piggyBac-Based Insertional Mutagenesis and Enhancer Detection as a Tool for Functional Insect Genomics

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ABSTRACT

Transposon mutagenesis provides a fundamental tool for functional genomics. Here we present a non-species-specific, combined enhancer detection and binary expression system based on the transposable element *piggyBac*: For the different components of this insertional mutagenesis system, we used widely applicable transposons and distinguishable broad-range transformation markers, which should enable this system to be operational in nonmodel arthropods. In a pilot screen in *Drosophila melanogaster*, *piggyBac* mutator elements on the X chromosome were mobilized in males by a *Hermes*-based jumpstarter element providing *piggyBac* transposase activity under control of the α*1-tubulin* promoter. As primary reporters in the *piggyBac* mutator elements, we employed the heterologous transactivators GAL4Δ or tTA. To identify larval and adult enhancer detectors, strains carrying *UASp-EYFP* or *TRE-EYFP* as secondary reporter elements were used. Tissue-specific enhancer activities were readily observed in the GAL4Δ/*UASp*-based systems, but only rarely in the tTA/*TRE* system. Novel autosomal insertions were recovered with an average jumping rate of 80%. Of these novel insertions, 3.8% showed homozygous lethality, which was reversible by *piggyBac* excision. Insertions were found in both coding and noncoding regions of characterized genes and also in noncharacterized and non-*P*-targeted CG-number genes. This indicates that *piggyBac* will greatly facilitate the intended saturation mutagenesis in Drosophila.

THE genome sequence of *Drosophila melanogaster* and its annotation are nearly complete (Adams *et al.* 2000; http://www.fruitfly.org/annot/release3.html). For the malaria mosquito *Anopheles gambiae* the genome is sequenced (Holt *et al.* 2002), and its annotation is in preparation (http://www.ncbi.nlm.nih.gov/cgi-bin/Entrez/map_search?chr=agambiae.inf; http://www.ensembl.org/Anopheles_gambiae/). The genome of the lepidopteran pest species *Heliothis virescens* is sequenced (http://gnn.tigr.org/articles/04_02/moth_dna.shtml) and for the silkmoth, *Bombyx mori* (http://www.ab.a.u-tokyo.ac.jp/lep-genome/; http://www.ab.a.u-tokyo.ac.jp/silkbase/), as well as the honey bee, *Apis mellifera ligustica* (http://www.nhgri.nih.gov/NEWS/sequencing.html), genome sequencing is planned. More insect species will follow. Nonetheless, within functional genomics it is necessary to determine not only the sequences of coding and noncoding regions but also the corresponding biological functions. Transposon-based insertional mutagenesis (Cooley *et al.* 1988; Robertson *et al.* 1988) and enhancer detection (O'Kane and Gehring 1987; Bellen *et al.* 1989; Bier *et al.* 1989; Wilson *et al.* 1989) will provide ideal means for the identification of gene functions.

The principle of transposon mutagenesis relies on the mobilization of transposable elements that can insert into new genomic loci and disrupt gene activities. A "jumpstarter" element providing transposase activity is used to mobilize a visibly marked, nonautonomous "mutator" element. If the mutator is equipped with an enhancer-sensitive reporter, enhancer activities can be identified on the basis of tissue-specific expression patterns ("enhancer detection") at the same time. Moreover, by using a heterologous transactivator gene as a reporter, the insertion will become a tool for tissue-specific expression studies (Brand and Perrimon 1993; Bello *et al.* 1998). In addition, the mutator insertion serves both as a visible label for stock keeping of the mutant or enhancer detector lines and as a molecular tag to facilitate cloning of the mutated gene or detected enhancer. This allows for rapid correlation of sequence data with biological functions.

In functional genomics, insertional mutagenesis has been applied most extensively in *D. melanogaster*, where ~25% of all vital genes have been disrupted by transposon insertions (Spradling *et al.* 1999; Peter *et al.* 2002; http://flypush.imgen.bcm.tmc.edu/pscreen/). However, most transposon mutagenesis screens in *D. melanogaster* rely on *P*-element-derived vectors, which are nonfunctional outside of the Drosophilids (Handler *et al.* 1993). Recently, more promiscuous transposable elements have been identified (reviewed in Handler and James 2000; Atkinson *et al.* 2001; Handler 2001), of which *Hermes* (Warren *et al.* 1994), *mariner* (*Mos1*; Medhora *et*

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al. 1988), *Minos* (Franz and Savakis 1991), and *piggyBac* (Cary *et al.* 1989) are most notable. *Minos* has been shown to be operational for genomewide insertional mutagenesis in mammalian tissues (Zagoraiou *et al.* 2001) and *Mos1* in the nematode worm *Caenorhabditis elegans* (Bessereau *et al.* 2001).

Fluorescence-based transformation markers that reliably identify transposon insertions have been established and their functionality has been demonstrated in at least three different insect orders (reviewed in Horn *et al.* 2002). One of the most widely used transformation markers, *3xP3-EGFP*, is based on the enhanced green fluorescent protein (EGFP) and an artificial promoter (*3xP3*) that is responsive to the phylogenetically conserved "master regulator" of eye development, Pax-6 (Berghammer *et al.* 1999; Horn *et al.* 2000). By replacing the coding region for EGFP with the coding region for its yellow (EYFP) or cyan (ECFP) spectral variants, or the red fluorescing protein DsRed, three completely distinguishable transformation markers have been established (Horn and Wimmer 2000; Horn *et al.* 2002). Thus, the basic tools needed to develop insertional mutagenesis systems for functional genomics in nonmodel insects are in place.

Here, we present a non-species-specific insertional mutagenesis and enhancer detection system that is based on derivatives of the transposable element *piggyBac* (Cary *et al.* 1989) and the potentially universal transformation markers *3xP3-ECFP*, *3xP3-EYFP*, and *3xP3-DsRed* (Horn *et al.* 2002). *piggyBac* mobility was originally shown in insect cell lines (Fraser *et al.* 1995) and has been successfully employed for germline transformation of dipteran, lepidopteran, and coleopteran species (reviewed in Handler 2002). To test the efficiency of our insertional mutagenesis system, we performed a pilot screen in *D. melanogaster*. We established stable *Hermes*-, *Minos*-, or *Mos1*-based jumpstarter elements that provide functional *piggyBac* transposase. We generated nonautonomous *piggyBac* mutator elements that allow gene disruption and enhancer detection in a way that permits enhancer-detecting insertions to be directly employed for misexpression studies using heterologous transcriptional activators. We chose (i) a deletion variant of the yeast transactivator GAL4 (Brand and Perrimon 1993) and (ii) the tetracycline-controlled transactivator tTA (Gossen and Bujard 1992). To identify larval or adult enhancer activities, we utilized reporter elements driving *EYFP* as a secondary reporter gene under the control of a GAL4-bound upstream activation sequence (*UASp*; Rørth 1998) or a tTA-response element (*TRE*; Gossen and Bujard 1992), respectively. For the different types of elements, we used distinguishable fluorescent markers (Horn and Wimmer 2000; Horn *et al.* 2002) that allow crosses of insect strains carrying the different elements without the need of balancer chromosomes. All components are therefore derived from broad range transposable elements and transformation markers, which should enable this insertional mutagenesis and enhancer detection system to be operational in nonmodel insects.

MATERIALS AND METHODS

Versatile two-step cloning procedure: In principle, we compose our constructs in the cloning shuttle vector pSLfa1180fa (Horn and Wimmer 2000). From the resulting progenitor vectors, the construct can be easily placed in a set of diverse transformation vectors on the basis of different transposable elements and transformation markers (Horn and Wimmer 2000). Table 1 provides an overview of the progenitor and final constructs generated. Not all of the constructs have yet been functionally tested in transgenic insects, but are listed since they are available. The two-step cloning procedure allows for many more constructs to be easily generated.

Jumpstarter elements: pBac{3xP3-ECFP, hsp70-Hermes} was generated by cloning into the *Fse*I site of pBac{3xP3-ECFPafm} (Horn and Wimmer 2000) an *Fse*I fragment from pSLfa_hsp70-Hermes_fa, which resulted from an *Asp*718/*Spe*I fragment from pKHSHH (Sarkar *et al.* 1997) cloned into *Asp*718/*Spe*I-cut pSLfa1180fa.

pHer{3xP3-ECFP, hsp70-piggyBac} and pMi{3xP3-DsRed, hsp70-piggyBac} were generated by cloning into the *Asc*I site of pHer{3xP3-ECFPaf} (Horn and Wimmer 2000) and pMi{3xP3-DsRed}, respectively, an *Asc*I fragment from pSLfa_hsp70-piggyBac_fa, which was the result of an *Eco*RI/*Hin*dIII fragment from phspBac (Handler and Harrell 1999) cloned into *Eco*RI/*Hin*dIII-cut pSLfa1180fa. pMi{3xP3-DsRed} was constructed by cloning an *Eco*RI/*Asc*I-fragment from pSL-3xP3-DsRedaf (Horn *et al.* 2002) into pMi{3xP3-EYFP} (provided by C. Savakis), thereby replacing *eyfp* with *DsRed*. For injection of pMi{3xP3-DsRed, hsp70-piggyBac}, a construct with the transposase gene in opposite orientation to the marker gene was chosen.

pHer{3xP3-ECFP, αtub-piggyBacK10}, pHer{3xP3-EYFP, αtub-piggyBacK10}, pMos{3xP3-ECFP, αtub-piggyBacK10}, and pMos{3xP3-EYFP, αtub-piggyBacK10} were generated by cloning into the *Asc*I site of pHer{3xP3-ECFPaf}, pHer{3xP3-EYFP-af}, pMos{3xP3-ECFPafm}, and pMos{3xP3-EYFPafm} (Horn and Wimmer 2000), respectively, an *Asc*I fragment from pSLfa_αtub-piggyBacK10_fa (provided by Exelixis, S. San Francisco). For injection, pHer{3xP3-ECFP, αtub-piggyBacK10} (transposase gene in opposite orientation to marker gene) and pMos{3xP3-ECFP, αtub-piggyBacK10} (transposase gene in same orientation as marker gene) were chosen.

Mutator elements: pHer{3xP3-EYFP, p-GAL4Δ-K10} and pBac{3xP3-EYFP, p-GAL4Δ-K10} were constructed by cloning into the *Asc*I site of pHer{3xP3-EYFPaf} and pBac{3xP3-EYFP afm} (Horn and Wimmer 2000), respectively, an *Asc*I fragment from pSLfa_p-GAL4Δ-K10_fa. For injection of pBac{3xP3-EYFP, p-GAL4Δ-K10}, a construct with the GAL4 gene in opposite orientation to the marker gene was chosen. To generate pSLfa_p-GAL4Δ-K10_fa, the coding region of a GAL4 deletion variant (II-9; Ma and Ptashne 1987) was cloned as a 1.1-kb *Asp*718 fragment from construct G610 (designed by G. Struhl, provided by G. Vorbrüggen) into the *Asp*718 site of pSLfa_p-K10_fa, which was obtained by inserting the 3′ untranslated region (UTR) of *K10* as an *Asp*718/*Pst*I-fragment from pUASp (Rørth 1998) into *Asp*718/*Pst*I-opened pSLfa_p_fa, which was generated by inserting the *P*-element promoter and first intron as a *Xho*I/*Asp*718-cut PCR fragment from pBluescript-GAGA-GAL4-Pprom+intron (primers 5′-CCGCTCGAGTCG ATAGCCGAAGCTTACC-3′ and 5′-GGGGTACCAATGAACA GGACCTAACGCA-3′; Rørth 1998) into *Sal*I/*Asp*718-cut pSLfa1180fa.

pBac{UASp-3xP3-EYFP, p-GAL4Δ-K10} was generated by cloning an *Asc*I/*Fse*I fragment from pSLfa_p-GAL4Δ-K10_fa into the *Asc*I/*Fse*I-sites of pBac{UASp-3xP3-EYFPafm} that resulted from the cloning of the 0.1-kb *Fse*I/*Bgl*II fragment from pSLfa1180fa into *Fse*I/*Bgl*II-opened pBac{UASp-3xP3-EYFPaf}, which was cloned by inserting into the *Hpa*I site of p3E1.2 (Cary *et al.* 1989) the *Eco*RI (Klenow-blunted)/*Nru*I fragment from pSL-UASp-3xP3-EYFPaf, which was the result of cloning a *Eco*RI/*BstZ*17I fragment from pSLfa_UASp_fa into *Eco*RI/*Sma*I-opened pSL-3xP3-EYFPaf (Horn and Wimmer 2000). pSLfa_UASp_fa was constructed by inserting into *Sal*I (Klenow-blunted)/*Asp*718-cut pSLfa1180fa a *Not*I (Klenow-blunted)/*Asp*718 fragment from pBluescriptGAGA-GAL4-Pprom+intron (p1396, provided by P. Rørth).

pBac{3xP3-EYFP, p-tTA-K10} was generated by cloning into the *Asc*I/*Fse*I sites of pBac{3xP3-EYFPafm} an *Asc*I/*Fse*I fragment from pSLfa_p-tTA-K10_fa, which was constructed by inserting an *Eco*RI (Klenow-blunted)/*Bam*HI fragment from pTet-Off (CLONTECH, Palo Alto, CA) into *Not*I (Klenow-blunted)/*Bam*HI-opened pSLfa_p-K10_fa.

pBac{TRE-3xP3-EYFP, p-tTA-K10} was generated by cloning an *Asc*I/*Fse*I fragment from pSLfa_p-tTA-K10_fa into the *Asc*I/*Fse*I sites of pBac{TRE-3xP3-EYFPafm} that resulted from the cloning of the 0.1-kb *Fse*I/*Bgl*II fragment from pSLfa1180fa into *Fse*I/*Bgl*II-opened pBac{TRE-3xP3-EYFPaf}, which was cloned by inserting into the *Hpa*I site of p3E1.2 the *Eco*RI (Klenow-blunted)/*Nru*I fragment from pSL-TRE-3xP3EYFPaf, which was the result of cloning the tTA response element (TRE) as a 0.3-kb *Xho*I (Klenow-blunted)/*Sma*I fragment from pUHD10-3 (Gossen and Bujard 1992) into *Sma*I-opened pSL-3xP3-EYFPaf.

Reporter elements: pBac{3xP3-EYFP, UASp-EYFP-K10}, pBac{3xP3-ECFP, UASp-EYFP-K10}, pHer{3xP3-EYFP, UASp-EYFP-K10}, pHer{3xP3-ECFP, UASp-EYFP-K10}, pBac{3xP3-EYFP, UASp-lacZ-K10}, pBac{3xP3-ECFP, UASp-lacZ-K10}, pHer{3xP3-EYFP, UASp-lacZ-K10}, pHer{3xP3-ECFP, UASp-lacZ-K10}, pBac{3xP3-DsRed, UASp-EYFP-K10}, and pBac{3xP3-DsRed, UASp-lacZ-K10} were generated by cloning an *Asc*I/*Fse*I fragment from pSLfa_UASp-eyfp-K10_fa or pSLfa_UASp-lacZ-K10_fa, respectively, into the *Asc*I/*Fse*I sites of the *eyfp*- or *ecfp*-marked *piggyBac* or *Hermes* transformation vectors (Horn and Wimmer 2000) or pBac{3xP3-DsRedaf} (Horn *et al.* 2002), respectively. pSLfa_UASp-eyfp-K10_fa and pSLfa_UASp-lacZ-K10_fa result from the insertion of *eyfp* as an *Asp*718/*Not*I fragment from pEYFP-1 (CLONTECH) or *lacZ* as an *Xba*I fragment from pCHABΔSal (Wimmer *et al.* 1993, 1995) into pSLfa_UASp-K10_fa opened with *Asp*718/*Not*I or *Xba*I, respectively. pSLfa_UASp-K10_fa was generated by opening pSLfa_UASp_fa by an *Asp*718/*Pst*I digest and inserting the 3′ UTR of *K10* as an *Asp*718/*Pst*I fragment from pUASp.

pBac{3xP3-DsRed, TRE-EYFP-SV40} and pBac{3xP3-ECFP, TRE-EYFP-SV40} or pBac{3xP3-DsRed, TRE-lacZ-SV40} and pBac{3xP3-ECFP, TRE-lacZ-SV40} were generated by cloning into the *Asc*I site of pBac{3xP3-DsRedaf} and pBac{3xP3-ECFPafm} (Horn and Wimmer 2000) the *Asc*I fragment from pSLfa_TRE-EYFP-K10_fa or pSLfa_TRE-lacZ-K10_fa, respectively. For all four constructs the orientation in which the reporter is transcribed in the same direction as the transformation marker was chosen (Figure 2). pSLfa_TRE-EYFP-K10_fa or pSLfa_TRE-lacZ-K10_fa resulted from the insertion of *eyfp* as an *Eco*RI/*Not*I (Klenow-blunted) fragment from pEYFP-1 or of *lacZ* as an *Xba*I fragment from pCHABΔSal into pSLfa_TRE-SV40_fa opened with *Eco*RI/*Xba*I (Klenow-blunted) or *Xba*I, respectively. pSLfa_TRE-SV40_fa was cloned by inserting the 0.9-kb *Xho*I (Klenow-blunted)/*Hin*dIII fragment from pUHD10-3 into *Eco*RI (Klenow-blunted)/*Hin*dIII-opened pSLfa1180fa.

Drosophila culture: Fly strains were reared under standard laboratory conditions (Roberts 1998). Drosophila germline transformation employing *piggyBac*, *Hermes*, and *Mos1* vectors and transgenic strain establishment was performed as described (Horn *et al.* 2000). For *Minos*-derived transformation vectors, injection mixes were at final concentrations of 500 ng/μl construct and 300 ng/μl helper pHSS6hsILMi20 (Klinakis *et al.* 2000).

In crosses to test the heat-shock-controlled jumpstarter strains, heat shocks were performed on 3 consecutive days during second and third larval stages at 37° for 3 hr each day. Remobilization of genome-integrated, nonautonomous *Hermes* or *piggyBac* elements was scored by novel integration of respective *3xP3-EGFP*- or *3xP3-EYFP*-marked elements onto balancer chromosomes.

Crosses for insertional mutagenesis were carried out as described in Figure 1. Since G_3 brothers might contain the same insertion event, when establishing novel insertion lines we ensured independence by selecting only one *3xP3-E*YFP$^+$/*3xP3-ECFP*$^-$ G_3 male of each single male G_2 cross. Exceptions were made when clear differences in the level of *3xP3-EYFP* marker expression or distinct enhancer activities were observed, thus indicating different insertion sites. To genetically identify the localization of the novel insertions and to test for lethality, mutator-carrying G_3 males were crossed to *w*; *SM5*;*TM3*/*T(2;3)ap*^{Xa} virgins. Segregation of the *EYFP* fluorescence compared to *DsRed* fluorescence was used to identify the chromosomal localization indicated in Table 3 by roman numerals. Of each insertion line, G_4 mutator-containing males and virgins carrying both *SM5* and *TM3* were intercrossed and lethality was scored on the basis of the presence of *SM5* or *TM3* in all G_5 progeny.

Epifluorescence microscopy: Filter sets required for the identification of the different fluorescent transformation markers have been described (Horn *et al.* 2002). Detection of adult and larval enhancer activities based on *EYFP* fluorescence was conducted employing the Leica MZ FLIII fluorescence stereomicroscope and the GFP2 (GFP Plus) filter set (excitation filter, 480/40 nm; barrier filter, 510 nm). For immobilization, larvae were mounted in 65° hot glycerol and documented after 5 min.

Embryo analysis: X-Gal stainings for the detection of embryonic enhancer activities were performed essentially as described (O'Kane 1998).

Inverse PCR and sequence analysis: To recover DNA sequences flanking *piggyBac* insertions, inverse PCR was performed as described (Huang *et al.* 2000). Genomic DNA of new insertion lines was digested with *Hae*III, *Msp*I, or *Taq*I. Restriction fragments were circularized by ligation. PCR reactions [5 min 95°, 35 times (30 sec 95°, 1 min 65°, 2 min 72°), 7 min 72°] were performed for the 5′ junction with the forward primer (PLF) 5′-CTTGACCTTGCCACAGAGGACTATTAG AGG-3′ and reverse primer (PLR) 5′-CAGTGACACTTACC GCATTGACAAGCACGC-3′ and for the 3′ junction with the forward primer (PRF) 5′-CCTCGATATACAGACCGATAAAA CACATGC-3′ and reverse primer (PRR) 5′-AGTCAGTCA GAAACAACTTTGGCACATATC-3′. The purified fragments were directly sequenced for the 5′ junction with primer CH_PLSeq 5′-CGGCGACTGAGATGTCC-3′ and for the 3′ junction with primer CH_PRSeq 5′-TACGCATGATTAT CTTTAACG-3′. The obtained sequences were used in BLAST searches against the Drosophila Genome Database (http://www.fruitfly.org/blast/).

RESULTS AND DISCUSSION

Insertional mutagenesis and enhancer detection for nonmodel insects: To develop insertional mutagenesis systems for nonmodel arthropods, we employed the

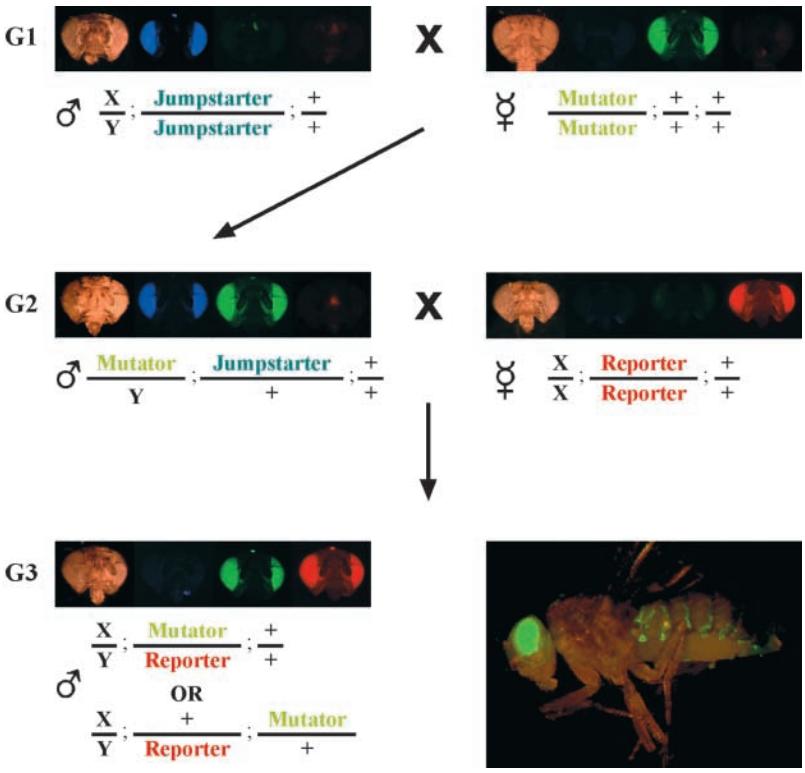

FIGURE 1.—Crossing scheme for the identification of new mutator insertions and adult enhancer activities without the use of balancer chromosomes (see text for details). The use of distinguishable fluorescent transformation markers makes it possible to follow the different jumpstarter (*3xP3-ECFP*), mutator (*3xP3-EYFP*), and reporter (*3xP3-DsRed*) elements independently. To demonstrate the presence of the different elements, fly heads were photographed without filter and with *ECFP*-, *EYFP*-, or *DsRed*-specific filters (HORN *et al.* 2002). From G_2 males carrying the mutator on the X chromosome and a jumpstarter on an autosome, all male progeny showing *EYFP* fluorescence must carry new mutator insertions on autosomes. Some of them also detect adult enhancer activities, like the male showing segmental fluorescence in the abdomen. Note that it is not important for the scheme on what autosome the jumpstarter or reporter are inserted. For the integrated mutator-reporter *{GAL4Δ+UAS}*, G_2 males were crossed to *white* instead of reporter strain virgins.

widely applicable transposable elements *Hermes*, *Minos*, *Mos1*, and *piggyBac*. Due to the absence of marked "balancer" chromosomes in most nonmodel arthropods, several reliable and distinguishable transformation markers are necessary when insertional mutagenesis and enhancer detection screens are combined with binary ectopic expression systems. To clearly identify, separate, and stably establish novel mutator insertion lines without the need of balancers, we have used the three independent and distinguishable fluorescent transformation markers *3xP3-ECFP*, *3xP3-EYFP*, and *3xP3-DsRed* (HORN *et al.* 2002) to mark (i) the jumpstarter, (ii) the mutator, and (iii) the reporter strains of enhancer detection expression systems (Figures 1 and 2).

After crossing jumpstarter and mutator strains (Figure 1, G_1), insects carrying both elements can be identified on the basis of the eye-specific *ECFP* and *EYFP* expression (Figure 1, G_2). In the next generation, the *3xP3-ECFP*-marked jumpstarter can be crossed out to allow stable inheritance of a novel *3xP3-EYFP*-marked mutator insertion. At the same time a *3xP3-DsRed*-marked reporter can be crossed in to detect adult enhancer activities that mediate expression of a heterologous transactivator encoded by the mutator (Figure 1, G_3). When both mutator and reporter are based on the same transposon, the *3xP3-DsRed*-marked reporter can be crossed out again to allow for molecular analysis of the novel insertion site. Since each construct can be followed independently, there is no need for balancer chromosomes. Moreover, the dominant fluorescent marker serves as a visible label for the novel insertions in both larval and adult stages and therefore facilitates stock keeping. Males and females carrying a novel insertion can be mated and their progeny analyzed for recessive phenotypes. Furthermore, the transposon insertion molecularly tags the mutated gene, which assists in its cloning.

Jumpstarter elements: Jumpstarter strains provide transposase activity to mobilize nonautonomous mutator elements. The jumpstarter constructs therefore contain an active transposase gene. However, to keep jumpstarter strains stable, the transposable element backbone that is used to introduce the active transposase gene into the genome should be derived from a different transposon family, so that cross-mobilization can be excluded. To generate a jumpstarter for mutators based on the *hAT* element *Hermes*, we chose the *TTAA* element *piggyBac*, and to create jumpstarters for *piggyBac*-based mutators, we used the *hAT* element *Hermes* or the *Tc1/mariner* elements *Mos1* and *Minos*. To drive expression of the transposase gene either the inducible Drosophila *hsp70* promoter (LIS *et al.* 1983) or the constitutive Drosophila *α1-tubulin* promoter (THEURKAUF *et al.* 1986) was used (Table 1).

Of the different jumpstarter constructs generated (see MATERIALS AND METHODS), the heat-shock-inducible *Hermes* jumpstarter *Bac{3xP3-ECFP, hsp70-Hermes}* and *piggyBac* jumpstarter *Mi{3xP3-DsRed, hsp70-piggyBac}*, as well as the constitutive *piggyBac* jumpstarters *Her{3xP3-ECFP, αtub-piggyBacK10}* and *Mos{3xP3-ECFP, αtub-piggyBacK10}* were tested, respectively, for remobilization of genome-integrated, nonautonomous *Hermes*

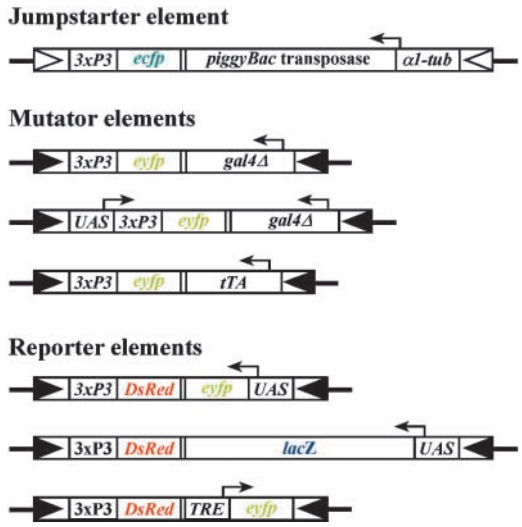

FIGURE 2.—Molecular structure of non-species-specific constructs for insertional mutagenesis and enhancer detection used in the Drosophila pilot screen. The *piggyBac*-jumpstarter element was integrated with a *Hermes* vector, whereas mutator and reporter elements used in this study were based on *piggyBac* vectors. For details on the different constructs see Table 1 and text.

or *piggyBac* elements (see MATERIALS AND METHODS). In this assay, all jumpstarter constructs proved functional (data not shown). Since stable and strongly expressed jumpstarter strains can be preselected before starting insertional mutagenesis screens, we chose to mark most of the jumpstarter constructs with the less-sensitive marker *3xP3-ECFP* (HORN and WIMMER 2000).

At what phylogenetic distance the Drosophila α*1-tubulin* promoter will stimulate sufficient transposase expression to mobilize mutator elements is difficult to estimate. However, the Drosophila *hsp70* promoter-based jumpstarters are also likely to work in non-Dipteran insects, since the *hsp70* promoter has been shown to mediate heat-shock-inducible gene expression in the lepidopteran silkmoth, *B. mori* (UHLÍŘOVA *et al.* 2002). Since *3xP3-ECFP* is a poor marker for *Tribolium castaneum* transgenesis (M. KLINGLER, personal communication) and since this might also hold true for other insect species, *Mi{3xP3-DsRed, hsp70-piggyBac}* was marked with the more easily selectable marker *3xP3-DsRed* (HORN *et al.* 2002). Moreover, this jumpstarter was specifically designed to contain the *hsp70* promoter toward the end of the transposon, which should allow for enhancer trap effects at different insertion sites. Therefore, even if the heat-shock promoter should be nonfunctional, the basal promoter region should suffice to obtain some functional jumpstarter strains based on suitable germline enhancers close to the insertion. It is interesting to note that even without heat-shock treatment, most Dm[*Mi{3xP3-DsRed, hsp70-piggyBac}*] strains mobilized nonautonomous, genome-integrated *piggyBac* elements, indicating that transposase activities are heat-shock independent and probably enhancer driven.

Mutator elements: To allow for effective insertional coverage in transposon mutagenesis screens, we marked the mutator elements with *3xP3-EYFP*, which represents a highly sensitive transformation marker (HORN *et al.* 2000; HORN and WIMMER 2000). When transposon mutagenesis is used *in vivo* to identify genes expressed in certain larval or adult tissues, novel insertions and enhancer activities should be detected simultaneously and noninvasively. To avoid frequent switching between different filter sets while screening, we also employed *EYFP* as a reporter gene either within the mutator itself or within separate reporter elements (see below). This excludes, however, *in vivo* enhancer detection in tissues such as the eyes and central nervous system and other tissues in which the artificial *3xP3* promoter drives marker gene expression (HORN *et al.* 2000).

To allow enhancer detectors to be directly employed for misexpression studies, we included genes in the mutators that encode heterologous transactivators (BRAND and PERRIMON 1993; BELLO *et al.* 1998). These primary reporter genes are controlled by the weak minimal promoter of the *P*-element transposase gene (RØRTH 1998), which is transcriptionally silent unless activated by an enhancer element (O'KANE and GEHRING 1987). The mutator elements are constructed with the basal promoter toward the end of the transposon (Figure 2). Genomic integration of the mutator near a tissue-specific enhancer will then allow the transactivator to be expressed in patterns similar to that of the gene normally under control of the detected enhancer.

In contrast to BRAND and PERRIMON (1993), we did not use the full-length yeast GAL4 gene when constructing GAL4-based mutators, but employed the coding region of the GAL4 deletion variant II-9 (MA and PTASHNE 1987). This deletion variant (GAL4Δ) contains both the amino-terminal DNA-binding domain and the carboxy-terminal activation domain of GAL4. GAL4Δ is almost as good an activator as GAL4 itself but supposedly more stable (G. STRUHL, personal communication). GAL4Δ-based mutators were generated in both *piggyBac* and *Hermes* backgrounds (see MATERIALS AND METHODS), but only *Bac{3xP3-EYFP, p-GAL4Δ-K10}*, hereafter abbreviated as *{GAL4Δ}*, was used in this study.

For *in vivo* identification of an enhancer's activity, GAL4Δ needs to drive the expression of a visibly detectable secondary reporter such as *EYFP*, whose coding region must therefore be placed under the control of GAL4-binding sites (referred to as *UAS*, for upstream activation sequence; BRAND and PERRIMON 1993). This can either be provided by a separate reporter element (Figure 1 and see below) or somehow be integrated into the mutator element itself. To employ *EYFP* as both a marker for novel insertions and as a reporter for enhancer detection, we placed an additional *UASp* promoter (RØRTH 1998) upstream of the *3xP3* promoter

TABLE 1

Summary of generated jumpstarter, mutator, and reporter constructs

Jumpstarter	*hsp70-Hermes*	*hsp70-piggyBac*	*αtub-piggyBac*	
Progenitors	pSLfa_hsp70-Hermes_fa	pSLfa_hsp70-piggyBac_fa	pSLfa_αtub-piggyBacK10_fa	
Final constructs	pBac{3xP3-ECFP, hsp70-Hermes}[a]	pHer{3xP3-ECFP, hsp70-piggyBac} pMi{3xP3-DsRed, hsp70-piggyBac}[a]	pHer{3xP3-ECFP, αtub-piggyBacK10}[a] pHer{3xP3-EYFP, αtub-piggyBacK10} pMos{3xP3-ECFP, αtub-piggyBacK10}[a] pMos{3xP3-EYFP, αtub-piggyBacK10}	
Mutator	*{GAL4Δ}*	*{GAL4Δ+UAS}*	*{tTA}*	*{tTA+TRE}*
Progenitors	pSLfa_p-GAL4Δ-K10_fa	pSLfa_p-GAL4Δ-K10_fa pBac{UASp-3xP3-EYFPafm}	pSLfa_p-tTA-K10_fa	pSLfa_p-tTA-K10_fa pBac{TRE-3xP3-EYFPafm}
Final constructs	pHer{3xP3-EYFP, p-GAL4Δ-K10} pBac{3xP3-EYFP, p-GAL4Δ-K10}[a]	pBac{UASp-3xP3-EYFP, p-GAL4Δ-K10}[a]	pBac{3xP3-EYFP, p-tTA-K10}[a]	pBac{TRE-3xP3-EYFP, p-tTA-K10}[a]
Reporter	*UASp-EYFP*	*UASp-lacZ*	*TRE-EYFP*	*TRE-lacZ*
Progenitors	pSLfa_UASp-eyfp-K10_fa	pSLfa_UASp-lacZ-K10_fa	pSLfa_TRE-EYFP-K10_fa	pSLfa_TRE-lacZ-K10_fa
Final constructs	pBac{3xP3-ECFP, UASp-EYFP-K10} pBac{3xP3-EYFP, UASp-EYFP-K10}[a] pBac{3xP3-DsRed, UASp-EYFP-K10}[a] pHer{3xP3-ECFP, UASp-EYFP-K10}[a] pHer{3xP3-EYFP, UASp-EYFP-K10}	pBac{3xP3-ECFP, UASp-lacZ-K10}[a] pBac{3xP3-EYFP, UASp-lacZ-K10} pBac{3xP3-DsRed, UASp-lacZ-K10} pHer{3xP3-ECFP, UASp-lacZ-K10}[a] pHer{3xP3-EYFP, UASp-lacZ-K10}	pBac{3xP3-ECFP, TRE-EYFP-SV40}[a] pBac{3xP3-DsRed, TRE-EYFP-SV40}[a]	pBac{3xP3-ECFP, TRE-lacZ-SV40}[a] pBac{3xP3-DsRed, TRE-lacZ-SV40}

[a] Constructs have been proven functional in transgenic Drosophila strains.

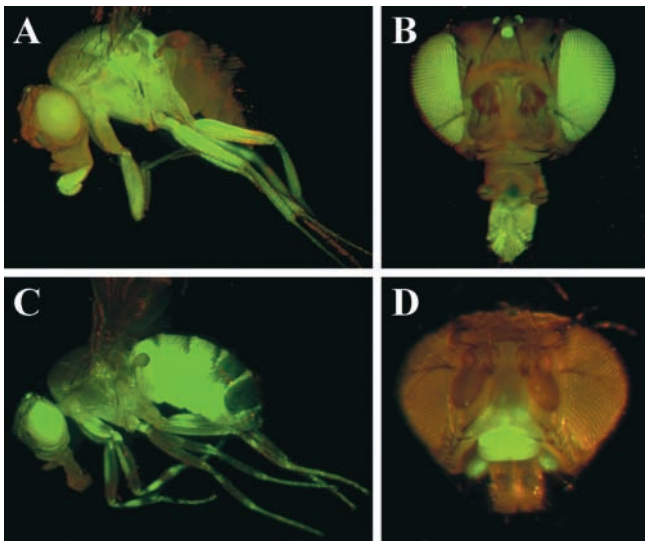

Figure 3.—Examples of adult enhancer activities isolated with the mutator-reporter element *{GAL4Δ+UAS}*. (A and B) Strain MM12.III detects an enhancer activity for the distal and medial part of the proboscis, the thorax, and the legs, (C) strain MM12.II for the abdomen and a specific pattern in the legs, as well as (D) strain MM12.II_2 for the basal part of the proboscis and the maxillary palps. Note that in this particular insertion strain the eye-specific marker expression is suppressed.

to generate *Bac{UASp-3xP3-EYFP, p-GAL4Δ-K10}*, hereafter referred to as *{GAL4Δ+UAS}*. Effective adult enhancer detection by this mutator could be observed when establishing transgenic strains, since many of them detected different enhancer activities (Figure 3). The high efficiency of visibly detecting enhancer activities with *{GAL4Δ+UAS}* might be due to the fact that in this construct the basal *P*-element promoter is actually placed at both ends of the mutator. This allows the genomic integration region to be scanned for enhancers with differently oriented basal promoters. In addition, the *3xP3* promoter (Horn *et al.* 2000) also occasionally serves as an enhancer detector. However, only the enhancer acting on the promoter driving *GAL4Δ* will serve as an expression tool for effector constructs.

At rare insertion sites of *{GAL4Δ+UAS}*, the *3xP3*-mediated eye expression of *EYFP* can actually be suppressed while another enhancer drives *EYFP* expression (Figure 3D). Should no defined sex-chromosomal insertions (Figure 1 and see below) be available in nonmodel organisms, such partially suppressed insertions could actually serve as ideal launching pads for insertional mutagenesis. After remobilization from such an insertion site, the restoration of eye-specific *EYFP* expression indicates that the mutator element has moved its position and thus novel insertions can be isolated. Similarly, the loss or change of a particular enhancer activity at the launching pad insertion could also serve this purpose.

We employed the bacterial-viral fusion tTA (Gossen and Bujard 1992), which has successfully been used to generate tetracycline-controlled binary expression systems in Drosophila (Bello *et al.* 1998; Thomas *et al.* 2000; Heinrich and Scott 2000; Horn and Wimmer 2003) as another transcriptional activator. The major advantage of this system is that targeted gene expression can be additionally controlled by a food supplement. The primary spatiotemporal control is provided by the detected enhancer as in the GAL4/*UAS* system, but here a secondary temporal control due to tetracycline-dependent inactivation of tTA makes it possible to switch off the system. Tetracycline and tTA form a complex, which prevents tTA from binding to its response element, which therefore becomes inactive (Gossen and Bujard 1992). The tTA-based mutator used in this study, *Bac{3xP3-EYFP, p-tTA-K10}*, is hereafter abbreviated as *{tTA}*.

As in the case of *{GAL4Δ+UAS}*, an integrated mutator-enhancer-detector for the binary tTA expression system was generated by placing a *TRE* promoter upstream of the *3xP3* promoter. However, when strains were generated with this *Bac{TRE-3xP3-EYFP, p-tTA-K10}* mutator (abbreviated *{tTA+TRE}*), enhancer activities were not detected. When driven by defined tTA driver constructs, *{tTA+TRE}*-mediated *EYFP* expression was actually enhanced compared to simple *TRE-EYFP* reporter constructs (data not shown). This suggests a positive feedback loop between the *TRE* sites and the basal promoter driving tTA expression and indicates that both *TRE* and tTA are functional in this construct. The lack of enhancer detection suggests a lower sensitivity of the tTA/*TRE* system compared to GAL4/*UASp*. *{tTA+TRE}* was therefore not used further in this study.

For the mutators *{GAL4Δ}*, *{GAL4Δ+UAS}*, and *{tTA}*, several X chromosomal insertions were obtained either directly after injection or after remobilization from autosomal insertion sites. For each mutator, three independent remobilizable and homozygous viable X chromosomal insertion strains were used in the Drosophila pilot screen (see below). During these pretests one X chromosomal *{tTA}* insertion was identified that could not be efficiently remobilized. The respective strain was not further analyzed but excluded from this study.

Reporter elements: To easily detect enhancer activities by *{GAL4Δ}* or *{tTA}* insertions, the transactivator needs to drive the expression of a secondary reporter, *e.g.*, *EYFP* for *in vivo* analysis or the bacterial *lac*Z gene for *in situ* analysis (O'Kane 1998). For this purpose, we generated separate reporter elements by placing these secondary reporter genes under the control of the *UASp* promoter (Rørth 1998) or the *TRE* promoter (Gossen and Bujard 1992). Of the different *3xP3-ECFP*- and *3xP3-EYFP*-marked reporters generated, *Bac{3xP3-EYFP, UASp-EYFP-K10}*, *Her{3xP3-ECFP, UASp-EYFP-K10}*, *Bac{3xP3-ECFP, UASp-lacZ-K10}*, and *Her{3xP3-ECFP, UASp-lacZ-K10}* were injected and the resulting strains were successfully tested (Table 1).

When testing strains for the reporter *Her{3xP3-ECFP, UASp-EYFP-K10}* by using defined GAL4 driver strains, we noted that not only *EYFP* was expressed as expected, but also *ECFP* (data not shown). This indicates that UAS-bound GAL4 can also activate transcription at the *3xP3* promoter, despite the respective downstream and distant location (see Figure 2 for composition of UAS reporters). In contrast, strains carrying the reporter *Her{3xP3-ECFP, UASp-lacZ-K10}* did not mediate GAL4-driven *ECFP* expression, suggesting that the larger distance between the *UASp* sites and the *3xP3* promoter (due to the longer *lac*Z gene; Figure 2) prevents activation of the *3xP3* promoter by *UASp*-bound GAL4. In these constructs, *3xP3* promoter activity seems to drive only the fluorescent marker placed directly downstream, which suggests that *3xP3* represents a proximal promoter element that cannot function as an enhancer element at greater distances.

Nonetheless, these results indicate that in the mutator element *{GAL4Δ+UAS}*, positive feedback loops can occur, which could allow strong and enduring enhancer detection after the loop has first been initiated. *{GAL4Δ+UAS}* could thus serve as an "enhancing reporter" that would allow (i) a more sensitive detection of enhancers and (ii) the detection of enhancers active at earlier stages. The enhancer activity might actually have already stopped, but it is still detectable due to the positive feedback loop of the reporter. Hassan *et al.* (2000) have previously described the use of two independent constructs, an *enhancer-GAL4* and a *UAS-GAL4* construct, to create a positive feedback loop for fate mapping and signal amplification. *{GAL4Δ+UAS}* differs in that it combines enhancer detection and signal amplification within one single construct. The positive feedback can be an advantage, since it allows screening for genes active at early stages of organogenesis during late developmental stages of that same organ. However, for tissues that are sensitive to high levels of GAL4Δ expression, this enhancing reporter might be detrimental and respective enhancers will not be detectable due to toxicity.

Strains with the *3xP3-DsRed*-marked reporters *Bac{3xP3-DsRed, UASp-EYFP-K10}*, *Bac{3xP3-DsRed, UASp-lacZ-K10}*, *Bac{3xP3-DsRed, TRE-EYFP-SV40}*, and *Bac{3xP3-DsRed, TRE-lacZ-SV40}* have been further used in this study (Figure 1 and 2). When we used *EYFP* as the reporter gene (Figure 1), adult enhancer activities could be detected noninvasively while screening for novel *3xP3-EYFP*-marked insertions without the need for switching fluorescence filter sets. This allowed straightforward screening of enhancer activities for specific tissues without the need of establishing individual insertion strains beforehand. Nevertheless, the maturation time for internal cyclization and oxidation causes a delay of several hours before *EYFP* fluorescence can be detected (Davis *et al.* 1995). This usually presents no problem when screening for larval or adult enhancer activities, but when we were interested in embryonic enhancer activities, we applied the more sensitive and faster-responding reporter gene *lac*Z. This, however, required the establishment of individual insertion strains first, since the embryos do not survive the necessary fixation and staining procedures.

Pilot screen in *D. melanogaster*: To test the balancer-free insertional mutagenesis scheme for enhancer detection (Figure 1), we performed a small pilot screen in *D. melanogaster*. We decided against *Mos1*-based mutators, since it was shown that genomic insertions of transgenic *Mos1* constructs can rarely be remobilized in Drosophila (Lozovsky *et al.* 2002). We decided against *Hermes*-based mutators, despite the fact that remobilization actually works well in Drosophila (data not shown), only because *Hermes* insertions can be remobilized by *hobo* elements (Sundararajan *et al.* 1999). Since not all Drosophila stocks are free of *hobo* (Blackman *et al.* 1989), novel *Hermes* insertions might become unstable in future analyses. For the application of *Hermes*-based insertional mutagenesis in Drosophila, special care should be taken to use *hobo*-free strains only.

In our Drosophila pilot screen, we chose the *3xP3-EYFP*-marked *piggyBac*-based mutators *{GAL4Δ}*, *{GAL4Δ+UAS}*, and *{tTA}*, for which we used three homozygous X chromosomal launching integrations each. To remobilize these mutators from the X chromosomes, we selected three different strains carrying the homozygous constitutive jumpstarter *pHer{3xP3-ECFP, αtub-piggyBacK10}* on the second or third chromosome. This allowed us to dispense with heat-shock protocols. In addition, to detect potential enhancer activities by novel *{GAL4Δ}* and *{tTA}* insertions, we employed homozygous strains carrying, on the second or third chromosome, the *3xP3-DsRed*-marked reporters *Bac{3xP3-DsRed, UASp-EYFP-K10}*, *Bac{3xP3-DsRed, UASp-lacZ-K10}*, or *Bac{3xP3-DsRed, TRE-EYFP-SV40}*, respectively (Figure 2).

The different fly strains were crossed as depicted in Figure 1: For each mutator, nine different mutator-jumpstarter-strain combinations were set up (Figure 1, G_1 cross; see also legend to Table 2). To maximize identification of independent transposition events for each combination, 27 single males carrying both jumpstarter and mutator were crossed to virgins of the respective EYFP-reporter strains Dm[*Bac{3xP3-DsRed, UASp-EYFP-K10}*] or Dm[*Bac{3xP3-DsRed, TRE-EYFP-SV40}*] (Figure 1, G_2 cross) or a *white* strain in the case of *{GAL4Δ+UAS}*. All male G_3 progeny that show the mutator marker *3xP3-EYFP* must carry novel autosomal insertions, since the originally X chromosomal launching insertion is not paternally inherited by males. This scheme, therefore, allows for straightforward identification of novel insertion events, which in some cases also led to the detection of adult enhancer activities that could be identified concurrently (Figure 1, G_3).

For each mutator, the results of nine combinations of jumpstarter and mutator strains are presented together, since no significantly different performance rates were observed between the individual combinations (Table 2): Simple excision events observed in the female prog-

TABLE 2

Transposition frequencies of *{GAL4Δ}*, *{GAL4Δ+UAS}*, and *{tTA}* mutator elements

Mutator element	G_2 crosses[a]	Jumping rate[b] (%)	Enhancer detection[c] (%)	Stable new insertions[d] (%)	Σ males[e]	Transposition frequency[f]	Excision rate[g] (%)
{GAL4Δ}	237	92 (81–100)	47	67	11,405	0.123 ± 0,031	96–100
{GAL4Δ+UAS}	225	72 (36–96)	51	45	9,153	0.084 ± 0,048	48–100
{tTA}	227	80 (60–96)	2	41	9,500	0.130 ± 0,055	56–100

For each mutator element, three independent homozygous strains were crossed to three independent homozygous jumpstarter strains (nine combinations as in Figure 1, G_1 cross): Dm[*{GAL4Δ}*] strains: M8-2x, M19-2x, M19-3x. Dm[*{GAL4Δ+UAS}*] strains: M5.x, FM26.x, FF36.x. Dm[*{tTA}*] strains: x.2, x.5, x.7. Dm[*Her{3xP3-ECFP, αtub-piggyBacK10}*] jumpstarter strains: M1.III, M6.II, M10.III. Per combination, 27 single male crosses were set up as shown in Figure 1 (G_2 cross).

[a] Total number of fertile single male G_2 crosses (of 243 crosses per mutator).

[b] Jumping rate is the percentage of (G_2 crosses with at least one 3xP3-EYFP$^+$ son)/(fertile G_2 crosses) (Berg and Spradling 1991); the mean value and the range of nine independent combinations are indicated.

[c] Percentage of (G_2 crosses with at least one *3xP3-EYFP*$^+$ son detecting an adult enhancer activity)/(fertile G_2 crosses).

[d] Percentage of [G_2 crosses with at least one *3xP3-EYFP*$^+$ and *3xP3-ECFP*$^-$ (no jumpstarter) son]/(fertile G_2 crosses).

[e] Total number of G_3 males.

[f] Transposition frequency [(Σ *3xP3-EYFP*$^+$ males)/(Σ males)] × 9/4 (Berg and Spradling 1991), since only four of nine mutated chromosome arms have been recovered. Standard error of the mean is indicated.

[g] Excision rate is the percentage of (G_2 crosses with at least one *3xP3-EYFP*$^-$ daughter)/(fertile G_2 crosses), the range of nine independent combinations is indicated.

eny (loss of *3xP3-EYFP* marker) were between 48 and 100%. The observed transposition frequency was high with an average >10%, thus indicating that *piggyBac* remobilization was highly effective. In ~80% of the single G_2 crosses, novel autosomal insertions could be identified. This represents an average jumping rate for *piggyBac* mutators that lies within the best rates observed for *P*-element constructs (Berg and Spradling 1991). Due to concomitant jumpstarter presence in some of the mutator-carrying G_3 males, not all of the 80% could be used to establish stable new insertion lines. However, novel insertions could be stably established from ~50% of the single G_2 crosses. This suggests that *piggyBac* can serve as an excellent insertional mutagenesis agent in *D. melanogaster* and might actually outperform *P* elements.

Adult and larval enhancer detection: In ~50% of the crosses with the GAL4-based mutators *{GAL4Δ}* and *{GAL4Δ+UAS}*, adult enhancer activities could be detected (Table 2). These enhancers activated gene expression in diverse body parts. Head-specific, thorax-specific, abdominal-specific, or leg-specific enhancers were observed (Figure 3). This indicates that *EYFP* reporters can be used efficiently to noninvasively detect adult enhancer activities; thus, genes expressed specifically in adult organs can easily be screened for without having to establish numerous insertion lines beforehand. This will be of importance for nonmodel organisms, since keeping a substantial number of independent insertion lines might not be manageable for arthropod species that are more complicated than Drosophila to rear.

{GAL4Δ+UAS} shows slightly reduced jumping rates and transposition frequencies (Table 2). This might be due to some slight toxicity when positive feedback loops cause high GAL4Δ expression. Since this integrated *{GAL4Δ+UAS}* mutator-reporter allows enhancer detection without crossing to separate reporter strains, we also investigated the established *{GAL4Δ+UAS}* insertion lines for larval enhancer activities. Figure 4 shows that various tracheal, fat body, or muscle enhancers were detected, indicating that *{GAL4Δ+UAS}* also serves as an excellent enhancer detector for larval tissues in Drosophila.

When using the integrated *{GAL4Δ+UAS}* mutator-reporter, an additional reporter construct is not needed. Only two elements, a jumpstarter and *{GAL4Δ+UAS}*, are required to screen for enhancers. In this case, *{GAL4Δ+UAS}* can be combined with *3xP3-DsRed*-marked jumpstarters to avoid using the weak *3xP3-ECFP* marker, which seems not to be suitable for all insect species. *{GAL4Δ+UAS}* and the jumpstarter *Mi{3xP3-DsRed, hsp70-piggyBac}* might actually be an ideal starting system for a first insertional enhancer detection screen in a nonmodel arthropod.

In contrast, *{tTA}* mutators only rarely detected enhancers (2%; Table 2). This further indicates that the tTA/*TRE* system has a much lower sensitivity for enhancer detection compared to GAL4/*UASp*. tTA/*TRE*-based constructs are therefore not reliable enough to screen for tissue-specific enhancers. This is unlikely to be due to higher toxicity of the tTA transactivator, since *{tTA}* jumping rates and transposition frequencies were similar to *{GAL4Δ}*. The low sensitivity of the tTA/*TRE* system is probably due to a lack of effective expression amplification in this binary system.

Insertional mutagenesis to identify novel Drosophila gene functions: To determine if *piggyBac* insertional mutagenesis screens could advance the Drosophila gene

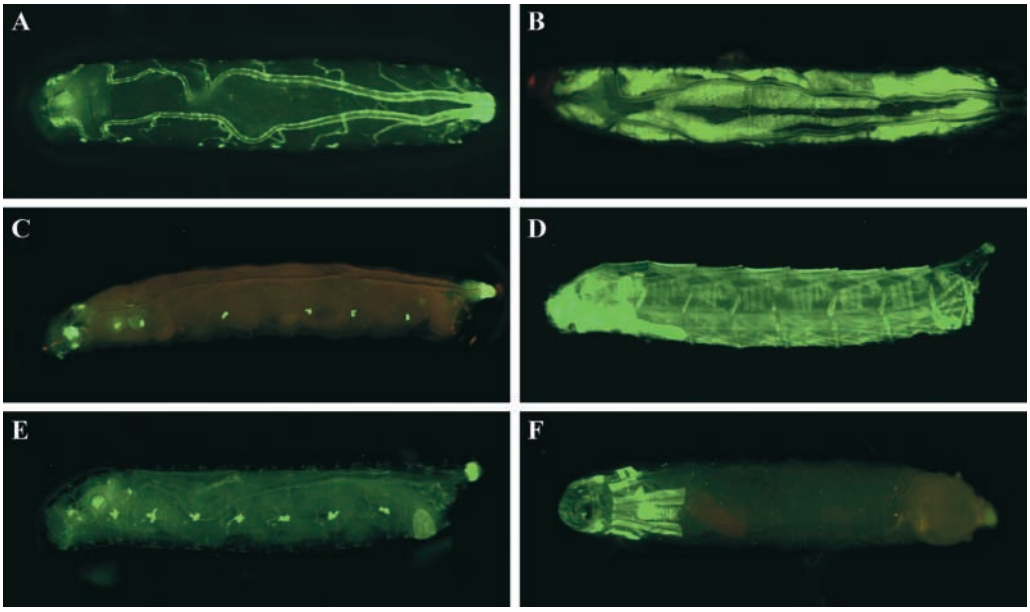

FIGURE 4.—Examples of larval enhancer activities isolated with the mutator-reporter element *{GAL4Δ+UAS}*. (A) Line 258 shows fluorescence in the tracheal system, (B) line 254 in the fat body, (C) line 249 in the posterior spiracles and in some unidentified lateral cells, (D) line 251 in muscles, (E) line 252 in the posterior spiracles and in a subset of the peripheral nervous system, which differs from marker-mediated fluorescence (HORN *et al.* 2000), and (F) line 257 in a subset of thoracic muscles.

disruption project, we further analyzed the autosomal *{GAL4Δ}* and *{tTA}* insertions. By segregation analysis, we identified the chromosomal location for 236 novel insertions and balanced them (see MATERIALS AND METHODS). When testing for recessive lethality, 4 insertions showed semilethality (homozygous progeny <10%) and 14 insertions were homozygous lethal (Table 3). This corresponds to a frequency of 7.6% for lethal or semilethal insertions, which is slightly lower than that observed in mutagenesis screens with *P* elements (BELLEN 1999; PETER *et al.* 2002). Of these 18 insertions, the genomic localization was determined by inverse PCR (HUANG *et al.* 2000), sequencing, and BLAST searches against the Drosophila genome sequence (Release 2; http://www.fruitfly.org/annot/release2.html). Molecular data confirmed the genetically determined chromosomal localization. The insertion in line 128 actually lies in an as-yet-unaligned scaffold, which, based on our genetic data, should be part of the third chromosome (Table 3).

Insertions could be identified in well-characterized genes, like *schnurri* (*shn*; line 166; STAEHLING-HAMPTON *et al.* 1995) or *tramtrack* (*ttk*; line 150; BROWN and WU, 1993), but also in noncharacterized *CG*-number genes or in genes for which no mutations have previously been identified, *e.g.*, line 52 insertion in gene *HLH106* (THEOPOLD *et al.* 1996; ROSENFELD and OSBORNE 1998). Within the genes, the insertions were found in the 5′ UTR, in exons, in introns, and downstream of them, but also in intergenic regions. The frequent insertion within introns actually makes *piggyBac* an ideal candidate for the design of protein trap systems (MORIN *et al.* 2001). For many of the targeted genes, no *P*-element alleles have previously been identified and sometimes the closest *P*-element insertion referenced in GadFly lies ~30 kb away. Table 3 shows further details on the different insertions.

To ascertain if the recessive lethality was correlated with the *piggyBac*-mutator insertion, we performed excision experiments by crossing the lethal lines with a jump-starter strain. We then isolated chromosomes carrying an excision event and tested them for lethality over the original insertion chromosome. In 9 of the 14 lethal insertion lines, the recessive lethality could be reversed, indicating that the mutator insertion was indeed the cause of the lethality phenotype. For the other 5 lines, the lethal mutation was not associated with the *piggyBac* insertion. This may be due to mutations in the background, since the employed chromosomes were not recently isogenized. Thus, 3.8% (9 out of 236) of the established novel insertion lines caused reversible lethal mutations, which is within the range observed in *P*-element mutagenesis screens (PETER *et al.* 2002).

The *ttk* insertion (line 150) is allelic to the lethal alleles ttk^{1} and ttk^{1e11}. The associated lethality can be reverted by mutator excision; thus, the intron-localized line 150 represents a true allele of *ttk*. This indicates that *piggyBac* insertional mutagenesis screens can be applied to mutate, to isolate, and to identify specific gene functions. In contrast, line 166, with the insertion in an intron of *shn*, is not allelic to the lethal alleles shn^{1} and shn^{04738}, and the lethality cannot be reverted by excision of the mutator. Therefore the lethality must derive from another recessive mutation on the chromosome.

To determine if *{GAL4Δ}* insertions could also serve as embryonic enhancer detectors, we crossed the lethal insertion lines (Table 3) to a strain carrying the reporter *Bac{3xP3-DsRed, UASp-lacZ-K10}* and performed X-gal stainings (O'KANE 1998) on their embryonic progeny. Figure 5 shows the detected enhancer activities for tis-

TABLE 3

Novel lethal and semi-lethal insertions of *{GAL4Δ}* and *{tTA}* mutator elements

Stock		Location of insertion[a]					Gene			Relation to *P* elements	
Line	Mutator	Genetic	Molecular			Reversion	Name		Functional part	*P* allele of gene?	Distance to next *P* insertion in GadFly
						A. Lethal					
29	*{GAL4Δ}*	II	2L	AE003628.1	118436	Yes	*CG5367*		Intron	No	27.7 kb, *l(2)k06709*
43	*{GAL4Δ}*	III	3R	AE003764.2	225029	Yes	*CG14066*	*larp*	Exon	No	3.2, kb, *l(3)06487*
51	*{GAL4Δ}*	III	3L	AE003532.2	126258	Yes	*CG13462*		Downstream	No	13.9 kb, *l(3)rO220*
52	*{GAL4Δ}*	III	3L	AE003515.2	16226	Yes	*CG8522*	*HLH106*	Exon	No	0.9 kb, *P{SUPor-P}KG03723*
80	*{GAL4Δ}*	II	2L	AE003669.2	232068	Yes	*CG8676*	*Hr39*	Intron	Yes	5.4 kb, *P{EP}Hr39[EP2490]*
127	*{GAL4Δ}*	II	2R	AE003795.2	3979	Yes	*CG9277*	*betaTub56D*	Intron	Yes	0.05 kb, *P{EP}betaTub56D[EP2640]*
128	*{GAL4Δ}*	III	U	AE003072.2	6877	Yes	not annotated[b]		EST	No	ND
139	*{GAL4Δ}*	II	2R	AE003462.1	161661	Yes	*CG3735*		Exon	No	0.5 kb, *P{EP}EP2311*
150	*{GAL4Δ}*	III	3R	AE003779.1	136330	Yes	*CG1856*	*ttk*	Intron	Yes	0.9 kb, *P{EP}ttk[EP621]*
151	*{GAL4Δ}*	II	2L	AE003623.1	248959	No	—		Intergenic	—	5.3 kb, *P{GT1}BG02711*
155	*{GAL4Δ}*	II	2L	AE003647.1	129102	No	*CG18482*		Intron	No	31.9 kb, *P{GT1}BG00946*
166	*{GAL4Δ}*	II	2R	AE003826.2	25487	No	*CG7734*	*shn*	Intron	Yes	1.3 kb, *P{EP}shn[EP2139]*
189	*{tTA}*	II	2R	DMU04853.3	11602	No	—		Intergenic	—	11.3 kb, *P{EP}EP2427*
233	*{tTA}*	III	3R	AE003770.1	165475	—[c]	*CG7581*	*Bub3*	5′ UTR	No	6.7 kb, *P{SUPor-P}KG01353*
						B. Semilethal					
185	*{tTA}*	II	2R	AE003799.2	60718	ND	*CG5170*	*Dp1*	Intron	Yes	0.4 kb, *P{EP}Dp1[EP2249]*
199	*{tTA}*	II	2L	AE003611.2	193888	ND	—		Intergenic	—	17.2 kb, *P{EP}Beach1[EP2299]*
214	*{tTA}*	II	2R	AE003785.2	3663	ND	—		Intergenic	—	1.7 kb, *P{EP}BcDNA[EP2646]*
236	*{tTA}*	III	3R	AE003749.2	46443	ND	*CG6875*	*asp*	Exon	Yes	11.3 kb, *P{GT1}BG02218*

ND, not determined. EST, expressed sequence tag.

[a] Sequence numbers and nucleotide positions refer to the Release 2 sequence of the Drosophila genome (http://www.fruitfly.org/annot/release2.html).

[b] Clot of 21 ESTs annotated; no *P* elements in genomic scaffold (length 12,500 bp) of this "U-region".

[c] Excisions not obtained (600 flies screened for absence of *EYFP*).

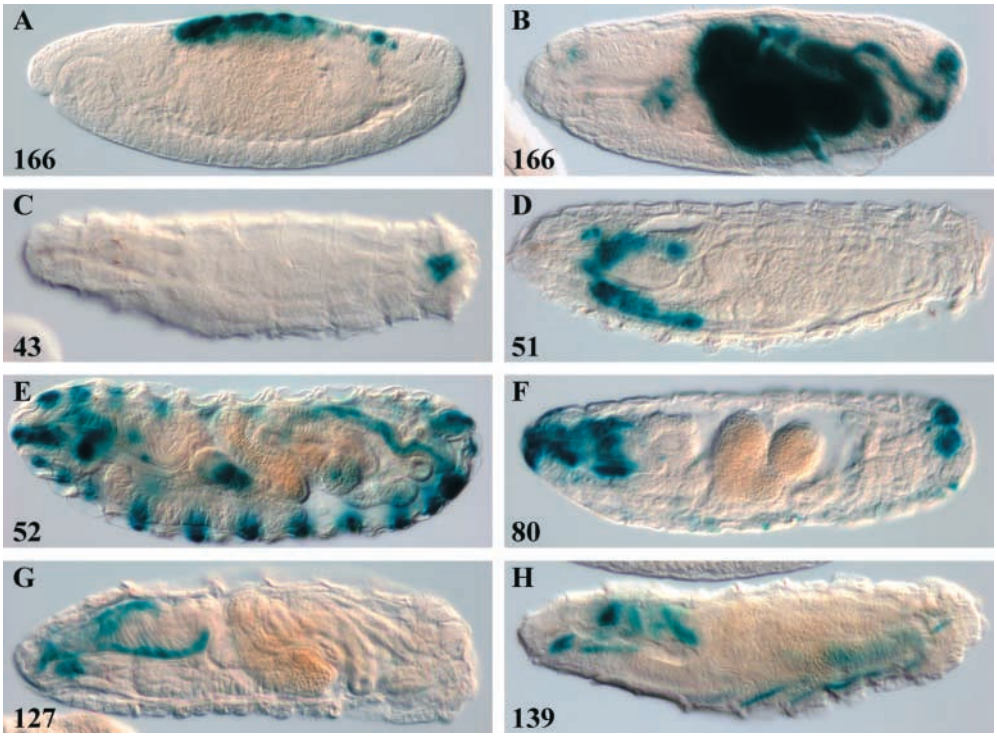

FIGURE 5.—Embryonic enhancer activities from lethal insertions (Table 2) of the *{GAL4Δ}* mutator element detected by X-gal staining. (A and B) Line 166 is an insertion into an intron of the *shn* gene. The insertion does not cause a mutation of the gene, but detects some of its enhancers. The enhancers are detected with significant delay: the dorsally restricted blastoderm expression can be detected in the amnioserosa at the germ-band retraction stage (A), and the endoderm-specific expression of *shn* at that stage can be detected in the gut after dorsal closure (B). (C) Line 43 shows an enhancer activity for the posterior spiracles, (D) line 51 for the salivary glands, (E) line 52 for a segment polarity pattern, (F) line 80 for the head and posterior spiracles, (G) line 127 for the pharynx, and (H) line 139 for specific muscles.

sues like salivary glands, specific muscles, segmental patterns, etc. Despite the fact that line 150 represents a *ttk* allele, no *ttk*-like expression pattern was observed. In this case *{GAL4Δ}* does not detect the enhancers of the gene in which it is inserted. In contrast, in line 166 *{GAL4Δ}* does detect *shn*-specific enhancers and drives *lac*Z expression in *shn*-like patterns. Thus, despite not creating a *shn* allele, *{GAL4Δ}* is picking up the enhancers of the gene. *{GAL4Δ}* can therefore be used to isolate genes on the basis of embryonic enhancer activities. However, when comparing the endogenous expression pattern of *shn* (STAEHLING-HAMPTON *et al.* 1995) with the GAL4-mediated *lac*Z expression, we recognized a significant time delay. While *shn* shows dorsal-specific expression during blastoderm stages, the matching enhancer-mediated *lac*Z expression cannot be detected until germ-band retraction (Figure 5A). *shn* is gut-specifically expressed during germ-band retraction, but the corresponding *lac*Z expression cannot be detected until dorsal closure and head involution are complete (Figure 5B). This delay might actually be the reason why most observed enhancer activities are detectable only at late embryonic stages (Figure 5).

Molecularly precise cut-and-paste mechanism of *piggyBac*: When the genomic localization of the insertions was determined by inverse PCR, the obtained genomic 5′ and 3′ sequences always matched to the same insertion site, indicating that only single insertions have been observed so far despite the high transposition rate of *piggyBac*. This is consistent with the nonreplicative, conservative cut-and-paste transposition mode described for *piggyBac* (LOBO *et al.* 1999). To molecularly analyze *piggyBac* excision sites, PCR reactions were performed on successfully excised chromosomes from lines 29, 51, and 139. Our data confirm that *piggyBac* excision is molecularly precise and does not leave a footprint behind, which is unique among eukaryotic class II transposons (ELICK *et al.* 1996). The original *TTAA* target site, which is duplicated upon insertion of the *piggyBac* element, is left as a single *TTAA* site after the element has been excised.

Imprecise excision events have not as yet been detected. This is disadvantageous compared to *P* elements, since it is unlikely that small deletions can be generated with *piggyBac*. Nevertheless, this might be overcome by including *P* ends into *piggyBac*-based mutators. Furthermore, the precise excision might actually be an advantage in the case that *piggyBac* would show hot-spot behavior like *P* elements (SPRADLING *et al.* 1999). This behavior causes problems with *P* elements, since often the induced mutation does not correspond to the final integration site but to a previous insertion site, where it causes a mutation by imprecise excision. Thus even if the *P* element is not finally localized at a hot spot, the caused mutation often maps to it. This makes the isolation of novel mutant insertions with *P* elements rather difficult. Molecularly precise excision events as observed with *piggyBac* will avoid these problems.

Over all, *piggyBac* insertions behave properly in Drosophila. The aforementioned X chromosomal insertion of *{tTA}* that was not efficiently remobilizable and line 233, in which the *{tTA}* mutator could not be excised (Table 3), are the only two cases we observed so far. We therefore conclude that *piggyBac* can serve as a reli-

able tool in insertional mutagenesis approaches for the Drosophila gene disruption project.

Concluding remarks: Despite the limitations of our small pilot screen, it can be expected that *piggyBac*-based mutator elements will allow the isolation of novel gene functions through the identification of insertions in previously untargeted gene loci of Drosophila. Even within our limited screen, which detected nine novel mutator-caused lethal insertions, genes and chromosomal regions were targeted to which no *P* element has gone before. Moreover, transposon mutagenesis with *piggyBac* mutators could even be carried out in the presence of *P* elements, as in *FRT*-based mosaic (Xu and Rubin 1993) or eye misexpression screens (Karim *et al.* 1996). This would facilitate the isolation of previously unidentified gene functions in organogenesis and other late developmental processes.

Furthermore, the presented *piggyBac*-based insertional mutagenesis and enhancer detection system has been generated using broad-range transposable elements and widely applicable fluorescent transformation markers (Horn *et al.* 2002). This allows for utilization of the system in nonmodel insects and will therefore enable the identification of gene functions in many different biological processes. Initial experiments to test for remobilization of genome-inserted *piggyBac* insertions have already been successfully performed in the red flour beetle *T. castaneum* (A. Berghammer and M. Klingler, personal communication). The visible markers will facilitate stock keeping of mutant and enhancer detector lines, which is especially important for "balancer-less" nonmodel arthropods. Identification and isolation of insertional mutations is much simpler than that of chemically induced mutations, since the mutated genes or detected enhancers are molecularly tagged. This will facilitate cloning of the affected genes and thus help to rapidly correlate sequence data with biological functions, which is of key importance for successful functional genomics approaches in insects.

In addition, insertional mutagenesis and enhancer detection systems will help to identify *cis*-regulatory sequences from important agricultural pest species such as the Mediterranean fruit fly *Ceratitis capitata*. The isolation of sex-specific enhancers would make it possible to develop female lethality systems, which could be employed to generate male-only strains (Heinrich and Scott 2000; Thomas *et al.* 2000). In addition, early embryonic enhancers could be isolated for the development of embryonic lethality systems to generate competitive males for a transgenic sterile insect technique (Horn and Wimmer 2003).

Moreover, a major advantage of this elaborate insertional mutagenesis system is that it not only identifies interesting enhancers, but also at the same time provides tools to drive gene expression. Once enhancers of interest have been identified, they can be used to express any cloned gene as an effector in the respective embryonic, larval, or adult tissues and the effect of the expression on the tissues can be examined. In medically important disease vectors like the yellow fever mosquito *Aedes aegypti* or in Anopheles malaria mosquitoes, this will make it possible (i) to identify gut or salivary gland specific enhancers and (ii) to employ them for the expression and examination of peptides regarding their ability to block the transmission of these diseases (Ito *et al.* 2002).

piggyBac seems a good candidate for insertional mutagenesis and enhancer detection screens in a broad range of insect species. It should be noted, however, *piggyBac* shows some phylogenetic distribution (Handler 2002) and might therefore not be usable in all insect species. Even though stable *piggyBac* integration can be obtained in species that carry closely related transposable elements (Handler and McCombs 2000), the introduction of an active *piggyBac* transposase in such a species might trigger an uncontrollable insertional mutagenesis. Moreover, insertions will be hard to characterize molecularly. Consequently, other broad-range transposable elements, like *Hermes*, *Minos*, and *Mos1*, should also be further developed for insertional mutagenesis and enhancer detection systems. These will present invaluable tools to correlate biological functions with the explosion of DNA sequence information soon to emerge from the field of insect genomics. Functional genomics will rely heavily on possibilities of identifying gene expression patterns and of characterizing mutations of the proposed genes to understand their molecular and physiological roles.

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