2 min and finally 20 units of SuperScript II (Invitrogen) was added. The reaction was incubated at 42°C for 50 minutes before inactivation at 70°C for 15 minutes. The first strand cDNA was mixed with 0.25 units Taq polymerase in 1 X reaction buffer, 0.5 M dNTPs, 25mM $MgCl_2$ and 25 pmoles primers. The amplification conditions were 94°C for 5 minutes followed by 35 cycles of 94°C for 15 sec, 55°C for 15 sec, and 72°C for1 min followed by 5 min extension step at 72°C. The primer set: SASFWD: 5'-TTAAGCCTTGATTTTCTTGCTGTG-3' and SASRVS; 5'-ATGCCGCTGGAACTGGAGCT-3' was used to amplify the sialic acid synthase. While the CMP-sialic acid synthetase was amplified using the primer set: CMP-SASFWD-2: 5'-ATGCGGGGGTCTTCCAGAGT-3' and CMP-SASRVS-2; 5'-GGCCATTGGTGAGACATCCA-3'. The marker gene encoding the enhanced yellow fluorescent protein was amplified using the primer set: EYFP-FWD:5'-ATGGTGAGCAAGGGCGAGGAGCTGTTC -3' and EYFP-REV: 5'-TTACTTGTACAGCTCGTCCATGCC-3'.

RESULTS

Transformation experiments

Drosophila melanogaster w^{1118} embryos were microinjected with equal concentration of 0.4µg/µl of pXLBacII-SAS/CMP.SAS-EYFP clone#42-3 and phspBac. The phspBac plasmid is used as a helper vector, it expresses the *piggyBac* transposase enzyme under the drosophila heat-shock inducer promoter *hs70*. The transposase cut *piggyBac* terminal repeats including the flanking sequences encoding CMP-SAS, SAS and the eye-specific marker EYFP. From five separate transformation experiments, a total of 2342 embryos were injected and 1081 larvae were hatched with percentage ranging between 26.6 and 61.92 (Table 1).

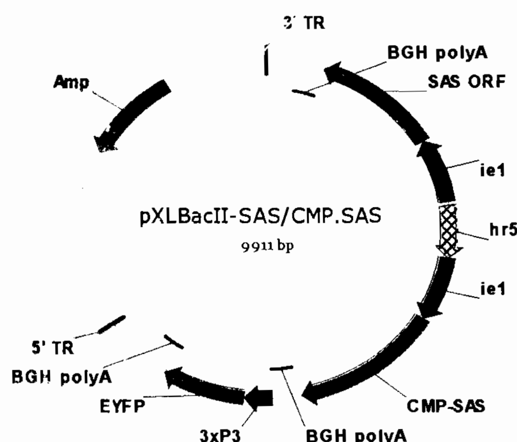

Fig. (1): Map of the donor plasmid, pXLBacII-SAS/CMP.SAS-EYFP clone#42-3.

Table (1): Transformation experiments.

Injected Experiment	Number of injected embryos	Number of hatched larvae	% hatching	Number of crosses	Number of cyan-expressing flies	Transformation frequency %
1	400	150	37.5	58	3	5.1
2	500	133	26.6	40	3	7.5
3	167	61	36.5	ND	0	0
4	625	387	61.92	46	0	0
5	650	350	53.84	45	0	0

The emerged adults were individually backcrossed with w^{1118} flies in separate families and the progenies (G0) were screened for EYFP expression. Two out of five injection experiments yielded six positive flies showing yellow fluoresces. The fluorescent protein expression was visible in their eyes (Fig. 2). The transformation frequency is calculated by dividing the number of the G0 transgenic individuals by the number of crossed families. The transformation frequencies ranged from 5.1-7.5%. However, the fertilities of the crossed families were not determined. Therefore, the frequencies are expected to be lower. The positive flies were backcrossed with w^{1118} flies for few generations. Every generation, the flies were selected for yellow expression then crossed with their siblings and the flies which did not show the marker expression were immediately isolated and destroyed. Simply, the putative transgenic individuals were marked as 42-1 through 42-6 and so, their progenies were designated as line. Positive six homozygous lines, 42-1 through 42-6 were established. Fly samples representing these lines were analyzed for legitimate *piggyBac* transposition.

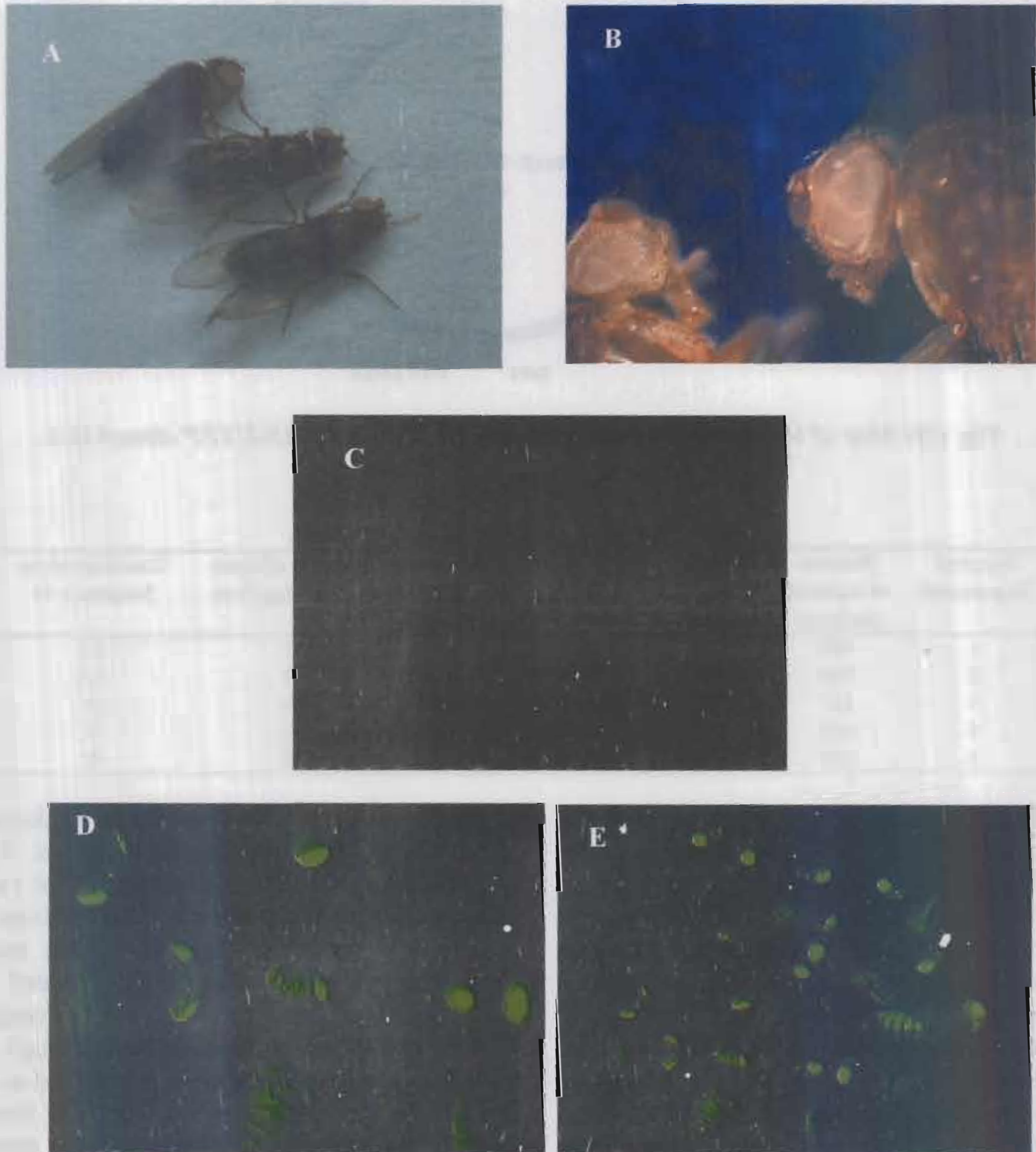

Fig. (2): Full body flies (A) and white eye color phenotype (B) of w^{1118} flies under white light and UV light (C). The transformant flies (D and E) under the exposure of UV light using fluorescent microscope equipped with YFP filter set.

Insertion analysis

Genomic transposition of the *piggyBac* was verified by Southern DNA hybridization. The genomic DNA extracted from positive flies, was digested with *Eco*RV restriction enzyme which has three sites on the vector sequence. Digestion with *Eco*RV enzyme results in three bands; two finger-print DNA fragments at 2.148 and 1.591kb. Both bands represent the two trangenes and the marker gene. A third fragment at 6.172kb represents the rest of the plasmid (Fig. 3.A). The *piggyBac*-transformed flies should contain such two internal signals at 2.148 and 1.591kb. The extra signal(s) is variable at sizes and depends on the insertion site(s) and number(s) within the insect genome. The donor plasmid DNA that is initially used for microinjection, is also used as a positive control as well as a probe. The positive control contains three signals at hybridization (Fig. 3.B). The probe did not hybridize with the DNA from wild type flies. Transgenic lines 42-1 through 42-6 showed the two predicted size fragments in addition to other fragments with variable sizes. The variable size of the third fragment indicates different insertion sites within the insect genome across the three transgenic lines. These lines are originated from different transposition events within the embryonic germ cells. With line 42-3, three DNA fragments were detected, indicating that this line has three separate insertions of the DNA fragment encoding SAS, CMP-SAS and the marker EYFP.

RT-PCR

The sialic acid synthase and CMP-sialic acid synthetase expression, in six transgenic lines, was confirmed using reverse transcriptase PCR. One microgram of DNA-free total RNA was used as a template for the synthesis of the first strand which was used for further amplification. ECFP-specific primers were used for positive control reaction. Both the sialytransferases were amplified using specific primer sets but with unequal expression within the same transgenic flies (Fig. 4). In all lines, the sialic acid synthase transcript is much more expressed than the CMP-sialic acid synthetase. In a previous study, the dual *piggyBac*-mediated vectors detected unequal transcriptional levels due to positional effect of the dual construct (Shi *et al.*, 2007). The heterologous genes adjacent to the 3'- terminal region are consistently expressed at higher levels than those adjacent to the 5'-terminal region of the *piggyBac* element.

Insertion site determination

The *piggyBac*-mediated chromosomal transposition was verified by inverse PCR. The PCR products were cloned and sequenced. The sequencing data were analyzed by DNA alignment with the *piggyBac* terminal sequences and identification of TTAA duplicate target site, the characteristic for all *piggyBac* integrations (Elik *et al.*, 1995). Finally, the DNA sequence proximal to the TTAA was Blast analysis (Altschul *et al.*, 1990) to identify genomic insertion site sequences. For lines 42-2 and 42-5, both 5' and 3' junctions yielded the *piggyBac* inverted terminal repeat sequences immediately adjacent to a TTAA sequence (Table 2). Although the insertion site for both lines are the right second chromosome, but their location is different. Only the 3' junction for lines 42-3 and 42-4 were recovered and it shows that they are located at the second and third chromosome, respectively. Due to failure of PCR reactions, neither the junction sequences nor the chromosome location were resolved for lines 42-1 and 41-6.

A.

EcoRV EcoRV EcoRV

5'TTR 3xP3 EYFP CMP-SAS IE1-hr5-IE1 SAS 3'TTR

2.148kb 1.591kb

B.

P 42-1 42-2 42-3 42-4 42-5 42-6 C

6.1 kb

2.1 kb

1.6 kb

Fig. (3): Southern DNA hybridization analysis of D. melanogaster transformants. A) Scheme of the pXLBacII-SAS/CMP.SAS-EYFP clone#42-3 vector showing the location of EcoRV restriction sites. B) DNA hybridization representing flies from each transgenic line (42-1 through 42-6). All lines have two internal fragments (2.1 and 1.6kb) and at least one variable size fragment. The positive control is the plasmid DNA (P) and the negative control is the w^{1118} flies (C).

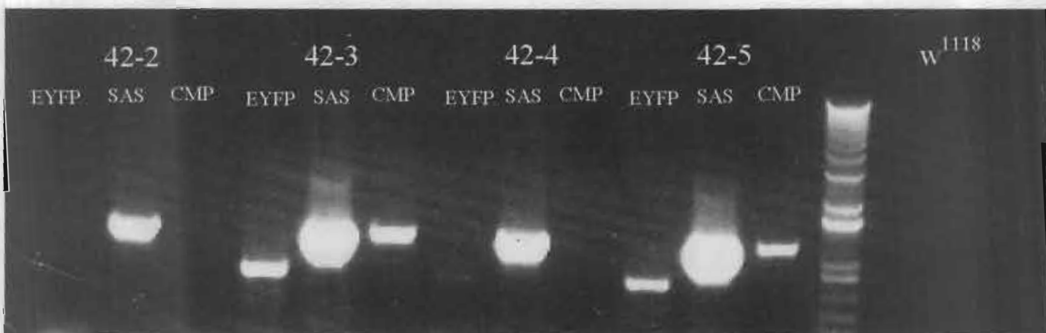

Fig. (4): The transcripts of enhanced yellow fluorescence protein (EYFP), sialic acid synthase (SAS) and CMP-sialic acid synthetase (CMP-SAS) were verified using RT-PCR for lines 42-2 through 42-5 as well as the negative control of w^{1118} flies.

Table (2): Insertion sites of transformed Drosophila lines.

Line	Chromosome location	Insertion site sequence 5' junction		Insertion site sequence 3' junction
pXLBacII -clone# 42-3	------	ctgatgcagctagaTTAA	..*piggyBac*..	TTAAtctagctgcgtgttctg
42-1	ND*	ND	..*piggyBac*..	ND
42-2	2R	taccagatatttcagTTAA	..*piggyBac*..	TTAAtgaagttaaggtaag
42-3	2L	ND	..*piggyBac*..	TTAAacataaaacaagaac
42-4	3L	ND	..*piggyBac*..	TTAAaaccctatactggc
42-5	2R	gccagtatagggttTTAA	..*piggyBac*..	TTAAtcgctgacagtggaa
42-6	ND	ND	..*piggyBac*..	ND

*Neither the junction sequences nor chromosome location resolved by inverse PCR.

DISCUSSION

Engineering the *Drosophila* with *piggyBac* construct encoding two mammalian sialyltransferase genes resulted in seven putative transgenic flies. The transformation frequencies ranged from 5.1-7.5%. However, the fertilities of the crossed families were not determined. Therefore, the frequencies are expected to be lower. Previously, the transformation frequencies using *piggyBac*-derived vectors in *Drosophila* are variable from 1 to 26% (Handler and Harrell 1999). In *Drosophila*, *piggyBac*-derived constructs were used as a genetic tool to evaluate or study certain sequences or features such as; promoter activity (Li *et al.*, 2001), insertional mutagenesis (Horn *et al.*, 2003 and Häcker *et al.*, 2003) and gene trapping for gene function and expression analysis (Bonin and Mann 2004). In the present study, *piggyBac*-based system is used to modify the N-glycosylation pathway in the model insect.

Previous studies on the insect cells resulted in transgenic SfSWT-3, Sf9 cells derivative, with total of seven N-glycosylation-related mammalian genes that could sialylate the recombinant glycoprotein (Aumiller *et al.*, 2003). In the effort to modify the insect N-glcyosylation process pathway, six mammalian genes have been selected to transform *Drosophila melanogaster*. The selected genes are N-acetylglucosaminylteransferase II, β 1,4-galactosyltransferase, α2,6-sialyltransferase (ST6GalI), α2,3-sialyltransferase (ST3GalIII), sialic acid synthase (SAS) and CMP-sialic acid synthetase (SMP-SAS). Three piggyBac-bsed vectors, each carries dual genes, are used to engineer the N-glcyosylation process pathway. Finally, the three transgenic lines will be crossed to combine all six genes in one transgenic line. The purpose of this study is to develop insect line expressing SAS and CMP-SAS.

Sialic acids are typically found as terminal residues on the N-glycans of mammalian cells. These residues play an important role in glycoprotein biology and involved in cell-cell interaction, immunological reactions and clearance of circulating glycoproteins (Marchal *et al.*, 2001). The mammalian biosynthesis pathway of sialic acid and CMP-NeuAc (reviewed by Schauer and Corfield 1982) involves series of biochemical reactions which start with phosphrylation of N-acetylmannosamine by N-acetylmannosamine kinase and end by the

product N-acetylneuraminic acid, the most common form of sialic acid which, finally, is activated by CMP-N-acetylneuraminic acid synthetase (CMP-SAS) to produce CMP-N-acetylneuraminic acid. In either intact insect or insect cell lines, mostly but not all, the endogenous glycoproteins lack sialic acids. Previous results from studies on different insect orders and cell lines show inconsistency of detecting sialic acids within the insect cells. In *Drosophila melanogaster*, sialic acids have been detected by cytochemistry using LFA (Limax flavus agglutinin) and by a combination of gas-liquid chromatography and electron-impact mass spectrometry (Roth *et al.*, 1992). Using immuno-blotting analysis, anti-polysialic acid antibody demonstrated that endogenous sialic acids are expressed only in embryonic developmental stage between 14 and 18 hours. Such observation indicates that sialic acids expression in Drosophila is a developmental stage-specific fashion. However, the transgenic *Drosophila melanogaster* flies, produced in the current study, are constitutively expressing both SAS and CMP-SAS. The flies are capable to salvage the sialic acids during larval stage when the sugar precursor, N-acetylmannosamine, is supplemented in the insect diet. Glycoprotein sialylation occurs in the Golgi apparatus. Two putative CMP-sialic acid transporter genes have been identified in *Drosophila* (Ferraz *et al.*, 1999). It is predicted that CMP-sialic acid, expressed in transgenic flies, is transported into the Golgi. Sialylation of N-glycan with sialic acid residues require active transferases and that is presented in our next article somewhere else in the current volume.

ACKNOWLEDGEMENTS

We would like to thank Dr. Donald Jarvis, University of Wyoming, for kindly providing plasmid encoding SAS and CMP-SAS for this work. We gratefully acknowledge financial support from the National Institutes of Standards and Technology through an Advanced Technology Program grant to Chesapeake-PERL, Inc.

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الملخص العربي

التحول الوراثى للخلايا الجرثومية فى درسوفيلا ملانوجاستر لانتاج الأنزيمين الثديين سليك أسيد سنثيز و س.م.ب. سليك أسيد سنثيتيز

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كثيرا ما تستخدم ناقلات التعبير الجينى الباكولوفيرس التى تعتمد على الحشرات فى أنتاج الجليكوبروتين و غالبا ما تتضمّن هذة الجليكوبروتينات منتجات تستخدم في أنتاج بعض ألأدوية. نتيجة لأختلاف طرق معالجة البروتينات بالمواد الكربوهيدراتية فى أجسام الحشرات عنها فى الثدييات وبالتالى يكون تخليق هذا النوع من البروتينات محدود جدا فى الباكولوفيروس والتى تنتج جليكوبروتينات غير كاملة النشاط الحيوى. الدراسة الحاليّة هى جزء من سلسلة تجارب لأنتاج ذبابة فاكهه محوله وراثيا لها القدرة على معالجة الجليكوبروتينات كما يتم معالجتها فى الثدّييات. تم أجراء عدة تجارب تحول وراثى على سلالة طفرة العين البيضاء [و١١١٨] من ذبابة الفاكهة *درسوفيلا ملانوجاستر* بأستخدام العنصر القافز *بيجى باك* المحتوى على شظايا الحمض النووى الذى يرمز الى كل من الأنزيميين الثديين سليك أسيد سنثيز وس.م.ب. سليك أسيد سنثيتيز تحت تأثير العنصر الحافز المستمر المزدوج [إي١-هر٥-ي١]. أنتجت تجارب التحول الوراثى ست ذبابات إيجابيّة تظهرن الوميض الأصفر فى أعينهن عند تعرض أجسامهن للأشعة فوق البنفسجية. تم أخضاع الأجيال المستنبطة من هؤلاء الأفراد الستة الى أجراء تجارب تحليلية للتأكد من أن هذه الأفراد محولة وراثيا عن طريق العنصر القافز *بيجى باك* وتنتج الأنزيمات الثديية داخل أجسامها. أثبتت تجارب تهجين ساثرن وتفاعل السلسلة المتبلمر المعكوس وقراءة التتابع النيوكلتيدى للحمض النووى أن على الأقل أثنين من الأفراد الستة محولين وراثيا عن طريق *بيجى باك*.