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-tubulin promoter. As primary reporters in the piggyBac mutator elements, we employed the heterologous transactivators GAL4 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 GAL4/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=agambie.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 silkworm, Bombyx mori (http://www.ab.a.u-tokyo.ac.jp/lept-genome/; http://www.ab.a.u-tokyo.ac.jp/silkbase/), as well as the honey bee, Apis mellifera ligustica (http://www.ncbi.nlm.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.imagen 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...
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-EGFP, 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; Rorth 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 non-model insects.

MATERIALS AND METHODS

Versatile two-step cloning procedure: In principle, we compose our constructs in the cloning shuttle vector pSLfa1180 (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 Scal site of pbac[3xP3-ECFP, pm] (Horn and Wimmer 2000) an Fse fragment from pSLfa_hsp70-Hermes_pm, which resulted from an Asp718/SpeI fragment from pKHSHH (Sarkar et al. 1997) cloned into Asp718/SpeI-cut pSLfa1180 (Horn et al. 2000; Zagoraiou et al. 1998). pHer[3xP3-ECFP, hsp70-piggyBac] and pMi[3xP3-DsRed, hsp70-piggyBac] were generated by cloning into the AscI site of pHer[3xP3-ECFP, pm] (Horn and Wimmer 2000) and pMi[3xP3-DsRed], respectively, an AscI fragment from pSLfa_hsp70-piggyBac_pm, which was the result of an EcoRI/HindIII fragment from pbac (Handler and Harrell 1999) cloned into EcoRI/HindIII-cut pSLfa1180 (Horn et al. 2000). pMi[3xP3-DsRed] was constructed by cloning an EcoRI/Ard fragment from pSL-3xP3-DsRed (Horn et al. 2002) into pmi[3xP3-EYFP] (provided by C. Savakis), thereby replacing ryfp 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, atub-piggyBacK10], pHer[3xP3-EYFP, atub-piggyBacK10], pMos[3xP3-ECFP, atub-piggyBacK10], and pMos[3xP3-EYFP, atub-piggyBacK10] were generated by cloning into the AscI site of pHer[3xP3-ECFP, pm], pHer[3xP3-EYFP, pm], pMos[3xP3-ECFP, pm], and pMos[3xP3-EYFP, pm] (Horn and Wimmer 2000), respectively, an AscI fragment from pSLfa_atub-piggyBacK10_pm (provided by Exelixis, S. San Francisco). For injection, pHer[3xP3-ECFP, atub-piggyBacK10] (transposase gene in opposite orientation to marker gene) and pMos[3xP3-ECFP, atub-piggyBacK10] (transposase gene in same orientation as marker gene) were chosen.

Mutator elements: pHer[3xP3-EYFP, pGALA4-K10] and pbac[3xP3-EYFP, pGALA4-K10] were constructed by cloning into the AscI site of pHer[3xP3-EYFP, pm] and pbac[3xP3-EYFP, pm] (Horn and Wimmer 2000), respectively, an AscI fragment from pSLfa_pGALA4-K10_pm. For injection of pbac[3xP3-EYFP, pGALA4-K10], a construct with the GAL4 gene in opposite orientation to the marker gene was chosen. To generate pSLfa_pGALA4-K10_pm, the coding region of a GAL4 deletion variant (II9; Ma and Prashine 1987) was cloned as a 1.1-kb Asp718 fragment from construct G610 (designed by G. Struhl, provided by G. Vorbrüggen) into the Asp718 site of pSLfa_pK10_pm, which was obtained by inserting the 3′ untranslated region (UTR) of K10 as an Asp718/PstI fragment from pUASp (Rorth 1998) into Asp718/PstI-opened pSLfa_pK10_pm, which was generated by inserting the Kelement promoter and first intron as a XhoI/Asp718-cut PCR fragment from pBluescripts-GAGA-GAG4-Pprom+introns (primers 5′-CCGCTCGAGTCG ATAGCGGAAGCTTACC-3′ and 5′-GGGGTACCATGAAACA GGACCTAAGCA-3′; Rorth 1998) into SalI/Asp718-cut pSLfa1180 (Horn et al. 2000; Zagoraiou et al. 1998).
pBac[UA3p-3xP3-EYFP, p-GAL4a-K10] was generated by cloning an Adsl/Fsd fragment from pSLfa_p-GAL4a-K10_fa into the Asd/Fsd sites of pBac[UA3p-3xP3-EYFPam] that resulted from the cloning of the 0.1-kb Fsa/BglII fragment from pSLfa1180fa into Fsa/BglII-opened pBac[UA3p-3xP3-EYFPam], which was cloned by inserting into the HpaI site of pE3I.2 (Cary et al. 1989) the EcoRI (Klenow-blunted)/Nrdl fragment from pSLUA3p-3xP3-EYFPam, which was the result of cloning an EcoRI/BsiZ17I fragment from pSLfa_UASp_fa into EcoRI/Smal-opened pSL-3xP3-EYFPam (Horn and Wimmer 2000). pSLfa_UASp_fa was constructed by inserting into Smal (Klenow-blunted)/AspI-cut pSLfa1180a a Nodl (Klenow-blunted)/AspI fragment from blueScriptGAGA-GAL4a-prom-intron (p1936, provided by P. Rorth). pBac[3xP3-EYFP, p-TA-K10] was generated by cloning into the Asd/Fsd sites of pBac[3xP3-EYFPam] an Asd/Fsd fragment from pSLfa_p-TA-K10_fa, which was constructed by inserting an EcoRI (Klenow-blunted)/BamHI fragment from pTet-Off (CLONTECH, Palo Alto, CA) into Nodl (Klenow-blunted)/BamHI-opened pSLfa_p-K10_fa.

Reporter elements: pBac[3xP3-EYFP, UA3p-EYFP-K10], pBac[3xP3-ECFP, UA3p-EYFP-K10], pHer[3xP3-ECFP, UA3p-EYFP-K10], pHer[3xP3-ECFP, UA3p-EYFP-K10], pBac[3xP3-ECFP, UA3p-lacZ-K10], pBac[3xP3-ECFP, UA3p-lacZ-K10], pHer[3xP3-ECFP, UA3p-lacZ-K10], pBac[3xP3-DsRed, UA3p-EYFP-K10], and pBac[3xP3-DsRed, UA3p-EYFP-K10] were generated by cloning into an Asd/Fsd fragment from pSLfa_UA3p-eypf-K10_fa or pSLfa_UA3p-lacZ-K10_fa, respectively, into the Asd/Fsd sites of the eYfp- or eYfp-marked piggyBac or Hermes transformation vectors (Horn and Wimmer 2000) or pBac[3xP3-DsReda] (Horn et al. 2001), respectively. pSLfa_UA3p-eypf-K10_fa and pSLfa_UA3p-lacZ-K10_fa result from the insertion of eYfp as an Asp718/Nrdl fragment from pEYFP-I (CLONTECH) or lacZ as an XbaI fragment from pCHABA Sal (Wimmer et al. 1993, 1995) into pSLfa_UA3p-K10_fa opened with Asp718/Nrdl or XbaI, respectively; pSLfa_UA3p-K10_fa was generated by opening pSLfa_UA3p_fa by an Asp718/PslI digest and inserting the 3‘ UTR of K10 as an Asp718/PslI fragment from pUA3p.

pBac[3xP3-DsRed, TRE-EYFP-SV40] and pBac[3xP3-ECFP, TRE-EYFP-SV40] were generated by cloning into the Adsl site of pBac[3xP3-DsReda] and pBac[3xP3-ECFPam] (Horn and Wimmer 1998, 2000) the Asd 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 EcoRI/Nrdl (Klenow-blunted) fragment from pEYFP-I or of lacZ as an XbaI fragment from pCHABA Sal into pSLfa_TRE-SV40_fa opened with EcoRI/XbaI (Klenow-blunted) or XbaI, respectively; pSLfa_TRE-SV40_fa was cloned by inserting the 0.9-kb XbaI (Klenow-blunted)/HindIII fragment from pUDH10-3 into EcoRI (Klenow-blunted)/HindIII-opened pSLfa1180fa.

Drosophila culture: Fly strains were reared under standard laboratory conditions (Roberts 1998). Drosophila germline transformation employing piggyBac, Hermes, and Mos2 vectors and transgenic strain establishment was performed as described (Horn et al. 2000). For Minos-derived transformation vectors, injection mixtures were at final concentrations of 500 ng/μl construct and 300 ng/μl helper pHiS68aLIM20 (Kl-Nakis 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 h each day. Remobilization of genome-integrated, nonautonomous Hermes or piggyBac elements was scored by novel integration of respective 3xP3-EYFP or 3xP3-EYFP-marked elements onto balancer chromosomes.

Crosses for insertional mutagenesis were carried out as described in Figure 1. Since G2, brothers might contain the same insertion event, when establishing novel insertion lines we ensured independence by selecting only one 3xP3-EYFP/3xP3-EYFP+ G3 male of each single male G2 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 G3 males were crossed to w; SM5; TM3/ T(2; 3)a1api4v3 females. 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, G4, 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 G4 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).

Reverse PCR and sequence analysis: To recover DNA sequences flanking piggyBac insertions, reverse PCR was performed as described (Huang et al. 2000). Genomic DNA of new insertion lines was digested with Hpal, MspI, or TaqI. 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’-CTTGCACCTTGGACAGAGCATAGAGG-3’ and reverse primer (PLR) 5’-CAGTGCACATCTACGTTTGACAAGAACG-3’. The purified fragments were directly sequenced with the 5‘-UTR of K10 primer and for the 3‘ junction with the forward primer (PRF) 5’-CCTCGAGATTAGACGACCTCGGTAAG-3’ and reverse primer (PRR) 5’-GTGATCGTTTGAAACATTTGTTGCCATATT-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
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, G1), insects carrying both elements can be identified on the basis of the eye-specific ECFP and EYFP expression (Figure 1, G2). 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, G3). 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
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 α1-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 silkworm, B. mori (Uhlířová 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 Pelement 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 GALA gene when constructing GALA-based mutators, but employed the coding region of the GALA deletion variant II-9 (Ma and Ptashne 1987). This deletion variant (GALAΔ) contains both the amino-terminal DNA-binding domain and the carboxy-terminal activation domain of GALA. GALAΔ is almost as good an activator as GALA itself but supposedly more stable (G. Struhl, personal communication). GALAΔ-based mutators were generated in both piggyBac and Hermes backgrounds (see MATERIALS AND METHODS), but only Bac[3xP3-EYFP, p-GALAΔ-K10], hereafter abbreviated as /GALAΔ/, was used in this study.

For in vivo identification of an enhancer’s activity, GALAΔ 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 GALA4-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.
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<th>Jumpstarter</th>
<th>\textit{hsp70-\textit{Hermes}}</th>
<th>\textit{hsp70-piggyBac}</th>
<th>\textit{\textit{actub-piggyBac}}</th>
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<td>Progenitors</td>
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<td>pSL\textit{fa}_\textit{hsp70-piggyBac}-\textit{fa}</td>
<td>pSL\textit{fa}_\textit{actub-piggyBac}K10-\textit{fa}</td>
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<td>Final constructs</td>
<td>\textit{pBac}([3xP3-ECFP, \textit{hsp70-\textit{Hermes}}]-\textit{fa})</td>
<td>\textit{pHer}([3xP3-ECFP, \textit{hsp70-piggyBac}]-\textit{fa})</td>
<td>\textit{pMos}([3xP3-EYFP, \textit{actub-piggyBac}K10]-\textit{fa})</td>
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<th>Mutator</th>
<th>(\textit{GAL4}\Delta)</th>
<th>(\textit{GAL4}\Delta+\textit{UAS})</th>
<th>(\textit{\textit{tTA}})</th>
<th>(\textit{\textit{tTA+ TRE}})</th>
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<tr>
<td>Progenitors</td>
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<td>pSL\textit{fa}_\textit{p-GAL4\Delta-K10}-\textit{fa}</td>
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<tr>
<td>Final constructs</td>
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<td>\textit{pBac}([\textit{UASp-3xP3-EYFPafm}])</td>
<td>\textit{pBac}([\textit{3xP3-EYFP, p-GAL4\Delta-K10}]-\textit{fa})</td>
<td>\textit{pBac}([\textit{3xP3-EYFP, p-GAL4\Delta-K10}]-\textit{fa})</td>
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<th>\textit{UASP-lacZ}</th>
<th>\textit{TRE-EYFP}</th>
<th>\textit{TRE-lacZ}</th>
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</table>

* Constructs have been proven functional in transgenic Drosophila strains.
and Bujard launching pad insertion could also serve this purpose. {3xP3-ECFP, UASp-lacZ-K10} the loss or change of a particular enhancer activity at the integration site, the restoration of eye-specific mutagenesis. After remobilization from such an insertion and thus novel insertions can be isolated. Similarly, actually serve as ideal launching pads for insertional generated separate reporter elements by placing these e.g. insertions (Figure 1 and see below) be available in nonmodel organisms, such partially suppressed insertions could tend in situ for analysis or the bacterial lacZ 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 (Røth 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).

**Figure 3.** Examples of adult enhancer activities isolated with the mutator-reporter element {GALAΔ+ 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 UAS}, hereafter referred to as {GALAΔ+ 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 {GALAΔ+ 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 GALAΔ will serve as an expression tool for effector constructs.

At rare insertion sites of {GALAΔ+ 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 GALA/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 {GALAΔ+ 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/hancer detection by this mutator could be observed 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 Droso-

**Figure 3.** Examples of adult enhancer activities isolated with the mutator-reporter element {GALAΔ+ 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.
When testing strains for the reporter \textit{Her}(3xP3-ECFP, UASp-EYFP-K10) by using defined GAL4 driver strains, we noted that not only \textit{EYFP} was expressed as expected, but also \textit{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 \textit{Her}(3xP3-ECFP, UASp-lacZ-K10) did not mediate GAL4-driven \textit{ECFP} expression, suggesting that the larger distance between the UASp sites and the 3xP3 promoter (due to the longer \textit{lacZ} 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 \{GAL4\Delta + UAS\}, positive feedback loops can occur, which could allow strong and enduring enhancer detection after the loop has first been initiated. \{GAL4\Delta + 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 have already stopped, but it is still detectable due to the positive feedback loop of the reporter. \textit{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. \{GAL4\Delta + UAS\} differs in that it combines enhancer detection and signal amplification within a 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 GAL4 expression, this enhancing reporter might be detrimental and respective enhancers will not be detectable due to toxicity.

Strains with the 3xP3-DsRed-marked reporters \textit{Bac}[3xP3-DsRed, UASp-EYFP-K10], \textit{Bac}[3xP3-DsRed, UASp-lacZ-K10], \textit{Bac}[3xP3-DsRed, TRE-ECFP-SV40], and \textit{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 \textit{EYFP} fluorescence can be detected (\textit{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 \textit{lacZ}. 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 \textit{D. melanogaster}:** To test the balancer-free insertional mutagenesis scheme for enhancer detection (Figure 1), we performed a small pilot screen in \textit{D. melanogaster}. We decided against \textit{Mos1}-based mutators, since it was shown that genomic insertions of transgenic \textit{Mos1} constructs can rarely be remobilized in Drosophila (\textit{Lozovsky et al.} 2002). We decided against \textit{Hermes}-based mutators, despite the fact that remobilization actually works well in Drosophila (data not shown), only because \textit{Hermes} insertions can be remobilized by \textit{hobo} elements (\textit{Sundararajan et al.} 1999). Since not all Drosophila stocks are free of \textit{hobo} (\textit{Blackman et al.} 1989), novel \textit{Hermes}-based insertional mutagenesis in Drosophila, special care should be taken to use \textit{hobo}-free strains only.

In our Drosophila pilot screen, we chose the 3xP3-EYFP-marked piggyBac-based mutators \{GAL4\Delta\}, \{GAL4\Delta + UAS\}, and \{tTA\}, for which we used three homozygous X chromosomal launching integrations each. To remobilize these mutants from the X chromosomes, we selected three different strains carrying the homozygous constitutive jumpstarter \textit{pHer}[3xP3-ECFP, alex-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 \{GAL4\Delta\} and \{tTA\} insertions, we employed homozygous strains carrying, on the second or third chromosome, the 3xP3-DsRed-marked reporters \textit{Bac}[3xP3-DsRed, UASp-EYFP-K10], \textit{Bac}[3xP3-DsRed, UASp-lacZ-K10], or \textit{Bac}[3xP3-DsRed, TRE-ECFP-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\textsubscript{c} 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 \textit{Dm}[\textit{Bac}[3xP3-DsRed, UASp-EYFP-K10]] or \textit{Dm}[\textit{Bac}[3xP3-DsRed, TRE-ECFP-SV40]] (Figure 1, G\textsubscript{c} cross) or a \textit{white} strain in the case of \{GAL4\Delta + UAS\}. All male \textit{G\textsubscript{c}} 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\textsubscript{c}).

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-
For each mutator element, three independent homozygous strains were crossed to three independent homozygous jumpstarter strains (nine combinations as in Figure 1, G₂ cross): Dm[\textit{GAL4}] strains: M8-2x, M19-2x, M19-3x. Dm[\textit{GAL4} + \textit{UAS}] strains: M5.x, FM26.x, FF36.x. Dm[\textit{tTA}] strains: x.2, x.5, x.7. Dm[\textit{Her}/3xP3-ECFP, actub-piggyBacK10] jumpstarter strains: M1.III, M6.II, M10.III. Per combination, 27 single male crosses were set up as shown in Figure 1 (G₂ cross).

a Total number of fertile single male G₂ crosses (of 243 crosses per mutator).

b Jumping rate is the percentage of (G₂ crosses with at least one 3xP3-EYFP⁺ son)/(fertile G₂ crosses) (Berg and Spradling 1991); the mean value and the range of nine independent combinations are indicated.

c Percentage of (G₂ crosses with at least one 3xP3-EYFP⁺ son detecting an adult enhancer activity)/(fertile G₂ crosses).

d Percentage of [G₂ crosses with at least one 3xP3-EYFP⁺ and 3xP3-ECFP (no jumpstarter) son]/(fertile G₂ crosses).

e Total number of G₂ males.

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.

f Excision rate is the percentage of (G₂ crosses with at least one 3xP3-EYFP⁺ daughter)/(fertile G₂ crosses), the range of nine independent combinations is indicated.

For each transposon mutagenesis system, three independent homozygous strains were crossed to three independent homozygous jumpstarter strains (nine combinations as in Figure 1, G₂ cross): Dm[\textit{GAL4}] strains: M8-2x, M19-2x, M19-3x. Dm[\textit{GAL4} + \textit{UAS}] strains: M5.x, FM26.x, FF36.x. Dm[\textit{tTA}] strains: x.2, x.5, x.7. Dm[\textit{Her}/3xP3-ECFP, actub-piggyBacK10] jumpstarter strains: M1.III, M6.II, M10.III. Per combination, 27 single male crosses were set up as shown in Figure 1 (G₂ cross).

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c Percentage of (G₂ crosses with at least one 3xP3-EYFP⁺ son detecting an adult enhancer activity)/(fertile G₂ crosses).

d Percentage of [G₂ crosses with at least one 3xP3-EYFP⁺ and 3xP3-ECFP (no jumpstarter) son]/(fertile G₂ crosses).

e Total number of G₂ males.

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.

f Excision rate is the percentage of (G₂ crosses with at least one 3xP3-EYFP⁺ daughter)/(fertile G₂ crosses), the range of nine independent combinations is indicated.
disruption project, we further analyzed the autosomal \(\text{GAL4}\Delta\) and \(\text{tTA}\) 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 (BELEN 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 (\(\text{shn}\); line 166; STAELING-HAMPTON et al. 1995) or tramtrack (\(\text{ttk}\); 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 \(\text{HLH106}\) (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 \(\text{piggyBac}\) an ideal candidate for the design of protein trap systems (MORIN et al. 2001). For many of the targeted genes, no \(\text{P}\)-element alleles have previously been identified and sometimes the closest \(\text{P}\)-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 \(\text{piggyBac}\)-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 \(\text{piggyBac}\) 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 \(\text{P}\)-element mutagenesis screens (PETER et al. 2002).

The \(\text{ttk}\) insertion (line 150) is allelic to the lethal alleles \(\text{ttk}^{1}\) and \(\text{ttk}^{11}\). The associated lethality can be reverted by mutator excision; thus, the intron-localized line 150 represents a true allele of \(\text{ttk}\). This indicates that \(\text{piggyBac}\) 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 \(\text{shn}\), is not allelic to the lethal alleles \(\text{shn}^{1}\) and \(\text{shn}^{04738}\), 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 \(\text{GAL4}\Delta\) insertions could also serve as embryonic enhancer detectors, we crossed the lethal insertion lines (Table 3) to a strain carrying the reporter \(\text{Bac}\{3x\text{P3-DsRed, UASlp-lacZ-K10}\}\) 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 \(\text{GAL4}\Delta\) and \(\text{tTA}\) mutator elements

<table>
<thead>
<tr>
<th>Line</th>
<th>Mutator</th>
<th>Genetic</th>
<th>Molecular</th>
<th>Reversion</th>
<th>Gene</th>
<th>Functional part</th>
<th>P allele of gene?</th>
<th>Distance to next P insertion in GadFly</th>
</tr>
</thead>
<tbody>
<tr>
<td>29</td>
<td>(\text{GAL4}\Delta)</td>
<td>II 2L</td>
<td>AE003628.1</td>
<td>118436</td>
<td>Yes</td>
<td>CG5367</td>
<td>Intron</td>
<td>No</td>
</tr>
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<td>43</td>
<td>(\text{GAL4}\Delta)</td>
<td>III 3R</td>
<td>AE003764.2</td>
<td>225029</td>
<td>Yes</td>
<td>CG14066</td>
<td>larp</td>
<td>No</td>
</tr>
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<td>(\text{GAL4}\Delta)</td>
<td>II 2L</td>
<td>AE003609.2</td>
<td>232068</td>
<td>Yes</td>
<td>CG8676</td>
<td>Hr39</td>
<td>Yes</td>
</tr>
<tr>
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<td>AE003532.2</td>
<td>120258</td>
<td>Yes</td>
<td>CG3735</td>
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<td>248959</td>
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<td>CG7373</td>
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<td>CG5370</td>
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ND, not determined. EST, expressed sequence tag.

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

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

* Excisions not obtained (600 flies screened for absence of EYFP).
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 lacZ 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 lacZ expression, we recognized a significant time delay. While shn shows dorsal-specific expression during blastoderm stages, the matching enhancer-mediated lacZ expression cannot be detected until germ-band retraction (Figure 5A). shn is gut-specifically expressed during germ-band retraction, but the corresponding lacZ 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, this case /GAL4Δ/ does not detect the enhancers of the gene in which it is inserted. In contrast, in line 166 which is unique among eukaryotic class II transposons (Elick et al. 1996). The original TTAA target site, which /GAL4Δ/ does detect shn-specific enhancers and drives lacZ expression in shn-like patterns. Thus, despite not is duplicated upon insertion of the piggyBac element, is left as a single TTAA site after the element has been creating a shn allele, /GAL4Δ/ is picking up the enhancers of the gene. /GAL4Δ/ can therefore be used 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 male 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 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 MosI, 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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