

Short Communication

SCREEN FOR DOMINANT BEHAVIORAL MUTATIONS CAUSED BY GENOMIC INSERTION OF P-ELEMENT TRANSPOSONS IN *DROSOPHILA*: AN EXAMINATION OF THE INTEGRATION OF VIRAL VECTOR SEQUENCES

**LYLE E. FOX^a, DAVID GREEN^a, ZIYING YAN^b,
JOHN F. ENGELHARDT^b, and CHUN-FANG WU^c**

^aDepartment of Biological Sciences, University of Iowa,
Iowa City, Iowa

^bDepartment of Anatomy and Cell Biology, and Center
for Gene Therapy of Cystic Fibrosis and other Genetic
Diseases, Carver College of Medicine, University of
Iowa, Iowa City, Iowa

^cDepartment of Biological Sciences and Center for Gene
Therapy of Cystic Fibrosis and other Genetic Diseases,
Carver College of Medicine, University of Iowa, Iowa
City, Iowa

Received 1 November 2006; accepted 4 December 2006.

We would like to thank Jan Pettus and Wayne A. Johnson for assistance in producing the transgenic *Drosophila*. In addition we are grateful to James Brown, Katie Hanson, Kristin Hart, Catherine Jancila, Jisue Lee, Catie Sanders, Xiaotian Zhong, and Hongyu Ruan, for help screening the transgenic flies. This work was supported by NIH POI 18577 and University of Iowa Center for Gene Therapy P30 DK54759 to CFW, and NIH ROI HL 58340 to JFE.

Address correspondence to Lyle E. Fox, Department of Biological Sciences, University of Iowa, 231 Biology Building, Iowa City, Iowa, 52242. E-mail: lyle-fox@uiowa.edu

Here we report the development of a high-throughput screen to assess dominant mutation rates caused by P-element transposition within the *Drosophila* genome that is suitable for assessing the undesirable effects of integrating foreign regulatory sequences (viral cargo) into a host genome. Three different behavioral paradigms were used: sensitivity to mechanical stress, response to heat stress, and ability to fly. The results, from our screen of 35,000 flies, indicate that mutations caused by the random insertion of transposons in *Drosophila* are more effective at disrupting flight than stress sensitivity. This approach was used to ascertain the frequency of deleterious dominant mutations caused by viral vectors utilized in gene therapy.

Keywords: Adeno Associated Virus (AAV); Complex behaviors; Cytomegalovirus (CMV); Gene therapy; Mutagenesis; Simian Virus-40 (SV-40); Stress sensitivity

INTRODUCTION

Genetic techniques that deliver foreign genes into organisms are being used with increasing frequency by scientists to study the function of specific genes (Palmiter & Brinster, 1986, Janson et al., 2001 Duffy, 2002; Washbourne & McAllister, 2002) and by physicians to correct deficits or slow the progression of disease (Thomas et al., 2003; Verma & Weitzman, 2005). The vectors used to deliver these genes (transposons or viruses) have the potential to integrate into the host genome. This integration, however, has several potentially serious risks inherent in the process including the mutagenesis of critical host gene sequences or altered transcriptional regulation. These deleterious effects can be caused by a "loss-of-function" in which the transcription of the gene or the function of its protein product is reduced or eliminated, and a "gain-of-function" in which the transcription of the gene or the function of its protein product is increased or altered. In the most severe case, a dominant mutation, the disruption of one copy of a gene in a diploid genome is sufficient to cause serious functional deficits or even death. Because of the severity of dominant mutations, we were interested in developing a high-throughput model system, using P-element transformation in the fruit fly *Drosophila melanogaster* that is capable of assessing the frequency of dominant mutations. We were able to screen for dominant mutations in over 35,000 independent candidates with a limited budget and in a relatively short period of time.

This system also has the potential to determine whether specific gene sequences have detrimental effects. Most current gene therapy strategies rely on viral vectors to facilitate gene transfer. These virally derived elements such as promoter/enhancer sequences, long-terminal repeats (LTRs), and/or inverted terminal repeats (ITRs) are essential components of these vectors, and impart transcriptional activity to the transgene cassette. The harmful effects that may result from the insertion of such elements have been difficult to quantify. We constructed P-element transposons (Pirrotta, 1988) carrying elements from the recombinant adeno-associated virus (rAAV) proviral genome (Duan et al., 2000) to investigate the frequency of dominant mutations caused by the insertion of viral sequences. Two advantages of using this system are that the integration of P-elements with the viral cargo can be verified visually when they encode an endogenous *Drosophila* eye color gene as a marker and that rare dominant mutations generated by the P-elements can be identified readily with high-throughput behavioral screens, such as sensitivity to mechanical stress (Ganetzky & Wu, 1982), response to heat stress (Suzuki et al., 1971), and ability to fly (Green et al., 1986). These phenotypes are potentially controlled by multiple complex networks of highly regulated genes and provide a sensitive method for not only identifying new genes involved in these processes, but also quantifying mutational rates caused by certain vector sequences.

MATERIALS AND METHODS

Transgenic *Drosophila*

Construction of rAAV super-enhancer constructs containing the AV ITRs and SV40/CMV enhancer sequences was previously described (Duan et al., 2000). The rAAV super-enhancer constructs were cloned into P-element transformation vectors, pCaSpeR3 and pCaSpeR4 (Pirrotta, 1988), carrying the *Drosophila* ITRs required for integration into the *Drosophila* genome and a cDNA for an eye color gene, mini-white (w^+), as a reporter (Figure 1D).

Transgenic lines of *Drosophila* carrying the control P-element or the P-element construct carrying the rAAV ITRs and SV40/CMV enhancer sequences were generated using standard P-element-mediated transformation techniques (Rubin & Spradling, 1982). Briefly, w^{1118} embryos were coinjected with a solution containing one of the experimental constructs (0.5 $\mu\text{g/ml}$) and the $\Delta 2-3$ transposase helper plasmid

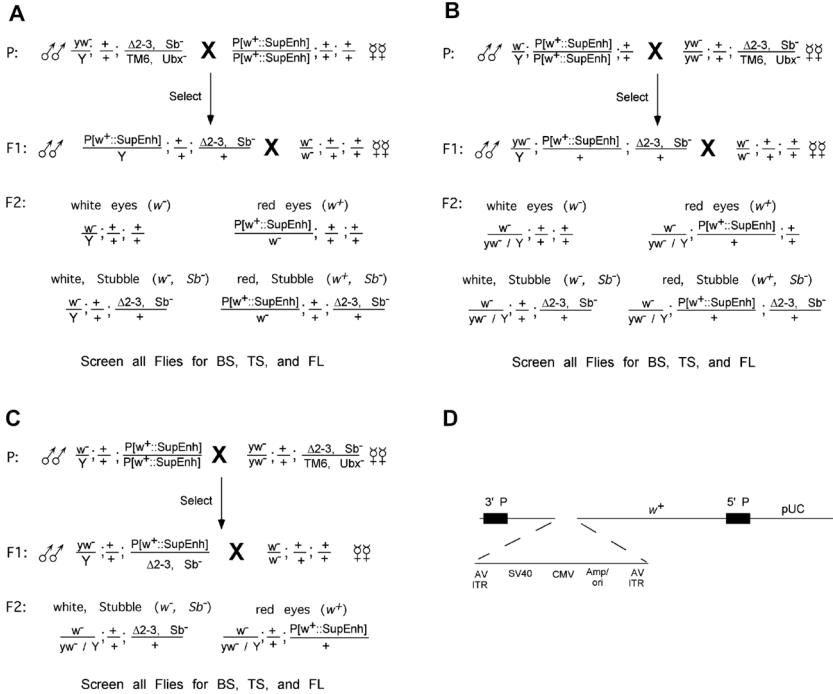


Figure 1. Construct design and crossing schemes for mobilization of P-elements. A–C. Crossing schemes for mobilization of P-elements from the first (A), second (B), or third (C) chromosome. P-elements were mobilized by mating flies from the transgenic lines to flies with a stable insertion of $\Delta 2-3$ transposase on the third chromosome. F1 males, carrying both the P-element (red eyed, w^+) and the $\Delta 2-3$ transposase (stubble bristles, Sb^-), were backcrossed to virgin w^{1118} females. The progeny of this cross were screened for dominant mutations using three behavioral paradigms, sensitivity to mechanical stimulation (BS), response to heat (TS), and ability to fly (FL). D. Two experimental constructs were prepared, the control transformation vectors (pCaSpeR3 or pCaSpeR4) and the recombinant adeno-associated virus (rAAV) super-enhancer constructs which consisted of AV inverted terminal repeats (ITR), the Simian virus-40 (SV40) and the cytomegalovirus (CMV) enhancer elements cloned into the control transformation vectors. Transgenic founders were created by injection of w^{1118} embryos with one of experimental constructs and the $\Delta 2-3$ transposase helper vector, necessary for the excision of the P-element from pCaSpeR and its integration into the host genome.

(0.25 $\mu\text{g}/\text{ml}$), which provides the necessary transposase activity for the integration as well as the excision of the P-element, dissolved in 0.1 mM NaH_2PO_4 , 5 mM KCl, pH 7.8. The rAAV super-enhancer and control constructs were devoid of the transposase sequence, and

the $\Delta 2-3$ helper construct carried the transposase sequence but lacked functional P-element ITRs necessary for genomic integration (Karess & Rubin, 1984). Embryos that survived and became adults were backcrossed to w^{1118} flies. The F_1 progeny were screened for red eyes, indicating the presence of the w^+ reporter gene and successful transformation. Each red-eyed F_1 fly was considered an independent transformant line and backcrossed to w^{1118} for line stabilization.

P-Element Mobilization

The crossing scheme for P-element inserts on the first, second, or third chromosomes is diagramed in Figure 1. P-elements were mobilized by mating males from the transgenic lines carrying the constructs on the second or third chromosome to virgin females with a stable insertion of $\Delta 2-3$ transposase on the third chromosome $y w^-$; $\Delta 2-3$, $Sb^-/TM6$, Ubx^- (Robertson et al., 1988) (Figure 1B and C). Virgin females were used from lines carrying the constructs on the first chromosome in order to generate F_1 males of the correct genotype (Figure 1A). F_1 males, carrying both the P-element (red eyed, w^+) and the $\Delta 2-3$ transposase (stubble bristles, Sb^-), were backcrossed to virgin w^{1118} females. The progeny of this cross were sorted according to visible markers and screened for dominant mutations using three behavioral paradigms (see Behavioral Screening). Transgenic lines carrying the P-element construct on the third chromosome were used to estimate the translocation rate of the P-element to another chromosome. For these lines, the eye color (w^+) and bristle (Sb^-) markers on homologous chromosomes should segregate independently (Figure 1C). Therefore, co-segregation of these markers in the progeny is most likely caused by translocation of the P-element to another chromosome providing an estimate of the frequency of translocation.

Behavioral Screening

Three well-established behavioral screens were used to identify dominant mutations generated by P-element mobilization. Progeny from the crosses were first assayed for sensitivity to mechanical stimulation (Bang Sensitivity, BS) by vortexing them for 10 s using a bench-top vortex (Ganetzky & Wu, 1982). The sensitivity of the flies to high temperature (Temperature Sensitivity, TS) was tested by incubating flies at 37°C for 5 mins (Suzuki et al., 1971). Using these tests, several mutant strains of flies have seizures or are immobilized, whereas wild-type are not.

Flightless flies were selected using a non-destructive flight-testing column (Green et al., 1986). Flies that were between 2 and 10 days old were introduced into the column from a funnel at the top and flightless flies were collected from the bottom. Flies were flight tested at least 3 times. Flies that failed three flight tests were characterized as “flightless” (FL).

RESULTS AND DISCUSSION

In the present study, we evaluated how random mobilization of a P-element transposon affected readily detectable behavioral phenotypes in *Drosophila*. In addition, we investigated whether the rAAV super-enhancer proviral genome containing the SV40 and CMV enhancers could alter the frequency of behavioral phenotypes. These two viral transcriptional elements are commonly used in gene therapy applications. Additionally, the CMV promoter/enhancer has been shown to be transcriptionally active in *Drosophila* (Sinclair, 1987). Initially, two experimental constructs were prepared, the control P-element transformation vector and the P-element construct carrying the rAAV ITRs and SV40/CMV enhancer sequences (Duan et al., 2000) (Figure 1D). Transgenic lines of *Drosophila* were generated in order to obtain a stable source of the constructs for generating new P-element insertions. Using standard P-element-mediated germ-line transformation techniques (Rubin & Spradling, 1982), we obtained 19 independent transgenic lines with stable integration of the constructs on the 1st, 2nd, and 3rd chromosomes of the host genome: 12 containing the AAV super-enhancer constructs and 7 containing the control P-element without viral DNA.

To initiate new insertions, the P-elements from these transgenic *Drosophila* lines were re-mobilized by crossing with the $\Delta 2-3$ helper line to create a large number of progeny, each potentially carrying the P-element at a new insertion site for high-throughput behavioral screening (Figure 1). Multiple transgenic lines with different initial insertion sites were used because new insertions tend to cluster around the original site (Tower et al., 1993). The minimum translocation rate of the P-elements to another chromosome was estimated to be in the range of 10%, by following the segregation of visible markers from maternal and paternal homologous third chromosomes (see MATERIALS AND METHODS). This rate of inter-chromosomal transposition was comparable to previously published rates (Robertson et al., 1988). The somatic mutation rate was presumably high as nearly all of the flies expressing both markers had a mottled eye color.

We tested $\sim 35,000$ heterozygous flies using the behavioral screens, 19,980 carrying the rAAV super-enhancer construct and 14,230 with the control construct lacking rAAV sequences. Only a few lines produced stress-sensitive (BS or TS) or flightless (FL) flies at higher frequencies than their parental w^{1118} strain (Table I) suggesting that the initial insertion site of individual transgenic lines had a larger effect on the occurrence of dominant mutants than the cargo. For example, the insertion site may be on or near a “hot spot” for generating stress sensitive or FL mutations. The observed dominant mutations were not caused by a general weakness of the parental transgenic flies because none of the lines performed poorly on all of the behavioral tests. In pooled data from all of the transgenic lines, the rAAV cargo had little effect on the mutation rate for any of the behavioral paradigms (Table I). The frequency of stress-sensitive and FL flies was also similar for red- and white-eyed flies, corresponding to lines carrying the rAAV super-enhancer construct vs. those without, respectively (Table II).

The potential stress-sensitive and FL flies were mated in order to generate stable lines. In all, 476 out of the 835 potential mutants successfully reproduced, with 12 stable FL lines being established from flies carrying the rAAV super-enhancer construct and 5 lines from those carrying the control construct. The frequency of stable FL lines was similar for the control construct ($0.05 \pm 0.03\%$) and the AAV super-enhancer constructs ($0.08 \pm 0.03\%$). However, none of the flies exhibiting stress sensitivity established stable lines. Our results suggest that it is more difficult to generate dominant mutants for simple behaviors like stress sensitivity than for complex behaviors like flight that require the exact coordination of many different components and probably the interaction of extended networks of genes.

Understanding the nature of dominant mutations is critical because of their ability to cause serious functional deficits or death after the disruption of only a single copy of a gene. Recently, retrovirus-mediated gene therapy for X-linked severe combined immunodeficiency (X-SCID) has been hindered by the induction of a leukemia-like disease in 3 out of 17 treated patients (Hacein-Bey-Abina et al., 2003; Couzin & Kaiser, 2005). The X-SCID clinical trials raised important questions about the frequency of deleterious mutations produced by alterations in gene transcription caused by vector insertion and demonstrated a need for a comprehensive understanding of the biology of viral and non-viral vectors. To this end, it is important to develop systems for studying

Table 1. Frequency of dominant behavioral mutants for heterozygous flies from transgenic lines carrying the AAV super-enhancer or control constructs

Line	#Tested	Stress sensitivity (BS + TS) ^{a,c}		Flight failures (FL) ^{b,c}	
		White eyes ^d	Red eyes ^d	White eyes ^d	Red eyes ^d
AAV Constructs/$\Delta 2-3$ transposase					
1146.1-1	2,068	0.0 ± 0.0 (0) ^c	0.04 ± 0.04 (1)	4.8 ± 3.9 (40)	3.7 ± 2.5 (28)
1146.1-8	2,221	0.21 ± 0.15 (2)	0.06 ± 0.06 (1)	6.4 ± 3.5 (56)	2.4 ± 0.6 (25)
1146.1-9	2,452	0.0 ± 0.0 (0)	0.03 ± 0.03 (1)	3.2 ± 0.9 (33)	0.9 ± 0.5 (15)
1149.13-3	2,002	0.65 ± 0.21 (10) ^{*,d,f}	0.78 ± 0.37 (7)*	1.0 ± 0.2 (13)	2.5 ± 1.3 (11)
1149.16-5	2,331	0.13 ± 0.13 (2)	0.27 ± 0.18 (2)	2.2 ± 0.5(33)	2.3 ± 0.5 (17)
1149.16-5A	2,160	0.09 ± 0.09 (1)	0.21 ± 0.13 (2)	5.0 ± 1.5 (70)	6.5 ± 1.8 (61)*
1149.26-2	2,266	0.0 ± 0.0 (0)	0.0 ± 0.0 (0)	1.3 ± 0.7 (17)	3.0 ± 1.3 (19)
Totals	15,500	0.15 ± 0.05 (15)	0.19 ± 0.07 (14)	3.4 ± 0.8 (262)	3.0 ± 0.6 (176)
Control Constructs/$\Delta 2-3$ transposase					
1166.1-2	2,154	0.0 ± 0.0 (0)	0.0 ± 0.0 (0)	3.8 ± 0.8 (57)	3.2 ± 1.8 (17)
1166.4-2	2,209	0.0 ± 0.0 (0)	0.06 ± 0.06 (1)	3.8 ± 0.7 (36)	4.0 ± 0.9 (37)
1166.9-3	2,426	0.28 ± 0.12 (5)	0.63 ± 0.38 (5)	2.0 ± 0.4 (32)	0.6 ± 0.3 (5)
1166.15-1	2,235	0.47 ± 0.14 (6) ^{**}	0.19 ± 0.19 (3)	6.3 ± 2.3 (74)	5.4 ± 1.7 (53)
1166.15-3	2,058	0.13 ± 0.08 (3)	0.22 ± 0.15 (4)	3.3 ± 1.7 (24)	5.0 ± 1.9 (51)
Totals	11,082	0.17 ± 0.05 (14)	0.22 ± 0.09 (13)	3.8 ± 0.7 (223)	3.8 ± 0.7 (163)
AAV Constructs/<i>w¹¹¹⁸</i>					
1146.1-8	1,159	n.a. ^g	0.11 ± 0.11 (2)	n.a.	1.3 ± 0.04 (15)
1149.13-3	1,017	n.a.	0.08 ± 0.08 (1)	n.a.	1.0 ± 0.2 (11)
1149.16-5	1,268	n.a.	0.32 ± 0.32 (5)	n.a.	1.1 ± 0.5 (12)
1149.26-2	1,036	n.a.	0.08 ± 0.08 (1)	n.a.	0.8 ± 0.5 (6)
Totals	4,480	n.a.	0.15 ± 0.08 (9)	n.a.	1.0 ± 0.2 (44)

Control Constructs/ w^{1118}					
1166.9-3	1,148	n.a.	0.18 ± 0.10 (2)	n.a.	0.6 ± 0.2 (6)
1166.15-1	949	n.a.	0.0 ± 0.0 (0)	n.a.	0.5 ± 0.4 (3)
1166.15-3	1,051	n.a.	0.0 ± 0.0 (0)	n.a.	0.7 ± 0.3 (9)
Totals	3,148	n.a.	0.06 ± 0.4 (2)	n.a.	0.6 ± 0.2 (18)
Parental Lines					
w^{1118}	2,161	0.07 ± 0.04 (2)	n.a.	2.0 ± 0.8 (22)	n.a.
$w^{w^{1118}, \Delta 2-3, Sb-1} / w^{1118, \Delta 2-3, Sb-1} / w^{1118, \Delta 2-3, Sb-1} / w^{1118, \Delta 2-3, Sb-1}$	1,071	0.0 ± 0.0 (0)	n.a.	11.3 ± 2.1 (122) ^{***f}	n.a.

^aMechanical and temperature stresses were used to test for dominant mutant flies. Stress sensitivity includes both the bang sensitive (BS) and temperature sensitive (TS) flies.

^bFlightless (FL) flies were selected using a non-destructive flight-testing column.

^cStress-sensitive and FL flies were mated to the parental w^{1118} strain. In all, 476 out of the 835 potential mutants successfully reproduced, establishing 12 stable FL lines (6 white eyes; 6 red) from flies carrying the rAAV super-enhancer construct, 5 lines (white eyes) from those carrying the control construct, and 1 line from the parental w^{1118} strain. The rest were too weak to propagate.

^dRed-eyed flies carry P-elements containing either the rAAV super-enhancer or control construct. White-eyed flies do not carry the constructs. ^eAverage frequency ± SEM for the 5 batches of flies screened for each line. The number of stress-sensitive or Flightless flies identified for each line is in parenthesis.

^fThe frequency of stress-sensitive and FL flies was calculated independently for each batch tested (n = 5 for AAV Constructs/ $\Delta 2-3$ transposase, Control Constructs/ $\Delta 2-3$ transposase, and Parental Lines; n = 3 for AAV Constructs/ w^{1118} and Control Constructs/ w^{1118}). All lines were initially screened using single factor analysis of variance to detect differences between lines (BOLD). Results were verified with one-tailed Student's t-test (***) means $p \leq 0.001$, ** means $p \leq 0.01$, * means $p \leq 0.05$.

^gn.a. means "not applicable." There were no flies with these markers.

Table II. Frequency of dominant behavioral mutants for flies carrying the P-element (w^+) or transposase (Sb^-) Markers^a

Phenotype	#Flies	BS ^b	TS ^c	FL ^d	Stable FL Lines ^e
AAV constructs					
w^-, Sb^+	2,906	0.12 ± 0.05 (6) ^f	0.03 ± 0.02 (2)	3.1 ± 1.5 (72)	0.04 ± 0.03 (2) ^g
w^-, Sb^-	5,936	0.11 ± 0.06 (6)	0.02 ± 0.02 (1)	3.4 ± 0.8 (190)	0.08 ± 0.05 (5)
w^+, Sb^+	4,942	0.14 ± 0.07 (7)	0.05 ± 0.05 (2)	3.3 ± 0.8 (130)	0.04 ± 0.03 (2)
w^+, Sb^-	1,714	0.19 ± 0.09 (5)	0.0 ± 0.0 (0)	2.3 ± 0.5 (46)	0.12 ± 0.09 (3)
Totals	15,500	0.14 ± 0.05 (24)	0.03 ± 0.01 (5)	3.1 ± 0.6 (438)	0.08 ± 0.03 (12)
Control constructs					
w^-, Sb^+	2,205	0.16 ± 0.08 (4)	0.06 ± 0.05 (2)	4.2 ± 1.0 (88)	0.21 ± 0.09 (5)
w^-, Sb^-	3,681	0.08 ± 0.05 (4)	0.03 ± 0.03 (1)	3.7 ± 0.5 (130)	0.0 ± 0.0 (0)
w^+, Sb^+	2,695	0.10 ± 0.07 (3)	0.07 ± 0.05 (2)	4.0 ± 0.8 (99)	0.0 ± 0.0 (0)
w^+, Sb^-	1,349	0.13 ± 0.11 (4)	0.06 ± 0.06 (1)	3.5 ± 1.2 (57)	0.0 ± 0.0 (0)
Totals	9,930	0.12 ± 0.05 (15)	0.06 ± 0.03 (6)	4.1 ± 0.7 (347)	0.05 ± 0.03 (5)

^aSomatic mutations could contribute to the behavioral phenotype of flies carrying the transposase.

^bBS means bang sensitivity.

^cTS means temperature sensitivity.

^dFL means flightless flies.

^eStable flightless lines established from mating the flies that failed the flight test. In all, 468 out of the 785 potential flight mutants successfully mated, with 12 stable FL lines being established from flies carrying the AAV super-enhancer construct and 5 lines from those carrying the control construct.

^fAverage frequency ± SEM for the 5 batches of flies screened for each line. The number of BS, TS, or FL flies identified for each genotype is in parenthesis.

^gThe number of stable FL lines is in parenthesis.

the effects of integration and expression of genetic material in model organisms. *Drosophila* is one such organism that allows effective screening of large populations of progeny with vector sequences integrated at different sites of the host genome. Transposons might also provide important genetic tools for functional genomic screens to examine this issue in other systems. They have the potential to delineate the effects of virally derived DNA sequences integrated into the host genome amongst complicating factors such as targeting, efficiency of integration, and immune reactions, that sometimes obscure results from viral vectors (Thomas et al., 2003). The mobilization of the Sleeping Beauty transposon harboring MSCV (murine stem cell virus) LTR has been used successfully to induce embryonic death and several types of cancer in mice leading to the discovery of unknown, as well as previously identified putative cancer genes (Collier et al., 2005; Dupuy et al., 2005).

Although our current study is limited to non-lethal mutations, these experiments do provide a first-order measure of the mutagenic efficiency caused by transcriptional elements contained within rAAV proviral genomes. The data indicated no compelling evidence that that transcriptional elements or ITR sequences from rAAV vector increase the rate of mutagenesis, or that these sequences preferentially interacted with specific subcategories of genes in *Drosophila*. It did, however, demonstrate that complex behaviors could be used as a sensitive and rapid screen for dominant mutations. In the future, as vectors with better coverage of the genome such as *piggyBack* become available and as libraries of transgenic flies with transposons inserted in identified genes are completed (Bellen et al., 2004), this model system may become a valuable tool for quantifying the risks involved in the integration of different virally-derived vectors into any locus in the *Drosophila* genome or the mutagenic effects of mobilizing P-elements from any gene of interest.

REFERENCES

- Bellen, H.J., Levis, R.W., Liao, G., He, Y., Carlson, J.W., Tsang, G., Evans-Holm, M., Hiesinger, P.R., Schulze, K.L., Rubin, G.M., Hoskins, R.A., & Spradling, A.C. (2004). The BDGP gene disruption project: single transposon insertions associated with 40% of *Drosophila* genes. *Genetics* **167**, 761–781.
- Collier, L.S., Carlson, C.M., Ravimohan, S., Dupuy, A.J., & Largaespada, D.A. (2005). Cancer gene discovery in solid tumours using transposon-based somatic mutagenesis in the mouse. *Nature* **436**, 272–276.

- Couzin, J. & Kaiser, J. (2005). Gene therapy. As Gelsinger case ends, gene therapy suffers another blow. *Science* **307**, 1028.
- Duan, D., Yue, Y., Yan, Z., & Engelhardt, J.F. (2000). A new dual-vector approach to enhance recombinant adeno-associated virus-mediated gene expression through intermolecular cis activation. *Nat. Med.* **6**, 595–598.
- Duffy, J.B. (2002). GAL4 system in *Drosophila*: a fly geneticist's Swiss army knife. *Genesis* **34**, 1–15.
- Dupuy, A.J., Akagi, K., Largaespada, D.A., Copeland, N.G., & Jenkins, N.A. (2005). Mammalian mutagenesis using a highly mobile somatic Sleeping Beauty transposon system. *Nature* **436**, 221–226.
- Ganetzky, B. & Wu, C.F. (1982). Indirect suppression involving behavioral mutants with altered nerve excitability in *Drosophila melanogaster*. *Genetics* **100**, 597–614.
- Green, C.C., Sparrow, J.C., & Ball, E. (1986). Flight testing columns. *Drosophila Inform. Serv.* **63**, 141.
- Hacein-Bey-Abina, S., Von Kalle, C., Schmidt, M., McCormack, M.P., Wulffraat, N., Leboulch, P., Lim, A., Osborne, C.S., Pawliuk, R., Morillon, E., Sorensen, R., Forster, A., Fraser, P., Cohen, J.I., de Saint Basile, G., Alexander, I., Wintergerst, U., Frebourg, T., Aurias, A., Stoppa-Lyonnet, D., Romana, S., Radford-Weiss, I., Gross, F., Valensi, F., Delabesse, E., Macintyre, E., Sigaux, F., Soulier, J., Leiva, L. E., Wissler, M., Prinz, C., Rabbitts, T.H., Le Deist, F., Fischer, A., & Cavazzana-Calvo, M. (2003). LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. *Science* **302**, 415–419.
- Janson, C.G., McPhee, S.W., Leone, P., Freese, A., & During, M.J. (2001). Viral-based gene transfer to the mammalian CNS for functional genomic studies. *Trends Neurosci.* **24**, 706–712.
- Karess, R.E. & Rubin, G.M. (1984). Analysis of P transposable element functions in *Drosophila*. *Cell* **38**, 135–146.
- Palmiter, R.D. & Brinster, R.L. (1986). Germ-line transformation of mice. *Annu. Rev. Genet.* **20**, 465–499.
- Pirrotta, V. (1988). Vectors for P-mediated transformation in *Drosophila*. In (R.L. Rogregez & D.T. Dengardt (Eds.), *Vectors: A Survey of Molecular Cloning Vectors and their Uses*, pp. 437–456, Butterworths: Boston.
- Robertson, H.M., Preston, C.R., Phillis, R.W., Johnson-Schlitz, D.M., Benz, W.K., & Engels, W.R. (1988). A stable genomic source of P element transposase in *Drosophila melanogaster*. *Genetics* **118**, 461–470.
- Rubin, G.M. & Spradling, A.C. (1982). Genetic transformation of *Drosophila* with transposable element vectors. *Science* **218**, 348–353.
- Sinclair, J.H. (1987). The human cytomegalovirus immediate early gene promoter is a strong promoter in cultured *Drosophila melanogaster* cells. *Nucleic Acids Res.* **15**, 2392.

- Suzuki, D.T., Grigliatti, T., & Williamson, R. (1971). Temperature-sensitive mutations in *Drosophila melanogaster*. VII. A mutation (*para-ts*) causing reversible adult paralysis. *Proc. Natl. Acad. Sci. USA*. **68**, 890–893.
- Thomas, C.E., Ehrhardt, A., & Kay, M.A. (2003). Progress and problems with the use of viral vectors for gene therapy. *Nat. Rev. Genet.* **4**, 346–358.
- Tower, J., Karpen, G.H., Craig, N., & Spradling, A.C. (1993). Preferential transposition of *Drosophila* P-elements to nearby chromosomal sites. *Genetics*. **133**, 347–359.
- Verma, I.M. & Weitzman, M.D. (2005). Gene therapy: twenty-first century medicine. *Annu. Rev. Biochem.* **74**, 711–738.
- Washbourne, P & McAllister, A.K. (2002). Techniques for gene transfer into neurons. *Curr. Opin. Neurobiol.* **12**, 566–573.