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# *Minos* as a Genetic and Genomic Tool in *Drosophila melanogaster*

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ABSTRACT

Much of the information about the function of *D. melanogaster* genes has come from *P*-element mutagenesis. The major drawback of the *P* element, however, is its strong bias for insertion into some genes (hotspots) and against insertion into others (coldspots). Within genes, 5′-UTRs are preferential targets. For the successful completion of the Drosophila Genome Disruption Project, the use of transposon vectors other than *P* will be necessary. We examined here the suitability of the *Minos* element from *Drosophila hydei* as a tool for Drosophila genomics. Previous work has shown that *Minos*, a member of the *Tc1/mariner* family of transposable elements, is active in diverse organisms and cultured cells; it produces stable integrants in the germ line of several insect species, in the mouse, and in human cells. We generated and analyzed 96 *Minos* integrations into the Drosophila genome and devised an efficient "jump-starting" scheme for production of single insertions. The ratio of insertions into genes *vs.* intergenic DNA is consistent with a random distribution. Within genes, there is a statistically significant preference for insertion into introns rather than into exons. About 30% of all insertions were in introns and ~55% of insertions were into or next to genes that have so far not been hit by the *P* element. The insertion sites exhibit, in contrast to other transposons, little sequence requirement beyond the TA dinucleotide insertion target. We further demonstrate that induced remobilization of *Minos* insertions can delete nearby sequences. Our results suggest that *Minos* is a useful tool complementing the *P* element for insertional mutagenesis and genomic analysis in Drosophila.

ONE of the main goals of modern genetics is to link the many thousands of genes identified through the sequencing of whole genomes of model organisms to gene function. The most powerful technique for this purpose so far has been transgenesis with mobile elements. This technique is a means to disrupt, overexpress, or misexpress single genes to identify expression patterns and also to characterize genetic pathways and their interactions. One of the main advantages of insertional mutagenesis over the classical method of chemical mutagenesis is the ease with which the targeted gene can be identified, since it carries an inserted tag.

The *P* element was the first mobile element that enabled germ-line transformation of an insect species (Rubin and Spradling 1982). Since then, thousands of single *P*-element insertions causing lethality, semilethality, sterility, semisterility, and visible phenotypes have been created and analyzed in Drosophila (Cooley *et al.* 1988; Bier *et al.* 1989; Gaul *et al.* 1992; Karpen and Spradling 1992; Chang *et al.* 1993; Török *et al.* 1993; Spradling *et al.* 1995, 1999; Rorth 1996). Furthermore, *P*-element-based enhancer and gene-trapping strategies (O'Kane and Gehring 1987; Bellen *et al.* 1989, 2004; Wilson *et al.* 1989; Brand and Perrimon 1993; Lukacsovich *et al.* 2001; Morin *et al.* 2001; Bourbon *et al.* 2002) have underlined the value of transposon mutagenesis for genome-wide functional analysis.

No other insect species were transformed for 13 years after the germ-line transformation of Drosophila, mainly because efforts were based on the *P*-element vector, which was subsequently found to be inactive in non-drosophilids (Handler *et al.* 1993). It was in 1995 when *Minos*, an element isolated from *Drosophila hydei* and belonging to the *Tc1/mariner* superfamily, was found to transform the medfly *Ceratitis capitata* (Loukeris *et al.* 1995b), an insect of great economical importance. This was the first report of a transposable element able to transform a species belonging to a genus other than that of the original host of the element. Since then, several insect species have been transformed by this and other mobile elements, some of which are active in organisms very phylogenetically distant (for review see Handler 2001). Interestingly, at least some members of the *Tc1/mariner* superfamily of transposable elements do not require any host-specific factors for transposition, since purified transposase is sufficient to catalyze *in vitro*

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transposition (Lampe *et al.* 1996; Vos *et al.* 1996). This property makes them potentially active in all organisms.

Despite the current existence of a diverse arsenal of transposable elements that can be used for the transformation of different species, certain features, such as efficiency of transposition and preference for integration into certain euchromatic regions, have to be considered for selection of the most appropriate transposon for functional genomic analysis. For example, although the *mariner* element is active in a broad spectrum of species, ranging from microorganisms (Gueiros-Filho and Beverley 1997) to human cells (Fadool *et al.* 1998; Sherman *et al.* 1998; Zhang *et al.* 1998), its transposition efficiency in Drosophila species is very low in comparison to other mobile elements with a more restricted spectrum, such as *P* and *hobo* (Garza *et al.* 1991; Lidholm *et al.* 1993; Lohe and Hartl 1996). Furthermore, different elements show distinctive intragenic preferences for integration. The *P* element inserts preferentially into the 5′-UTRs of genes in Drosophila (Spradling *et al.* 1995), while *Sleeping Beauty* has a preference for introns in human cells (Vigdal *et al.* 2002). The genomic insertional bias of the *P* element is such that integration preference is strong for some genes (hotspots), very low for the majority of genes (coldspots), and intermediate for a third group of loci (warmspots). This bias makes the mutagenesis of the entire Drosophila genome by the *P* element alone problematic. Therefore, the *piggyBac* element, which does not exhibit the same bias (Hacker *et al.* 2003), has recently been employed for the Drosophila gene disruption project and is greatly advancing its progress (Thibault *et al.* 2004).

In the context of functional genomic analysis we have further characterized the potential of the transposon *Minos* from *D. hydei* (Franz and Savakis 1991). *Minos* is a member of the *Tc1/mariner* family of transposable elements with 255-bp long terminal inverted repeats, which flank a single gene encoding transposase. The *Minos* transposase has been shown to catalyze, in most cases, precise excision and integration of the element without involvement of flanking DNA (Loukeris *et al.* 1995a; Arca *et al.* 1997). Most excision events either are precise (*i.e.*, the original, preinsertion sequence is restored) or leave behind a characteristic 6-bp footprint, consisting of four terminal nucleotides of the *Minos* element followed by the duplicated TA, which is generated by the element upon insertion; complex events involving partial loss of the element are rare (Arca *et al.* 1997). The *Minos* element is active in insect and mammalian cells in culture and leads to stable insertions into germ-line chromosomes of embryos of several insect species (Loukeris *et al.* 1995a,b; Catteruccia *et al.* 2000a,b; Klinakis *et al.* 2000a; Shimizu *et al.* 2000; Pavlopoulos *et al.* 2004) and of ascidians (Sasakura *et al.* 2003). It is also functional in somatic and germ cells of mice (Zagoraiou *et al.* 2001; Drabek *et al.* 2003). The wide range of host organisms that permit transposition of this element and the fact that transposition produces stable transformants with high efficiency (Kapetanaki *et al.* 2002), allowing genome-wide mutagenesis in mammalian cells (Klinakis *et al.* 2000b), suggests that it is a versatile tool for functional genomic analysis.

In this work, the ability of *Minos* transposons to insert into *Drosophila melanogaster* genes that have not been mutagenized by the *P* element is demonstrated, as is a preference of *Minos* to target introns. In contrast to other elements of the same family, *Minos* does not seem to have a strong preference for DNA sequences with certain primary motifs and its preferred insertion sites appear to have predicted physical properties that differ from those of other members of the *Tc1/mariner* superfamily. We also demonstrate the ability of *Minos* to produce deletions at the sites of integration upon remobilization in the germ line. In addition, a "jump-starting" scheme, efficiently producing reinsertions from the X chromosome to the autosomes, has been devised. We conclude that *Minos*-based mutagenesis has the properties required to approach saturation of the Drosophila genome with intragenic insertions useful for functional analysis.

## MATERIALS AND METHODS

**Plasmid constructions:** To generate pMiPR1, two annealed oligonucleotides containing *Kpn*I, *Sfi*I, *Bgl*II, *Xba*I, *Stu*I, *Eco*RV, *Sac*I, and *Ssp*I restriction sites were cloned between the *Hin*dIII and *Xma*I sites of vector pHSS6 (Seifert *et al.* 1986), resulting in pHSSK. The left *Minos* inverted repeat together with the 5′-UTR of *Minos* transposase and ~100 bp of flanking DNA from a *Minos* insertion in *D. hydei* were cloned into pHSSK as a *Cla*I-*Kpn*I fragment from pMiLRtetR (Klinakis *et al.* 2000a), resulting in pMiLori. Two other fragments of pMiLRtetR, an *Eco*RI-*Hin*dIII (blunted) fragment containing a tetracycline resistance gene and a *Sac*II-*Bst*NI (blunted) fragment containing the right *Minos* inverted repeat, with ~50 bp from *D. hydei* and 59 bp of the *Minos* transposase 3′-UTR, were cloned in the *Stu*I and *Ssp*I sites of pMiLori, respectively, resulting in pMiLRoriT. The enhanced green fluorescent protein (EGFP) gene flanked by the 3xP3 promoter and the SV40 polyadenylation signal was taken as an *Eco*RI-*Fse*I (blunted) fragment from plasmid pSL-3xP3-EGFP, which was kindly provided by E. Wimmer (Horn *et al.* 2000), cloned into *Eco*RI-*Sma*I-digested pBlueScript KSII+ (Stratagene, La Jolla, CA), and then recloned as an *Xba*I-*Xho*I fragment into pMiLRtetR, resulting in pMi3xP3-EGFP. An *Eco*RI-*Not*I fragment from pMi3xP3-EGFP, containing the eye-specific 3xP3-EGFP marker, was cloned into pMiLRoriT, resulting in transposon donor plasmid pMiPR1.

pMiET1 is based on transposon donor pMiPR1. The Gal4 gene, driven by the hsp70 minimal promoter and followed downstream by the hsp70 terminator, was amplified with Vent polymerase (New England Biolabs, Beverly, MA) from vector pGATN (Brand and Perrimon 1993). The primers contained an *Eco*RI site each, which were used to clone the PCR fragment into the unique *Eco*RI site of vector pMiPR1. Plasmid pPhsILMiT is a derivative of *P*-element vector pCaSper4 (Thummel and Pirrotta 1992), carrying in its unique *Not*I site a 2.3-kb *Not*I fragment from pHSS6hsILMi20 (Klinakis *et al.* 2000a), containing an intronless *Minos* transposase gene under control of the hsp70 promoter.

**Germ-line transformation and transposase mRNA synthesis:** Germ-line transformation was performed by microinjection of plasmid DNA or a mixture of plasmid DNA and RNA into *D. melanogaster* preblastoderm embryos of strain $yw^{67c23}$, as described (Rubin and Spradling 1982). Embryos were co-injected with 400 μg/ml of transposon donor and either 100 μg/ml of helper plasmid pHSS6hsMi2 (Loukeris *et al.* 1995a,b) or 100 μg/ml of *Minos* transposase mRNA, produced from vector pBS(SK)MimRNA (Pavlopoulos *et al.* 2004), using the message machine T7 kit (Ambion, Austin, TX), according to the manufacturer's instructions. $G_0$ males and females were individually backcrossed with four female or male flies, respectively. The $G_1$ progeny from these crosses were screened for green fluorescence of the eyes. Positive individuals were used to establish transgenic lines.

For the production of novel single insertions in flies, we used a so-called "jump-start" scheme (Cooley *et al.* 1988). We established a line producing *Minos* transposase from a balancer chromosome by co-injecting *D. melanogaster* embryos carrying the CyO balancer with helper plasmid Δ2-3 (Laski *et al.* 1986) and *P*-element-based plasmid pPhsILMiT. $G_1$ progeny that carried both the CyO balancer and the white gene were crossed individually to yw flies. Six lines that cotransmitted the balancer and the white marker gene were established.

**Plasmid rescue:** Purification of genomic DNA was after Holmes and Bonner (1973) and plasmid rescue was according to Pirrotta (1986). Genomic DNA was digested with *Bam*HI, *Xba*I, or double digested with *Xba*I and *Spe*I, diluted, and ligated. DH5α competent cells were transformed with the ligation products and plated onto Luria broth plates with kanamycin (25 μg/ml). Sanger sequencing was performed with primer IMio2 (Klinakis *et al.* 2000a).

**Computational analysis:** Analysis of the physical properties of *Minos* insertion sites was performed with the software by Liao *et al.* (2000). Flanking the TA insertion sites on either side, 50 bp each were aligned for 80 insertions and average values for GC content, DNA bendability, A-philicity, B-DNA twist, and protein-induced deformability were calculated. The values were predicted as previously described (Brukner *et al.* 1995; Gorin *et al.* 1995; Ivanov and Minchenkova 1995; Olson *et al.* 1998). H-bond view analysis was performed as previously described (Liao *et al.* 2000). The profiles were compared with those of 80 sequences randomly taken from the *D. melanogaster* genome, each centered around a TA dinucleotide. All calculations were performed using a 3-bp sliding window. For the determination of the consensus sequence of *Minos* insertions, 10 bp upstream and downstream of the TA insertion site were analyzed with the program SeqLogo (Schneider and Stephens 1990).

**Production and analysis of *Minos* excision events:** For the generation of excision events in the germ line of flies with single *Minos* insertions, flies homozygous for a MiET1 transposon insertion on the second chromosome were crossed with flies carrying helper chromosome PhsILMiT (cross 1). Two days after setting up the crosses, the flies were transferred to new vials and the old vials were heat-shocked daily for 1 hr in a 37° water bath until pupariation. Adults that expressed both the EGFP marker of the transposon and the white marker of the helper chromosome were crossed individually with flies carrying balancer chromosome SM6 over the marker Glaze (cross 2). Progeny with transposon excisions were identified as carrying the SM6 balancer but lacking the EGFP and white markers. One such fly was chosen per vial and crossed individually with flies carrying a chromosome 2 balancer over deficiencies covering the region of the initial transposon insertion (cross 3). DNA was extracted from flies that carried the chromosome with the excision event over the deficiency-carrying chromosome and used for PCR amplification of a 2-kb fragment centered around the TA insertion site. We analyzed excision events from introns of three different genes, CG4114 or *expanded* (AE003589.3, 132323 nt), CG5423 or *robo3* (AE003586.3, 55570 nt), and CG30497 (AE003840.3, 239589 nt). The deficiency-carrying flies were from the Bloomington Drosophila Stock Center. Df(2L)al, ds[al]/In(2L)Cy was used for excision events at CG4114, Df(2L)ast2/SM1 for excision events at CG5423, and Df(2R)NCX13/CyO for excision events at CG30497. The following three pairs of primers were used for the analytical PCR reactions:

1. Ex1: 5′ CGCTTGACAAACACACGCCC 3′ and Ex2: 5′ CG ATCGGACCGATCGGAGG 3′
2. Robo1: 5′ GCGTGCAGGAGCTCTTGCC 3′ and Robo2: 5′ AAGTGAGCAGTGGCAGGAAAG 3′
3. 30b1: 5′ TAAAGCCCGTGTGCCAAATGC 3′ and 30b2: 5′ CCATAGCCATACCCATACCAAG 3′.

PCR products that appeared larger or smaller than the expected 2 kb were cloned into vectors pBlueScript KSII+ (Stratagene) or PCRII (Invitrogen, San Diego) and sequenced.

## RESULTS

**Generation of *Minos* insertions in Drosophila:** Fifty-six Drosophila lines carrying *Minos* insertions were generated in three series of preblastoderm embryo injections. In experiment 1, donor plasmid pMiET1, carrying an enhancer trap transposon, was co-injected with *Minos* transposase mRNA; in experiment 2, donor plasmid pMiPR1 was used instead of pMiET1; and in experiment 3, donor pMiPR1 was co-injected with helper plasmid pHSS6hsMi2 (Loukeris *et al.* 1995a,b) (Figure 1). Transformation efficiencies ranged from 30% (with DNA helper) to 50% (with mRNA). Transformed flies were identified by eye-specific EGFP fluorescence in adults. The number of insertions per transformed line ranged in all experiments between one and four, as determined by Southern blot analysis (data not shown). Twenty-four additional lines, each carrying single autosomal insertions of transposon MiET1, were generated by the remobilization scheme described below.

**Mobilization of chromosomal insertions using endogenous transposase:** To establish an efficient source of transposase for transposon mobilization, six different lines carrying *P*-element-based helper PhsILMiT, encoding Minos transposase under control of a hsp70 promoter (Figure 1) on a balancer chromosome (hereafter called "helper chromosome"), were established. The helper chromosomes were tested for their ability to mediate remobilization of a single insertion of transposon MiPR1 from the X chromosome (X:8F3, hereafter called "transposon chromosome") to the autosomes. In a first experiment, "jump-start" males carrying both helper and transposon chromosomes were heat-shocked once during larval development. The highest remobilization efficiency observed was 24% (with line MiT2.4).

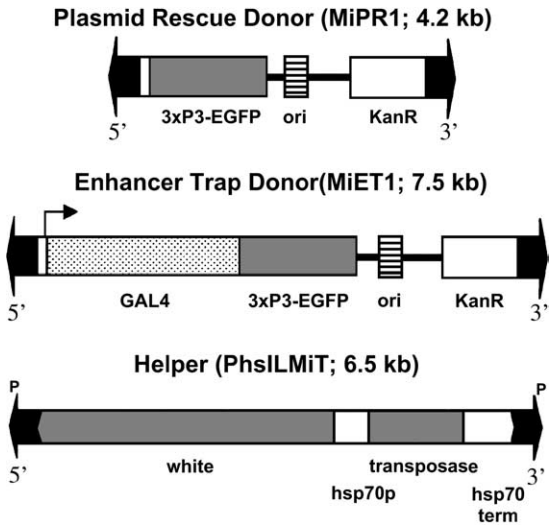


FIGURE 1.—*Minos* donor and helper constructs. Both donors contain the 3xPax6/EGFP dominant marker (BERGHAMMER *et al.* 1999) and allow plasmid rescue of insertions. The helper construct expresses *Minos* transposase under heat-shock control and is based on pCaSper. Only the transposon regions are shown.

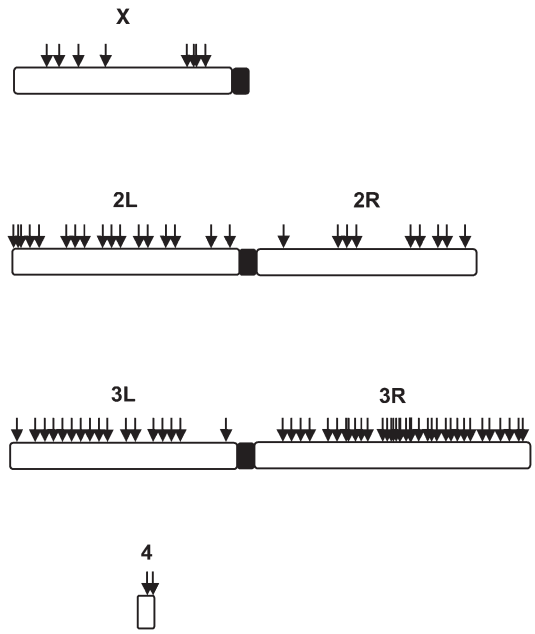


FIGURE 2.—Distribution of *Minos* insertions over the Drosophila genome. The number of insertions on the X chromosome is an underestimate, since 24 integration events were produced by mobilization of an X-linked insertion into the autosomes.

Flies from this line were then used to define optimal conditions for mobilization of a single insertion of transposon MiET1 (located at 17D3) from the X chromosome to the autosomes. The jump-start males were heat-shocked daily for 1 hr during the larval and pupal stages. Transposition efficiency in this experiment was 81%. No remobilization was detected when the jump-start males were kept continuously at 25° or 30°. Twenty-four of these reinsertions of MiET1 into autosomes were recovered and sequenced. The transposons MiET1 and MiPR1 used in these experiments allow recovery of the genomic DNA flanking the insertions on one side by plasmid rescue (PERUCHO *et al.* 1980). This enabled us to identify the exact insertion point of 92 different *Minos* insertions.

**Analysis of *Minos* insertion sites:** BLAST (ALTSCHUL *et al.* 1990) analysis was used to place the 92 insertions recovered plus 4 previously published insertions (LOUKERIS *et al.* 1995a) on the Drosophila genome, according to release 3 of the *D. melanogaster* database (CELNIKER *et al.* 2002). Seven insertions were in repetitive regions. One of these is found only on 3L while the others occur on more than one chromosomal arm. These were excluded from analysis of chromosomal distribution.

Figure 2 shows the distribution of the remaining *Minos* insertions over the chromosomal arms. A clear preference for 3R is apparent. Thirty-nine percent of *Minos* insertions lie on 3R, which contains only 24% of the euchromatin. The distribution of TA dinucleotides, which are a prerequisite for *Minos* insertion, does not explain this preference (Table 1). Chi-square analysis of the distribution of the 82 *Minos* insertions on the chromosomal arms *vs.* the distribution of TAs shows a significant bias ($P < 0.05$).

**TABLE 1**

**Observed and expected number of *Minos* insertions into the autosomes of *D. melanogaster* assuming all TA insertion targets are equally accessible**

| | Chromosome arm | | | | | |
|---|---|---|---|---|---|---|
| | 2L | 2R | 3L | 3R | 4 | Total |
| TA targets | 1,405,300 | 1,226,348 | 1,476,758 | 1,706,228 | 112,619 | 5,927,253 |
| Observed insertions | 18 | 9[a] | 18 | 35[a] | 2 | 82 |
| Expected insertions | 19 | 17 | 20 | 24 | 2 | 82 |

[a] $\chi^2$-test, $P \leq 0.05$.

Of the 96 characterized insertions, 58 were found to be within or close to (2 kb upstream or downstream) known or predicted genes (Table 2). A total of 30 insertions were in introns, 7 in exons (one of which was in a nested gene), 2 in 5′-UTRs, 2 in 3′-UTRs, and 1 in an intron/3′-UTR of an alternatively spliced gene. Sixteen insertions were located <2 kb from the closest gene. A total of 56 different genes were targeted by these insertions. Two genes, the *Dystrophin* gene and the predicted gene CG31000, were targeted twice. Additionally, one insertion occurred in the exon of a gene that lies nested within the intron of a second gene (Table 2). Thirteen of the insertions (~22%) occurred in genes that have not been hit either by the *P* element or by *piggyBac* (Bellen *et al.* 2004; Thibault *et al.* 2004).

Interestingly, introns were hit five times more frequently than exons. Chi-square analysis indicates that the preference of *Minos* for introns *vs.* exons is nonrandom ($P < 0.05$). This is not explained by the distribution of TA dinucleotides, the potential sites of *Minos* insertion, since the total number of TAs in introns is only twice the number of TAs in exons (Table 3).

No visible phenotypes, lethality, or semilethality were observed in 10 lines with single intronic insertions that were made homozygous, indicating that *Minos* insertions into introns are not likely to lead to a loss of function (data not shown).

**Lack of sequence bias at insertion sites:** An interplasmid transposition assay performed previously in insect cells failed to detect a sequence consensus at the insertion sites, beyond the actual TA target dinucleotide (Klinakis *et al.* 2000a). The sample of potential target sites in this assay was, however, rather limited, being restricted to a single 2-kb gene. As shown in Figure 3, analysis of the primary sequence of the *D. melanogaster* genomic integration reveals a very weak palindromic consensus around the actual insertion site. Interestingly, the corresponding sequence, ATA**TA**TAT, is also the consensus integration site for *Sleeping Beauty*, another transposon of the *Tc1/mariner* family; however, in the case of *Sleeping Beauty*, the consensus is considerably stronger (Vigdal *et al.* 2002).

**Physical properties of DNA at *Minos* insertions sites:** In addition to the primary sequence of the insertion site, structural properties of the target DNA are thought to determine the target preference of transposable elements (Craig 1997). We examined GC content and predicted bendability, B-DNA twist, A-philicity, and protein-induced deformability of 50 bp upstream and downstream of the *Minos* TA targets for 80 integration events. For comparison, 80 sequences of the same length and centered around a TA were taken randomly from the Drosophila genome (Figure 4).

Positions in which the *Minos* flanking sequences differ significantly from the random sequences (as judged by Student's *t*-test with a significance threshold of 0.01) are indicated by arrowheads in Figure 4. It appears that *Minos* insertion sites differ significantly from the random sequences only in predicted bendability and *P*-induced deformability.

H-Bond view analysis color codes the respective positions of base pairs according to their hydrogen bonding potential and generates average color values for a sequence alignment (Liao *et al.* 2000). This analysis reveals a weak conservation of potential hydrogen bonding at positions −3 to +3 flanking the target TA, presumably corresponding to the weak palindrome at these positions. Compared to *Sleeping Beauty* and *P*, however, *Minos* shows a much less pronounced hydrogen bonding pattern in the sequences flanking the TA target (Figure 5).

**Deletion of flanking genomic sequences through *Minos* excision:** The preference of *Minos* for introns, with the fact that the homozygous intronic *Minos* insertions examined did not have any detectable phenotype, raised the question of whether *Minos* transposons can mutate a gene by deleting flanking exonic sequences after mobilization from an initial insertion in an intron. We tested this by mobilizing, in the germ line of heterozygotes, the MiET1 transposon from intronic insertions in genes CG5423 (*robo3*), CG4114 (*expanded*), and CG30497 (a gene with no matches in the databases). Each excision-carrying chromosome was then made heterozygous with a deficiency spanning the respective locus. Genomic DNA from these heterozygous flies was subjected to PCR analysis with oligonucleotides priming at a distance of ~1000 bp on either side of the TA insertion site of *Minos*.

In 1 of 50 *Minos* excisions from the intron of gene *robo3*, the PCR product was 800 bp shorter than the expected 2000 bp. Sequence analysis showed a complex deletion/insertion event. A total of 800 bp of genomic DNA were deleted just adjacent to the TA insertion site, taking away almost the whole upstream exon, and 15 nucleotides of unknown origin were inserted instead. No transposon sequences were left behind.

Fifty excision events from the insertion in the *expanded* gene (*ex*, CG4114) were analyzed by PCR. The PCR product in all 50 cases had the expected size. Nevertheless, one of the excision chromosomes caused lethality over deletion Df(2L)al, ds[al]/In(2L)Cy. Certain mutations of the *ex* gene have been reported to cause lethality (Spradling *et al.* 1999). The initial insertion of *Minos* was 2 kbp upstream of the predicted ATG of *expanded*, and it is possible that a deletion, too small to be detected by our analysis, caused the lethality. Alternatively, the lethal mutation may have been the result of a "hit and run" event, where the excised element first reinserts into a nearby locus, from where it excises again, leaving behind its mutagenic footprint or a deletion.

In 2 of 89 excision events from orphan gene CG30497, the transposon was imprecisely excised, leaving behind 368 and 112 bp of the inverted repeat. In the latter case, 25 bp of genomic DNA were deleted

**TABLE 2**

***Minos* insertions into Drosophila genes**

| Genes with *Minos* hits | Position in gene | GenBank entry site (nucleotide position) | Cytogenetic site | Function | *piggy Bac* hits | *P*-element hits |
|---|---|---|---|---|---|---|
| CG5613 | Intron | AE003505.3: 190433 | X: 16A1-4 | — | + | – |
| CG32549 | Intron | AE003508.3: 254243 | X: 17A11 | 5′ nucleotidase activity | + | + |
| CG32498 | Intron | AE003426.2: 366742 | X: 3D1 | cAMP-specific phosphodiesterase activity | – | – |
| CG4114, Ex | Intron | AE003589.3: 132323 | 2L: 21C5-6 | Regulator of cell proliferation | – | + |
| CG5156 | 60 bp from 3′ end | AE003587.3: 309289 | 2L: 21F3 | — | + | – |
| CG5423, Robo-3 | Intron | AE003586.3: 55570 | 2L: 21F3-4 | Axon guidance receptor | + | – |
| CG17648 | 670 bp from 3′ end | AE003585.3: 212158 | 2L: 22B2 | — | + | – |
| CG16987, Alp23B | Intron | AE003583.3: 271193 | 2L: 23A3 | Metallopeptidase activity | – | + |
| CG31646 | Intron | AE003610.3: 217269 | 2L: 25F3 | Cell adhesion | + | + |
| CG11147 | Intron | AE003611.3: 43102 | 2L: 25F4 | ABC transporter | + | – |
| CG18340, Ucp4B | 110 bp from 3′ end | AE003612.2: 14760 | 2L: 26A5 | Mitochondrial transporter | + | – |
| CG7105 | Intron | AE003619.3: 47680 | 2L: 28D3-4 | Proctolin, neuropeptide hormone | + | – |
| CG8049, Btk29A | Intron | AE003620.3: 156821 | 2L: 29A1-3 | Tyrosine kinase | + | + |
| CG31719, RluA-1 | 500 bp from 5′ end | AE003628.2: 236953 | 2L: 31E6 | Deaminase activity | + | + |
| CG7294 | 160 bp from 5′ end | AE003629.2: 182506 | 2L: 32A2 | — | – | – |
| CG7147, Kuz | Intron | AE003640.3: 216217 | 2L: 34C4-6 | Metalloendopeptidase activity | + | + |
| CG4952, Dac | 400 bp from 3′ end | AE003651.2: 104793 | 2L: 36A1 | RNA pol II transcription factor | – | – |
| CG12508 | 590 bp from 3′end | AE003664.3: 237127 | 2L: 38B1 | — | + | – |
| CG30497 | Intron | AE003840.3: 239589 | 2R: 43E13 | — | + | + |
| CG12367 | 300 bp from 5′ end | AE003823.3: 149017 | 2R: 48E12 | — | + | + |
| CG17019 | 3′-UTR | AE003820.3: 115367 | 2R: 49E1-3 | Ubiquitin-protein ligase activity | + | – |
| CG6520 | 1300 bp from 5′ end | AE003803.3: 193893 | 2R: 54C5-6 | — | + | – |
| CG7020, DIP2 | Intron | AE003467.3: 238181 | 3L: 61B3 | — | + | + |
| CG16991, Tsp66A | Exon | AE003559.3: 258506 | 3L: 66A2 | Component integral to membrane | – | – |
| CG6718 | 3′-UTR | AE003550.3: 97483 | 3L: 67C9 | Ca-independent phospholipase A2 | + | + |
| CG7628 | Intron | AE003546.3: 242734 | 3L: 68A4-5 | Transporter activity | – | + |
| CG32146, dlp | Intron | AE003533.3: 35328 | 3L: 70E5-7 | Wnt receptor signaling pathway | + | + |
| CG13474 | Exon | AE003533.3:64573 | 3L: 70F1 | — | – | – |
| CG6117, Pka-C3 | Intron | AE003529.3: 158146 | 3L: 72A3-5 | cAMP-dependent protein kinase | + | + |
| CG7571, Oatp74D | Exon | AE003523.3: 24407 | 3L: 74D1 | Organic anion transporter | – | – |
| CG5582 | Intron | AE003522.3: 35487 | 3L: 75A4 | Transmission of nerve impulse | – | – |
| CG32457 | 700 bp from 3′ end | AE003599.2: 196361 | 3L: 80C2 | — | + | – |
| CG31519 | 400 bp from 5′ end | AE003607.3: 82270 | 3R: 82A1 | Olfactory receptor | – | – |
| CG32490, Complexin | Intron/3′-UTR | AE003606.3: 32995 | 3R: 82A3 | Syntaxin binding | – | + |
| CG1028, AntP | Intron | AE003673.3: 142252 | 3R: 84A6 | Transcription factor | + | + |
| CG1988 | 220 bp from 5′ end | AE003673.3: 283960 | 3R: 84C1 | — | – | + |
| CG31410 | Intron | AE003685.3: 109798 | 3R: 85F8-9 | — | – | – |
| CG7091 | 5′-UTR | AE003698.3: 62152 | 3R: 87D8 | Inorganic phosphate sodium symporter | + | – |
| CG17907, Ace | Intron | AE003699.3: 68737 | 3R: 87E2-3 | Acetylcholinesterase | + | + |
| CG31150 | Exon | AE003710.2: 194610 | 3R: 89A5-6 | Lipoprotein aminoterminal region | + | – |
| CG17562 | 140 bp from 3′ end | AE003714.2: 48639 | 3R: 89D5 | Oxidoreductase activity | + | – |
| CG3937, cherio | Intron | AE003716.3: 69050 | 3R: 89E13 | Actin binding | + | + |
| CG31243, Cpo | Intron | AE003720.3: 74671 | 3R: 90D1-6 | RNA binding | + | + |

(*continued*)

**TABLE 2**

**(Continued)**

| Genes with *Minos* hits | Position in gene | GenBank entry site (nucleotide position) | Cytogenetic site | Function | *piggy Bac* hits | *P*-element hits |
|---|---|---|---|---|---|---|
| CG18599 | 5′-UTR | AE003721.2: 107497 | 3R: 90F3 | Homeobox domain | – | – |
| CG7700 | Exon | AE003723.3: 59569 | 3R: 91B6-8 | SNAP receptor | – | + |
| CG31175, Dys | Intron | AE003726.3: 148326 | 3R: 92A6-7 | Cytoskeletal protein binding | + | – |
| CG31175, Dys | Intron | AE003726.3: 169013 | 3R: 92A6-7 | Cytoskeletal protein binding | + | – |
| CG5191 | 300 bp from 3′-end | AE003731.3: 49441 | 3R: 92F1-2 | Amidotransferase activity | + | – |
| CG5346 | Intron | AE003739.2: 102269 | 3R: 94A14 | — | + | + |
| CG13408 | 670 bp from 3′ end | AE003737.3: 128239 | 3R: 94A2 | — | – | – |
| CG4467 | Intron | AE003742.3: 124381 | 3R: 94E7-8 | Aminopeptidase activity | + | + |
| CG13624 | Intron | AE003748.3: 108349 | 3R: 96A3 | DNA binding | + | + |
| CR31382, tRNA Asp | Exon | AE003749.3: 204677 | 3R: 96B6 | tRNA Asp | – | – |
| CG31120 | Intron | AE003749.3: 204677 | 3R: 96B6 | Oxidoreductase activity | + | + |
| CG10001 | 1150 bp from 3′ end | AE003766.2: 166978 | 3R: 98E2 | Allatostatin receptor activity | – | – |
| CG31000 | Intron | AE003780.3: 91982 | 3R: 100F1 | Pre-mRNA splicing factor | + | + |
| CG31000 | Intron | AE003780.3: 151439 | 3R: 100F1 | Pre-mRNA splicing factor | + | + |
| CG31998 | Exon | AE003844.3: 44976 | 4: 102A1 | — | + | – |

The nucleotide sequences of the insertion sites are available upon request.

adjacent to the TA insertion site. Furthermore, three PCR reactions from gene CG30497 excisions did not give any product, indicating that deletions covering at least one of the primer-annealing sites may have taken place. Lethality was also observed in one excision event from gene CG30497. Again, Southern blot analysis of heterozygotes of this event did not reveal a deletion at the site of the initial *Minos* insertion.

## DISCUSSION

Transposon mutagenesis is an important tool in functional genomics. Mutagenesis of Drosophila with the *P* element has played a central role in elucidating the function and regulation of many genes. Constructs based on the *P* element are used to "trap" genes and enhancers, to produce loss-of-function mutations, to overexpress and ectopically express genes, and to study genetic interactions. Such studies have helped to unravel basic conserved genetic pathways, some of which are involved in human diseases and aging (for review see O'Kane 2003). It has been estimated that 77% of human genes implicated in specific diseases have one or more Drosophila homologs with considerable sequence similarity (Reiter *et al.* 2001).

**TABLE 3**

**Observed and expected number of *Minos* insertions into the TAs (potential sites of *Minos* insertions) of introns *vs.* exons**

| | Exons | Introns | Total |
|---|---|---|---|
| TA targets | 1,052,616 | 2,106,358 | 3,158,974 |
| Observed insertions | 6[a] | 30[a] | 36 |
| Expected insertions | 12 | 24 | 36 |

[a] $\chi^2$-test, $P \leq 0.05$.

An extensive body of data on the genomic distribution of *P*-element insertions accumulated over 20 years has revealed a high insertion preference for certain euchromatic areas, the so-called hotspots (Spradling *et al.* 1999). Consequently, so far only a fraction of all predicted genes in the Drosophila genome have been targeted by *P*, indicating that the *P* element alone is not sufficient for saturating mutational analysis of the Drosophila genome. Therefore, other mobile elements that also insert efficiently into the Drosophila genome and at the same time show a different insertional spectrum or even no insertion site preference at all are desirable as complementing mutagenesis tools.

We demonstrate here that the *Tc1/mariner*-like transposable element *Minos* is an efficient tool for generating insertions into the Drosophila genome, that *Minos* insertions show little sequence preference beyond the strictly required target dinucleotide TA, and that there is a significant bias for insertion into introns *vs.* exons. Furthermore, we show that while *Minos* inserts preferentially into introns, where it does not appear to interfere with expression of the target gene, it occasionally causes deletions of nearby exonic sequences upon subsequent excision. This property distinguishes *Minos* from *piggyBac*, which does not cause deletions upon excision (Horn *et al.* 2003).

**Efficiency of transposition:** *Minos* insertions were generated either through preblastoderm embryo injections or through remobilization of a *Minos* transposon from an X-linked insertion. Transposase expression in remobilization experiments was regulated by heat shock. Transformation efficiency varied between 30


| | -5 | -4 | -3 | -2 | -1 | 0 | 0 | +1 | +2 | +3 | +4 | +5 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | 20 | 21 | 31 | 26 | 31 | | 80 | 14 | 29 | 28 | 30 | 23 |
| T | 20 | 29 | 24 | 33 | 14 | 80 | | 42 | 27 | 31 | 18 | 25 |
| C | 19 | 17 | 12 | 15 | 17 | | | 12 | 11 | 14 | 16 | 22 |
| G | 21 | 13 | 13 | 6 | 18 | | | 12 | 13 | 7 | 16 | 10 |

Figure 3.—Sequence composition analysis of *Minos* insertion sites. (Top) Five nucleotides upstream and downstream of the insertions were aligned for 80 integrants and their informational content was plotted with the SeqLogo program (Schneider and Stephens 1990). (Bottom) Base distribution of the 80 insertion sites.

and 50%, with higher rates when the transposon was coinjected with transposase mRNA. Remobilization efficiency through expression of a chromosomally encoded transposase was at least 80%. Remobilization is thus more appropriate for genomic analysis, since it is easier to perform on a large scale and produces single insertions. One of the transposons used in this study (MiET1) was designed to function as a *GAL4*-based enhancer trap. Preliminary analysis of MiET1 insertions indicated that ~20% of insertions can be classified as *bona fide* enhancer traps (data not shown).

**Characterization of *Minos* insertions:** Alignment of 80 *Minos* insertions, centered around the target TA dinucleotide, revealed a weakly conserved palindromic motif, the sequence ATA**TA**TAT. Interestingly, the same consensus, although much more strongly conserved, was found for insertions of *Sleeping Beauty*, another member of the *Tc1/mariner* superfamily of mobile elements, into the mouse genome. A similar consensus (CAYA**TA**TRTY) has been reported for *Tc1* in *Caenorhabditis elegans* (Korswagen *et al.* 1996). Thus, these elements share a similar target site, albeit with a different degree of conservation. Compared to *Sleeping Beauty* and *Tc1*, *Minos* insertions seem to depend much less, if at all, on sequences beyond the TA insertion dinucleotide. This is strongly supported by H-bond view analysis, which reveals a very low degree of symmetric hydrogen bonding potential around the target TA. The *P* element, *Sleeping Beauty*, and *Tc1*, on the other hand, exhibit extensive conservation and strong symmetry in hydrogen-bonding potential within the DNA flanking their target sites (Liao *et al.* 2000; Vigdal *et al.* 2002).

High bendability (Figure 4) seems to be the DNA property that mainly distinguishes *Minos* target sites from randomly chosen TA-centered sequences. Additionally, the site of insertion has a significantly lower predicted protein-induced deformability than in the random sequences, a property not shared by other *Tc1/mariner* family members (Vigdal *et al.* 2002).

Analysis of genomic position of the retrieved *Minos* insertions reveals no apparent preference for insertions into genes. There is, however, a preference of *Minos* for introns *vs.* exons, which is not sufficiently explained by the respective frequency of TA dinucleotides in introns and exons ($P = 0.034$). However, the relative overall A/T richness may be a determinant of the integration site and may explain the observed bias. The sequences immediately flanking *Minos* insertions are A/T rich, and it is possible that such sites may be better targets either on the level of primary sequence or through their structural properties. Introns would thus be preferential targets since their A/T content, over all chromosomes, is ~10 percentage points above that of the exons.

A preference for insertions into introns has been reported for *piggyBac* in Drosophila (Hacker *et al.* 2003) and for the *Tc1*-like element *Sleeping Beauty* in mammalian cells (Vigdal *et al.* 2002). This property sets these elements apart from the yeast *Ty1* and *Ty2* elements and the *P* element, which preferably insert into 5′-UTRs of genes (Craig 1997).

Our analysis revealed no obvious hotspot for *Minos* integration. Two genes, which contain exceptionally large introns, were hit twice. A more thorough investigation of genome-wide insertional bias will require analysis of a much greater number of insertions. However, it can be inferred that *Minos* does not share the same bias of insertion as the *P* element; >55% of the genes with a *Minos* hit (30/56) have no known *P*-element insertions. Furthermore, the observation that ~20% of the genes hit by *Minos* have not been hit by

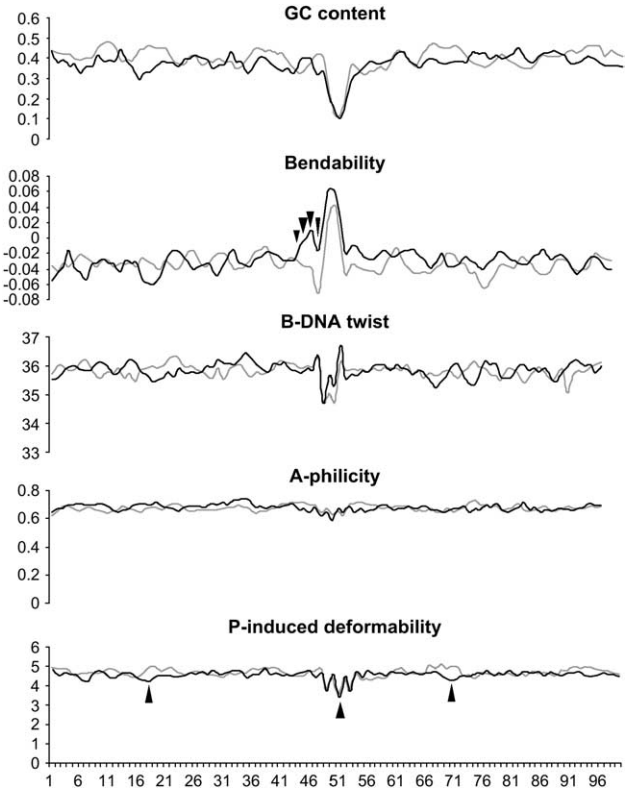


FIGURE 4.—Analysis of predicted physical properties of *Minos* insertion sites. One hundred nucleotides centered around each of the 80 *Minos* insertions were analyzed and averaged (solid lines). Eighty sequences, also centered around TAs, but randomly taken from the Drosophila genome, were coanalyzed (shaded lines). Arrowheads indicate nucleotide positions where the predicted values between the two groups are different according to a confidence level of >99% (Student's *t*-test). Bendability is the tendency of DNA to bend toward the major groove. B-DNA twist determines the tightness of the DNA coil. A-philicity is the tendency of the DNA double helix to form A-DNA. Protein-induced deformability describes the propensity of DNA to change conformation upon binding to a protein.

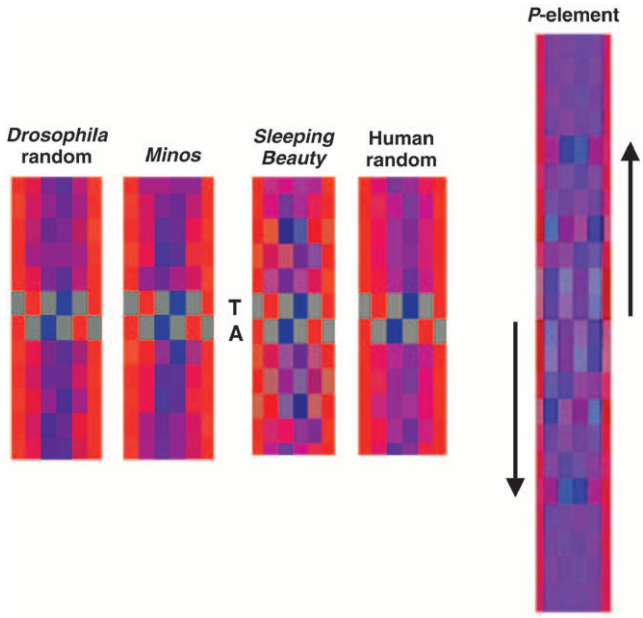


FIGURE 5.—Hydrogen-bonding analysis of *Minos* insertion sites compared to *Sleeping Beauty* and *P*-element insertion sites. Program HbondView (LIAO *et al.* 2000) was used to illustrate average hydrogen-bonding patterns of base pairs flanking the *Minos* insertion sites. Six positions for each base pair are color coded as potential hydrogen acceptor (red), donor (blue), or inert (gray). *Sleeping Beauty* and *P* graphs are from LIAO *et al.* (2000) and VIGDAL *et al.* (2002), respectively.

either *P* or *piggyBac* suggests that *Minos* will be invaluable in achieving saturation mutagenesis in Drosophila.

The *Minos* system has the key properties required of a tool for genome-wide insertional screens in Drosophila. First, high-efficiency germ-line transposition of a *Minos* insertion is achieved by expressing transposase in *trans*. Second, sequence conservation at the insertion site is considerably weaker compared to the *P* element. Third, since insertions in introns are more abundant than insertions in exons, *Minos* will be useful for developing true gene-trap constructs for genome-wide disruption screens. Additionally, *Minos* can be more useful as a mutagen compared to *piggyBac*, since new mutant alleles can be generated through imprecise excision of the element. One drawback of imprecise excision, however, is the possibility of "hit-and-run" events, which can complicate genetic analysis, as has been observed with *P*. All these features are, due to the broad host range of *Minos*, also potentially available for the genetic manipulation of many other species. We conclude that *Minos* can be instrumental for completion of the effort to introduce useful insertions into all known genes of *D. melanogaster*.

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