Efficient Transformation of the Beetle *Tribolium castaneum* Using the *Minos* Transposable Element: Quantitative and Qualitative Analysis of Genomic Integration Events

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ABSTRACT

Genetic transformation in insects holds great promise as a tool for genetic manipulation in species of particular scientific, economic, or medical interest. A number of transposable elements have been tested recently as potential vectors for transformation in a range of insects. *Minos* is one of the most promising elements because it appears to be active in diverse species and has the capacity to carry large inserts. We report here the use of the *Minos* element as a transformation vector in the red flour beetle *Tribolium castaneum* (Coleoptera), an important species for comparative developmental and pest management studies. Transgenic G0 beetles were recovered from 32.4% of fertile G1's injected with a plasmid carrying a 3xP3-EGFP-marked transposon and *in vitro* synthesized mRNA encoding the *Minos* transposase. This transformation efficiency is 2.8-fold higher than that observed when using a plasmid helper. Molecular and genetic analyses show that several independent insertions can be recovered from a single injected parent, but that the majority of transformed individuals carry single *Minos* insertions. These results establish *Minos* as one of the most efficient vectors for genetic transformation in insects. In combination with *piggyBac*-based transgenesis, our work allows the introduction of sophisticated multicomponent genetic tools in *Tribolium*.

THE use of transposable elements for genetic transformation in insects has attracted wide interest as a valuable tool for developmental studies, for biotechnological purposes, and for designing strategies to control important disease vectors and agricultural pests (Adams and Sekelsky 2002; Ito et al. 2002; Tomita et al. 2003). In 1982, the landmark transformation of *Drosophila melanogaster* using the *P* element (Rubin and Spradling 1982) raised the false expectation that genetic manipulation of other insects was close at hand. However, the *P* element proved to be inactive in non-drosophilid insects (Handler et al. 1993), and it took more than a decade to realize that other insect transposons have a broader host range. Using such transposons, it has been possible during the last 8 years to expand transgenic technology to several other insect taxa. Representatives of four insect orders (Diptera, Lepidoptera, Coleoptera, and Hymenoptera) have been genetically transformed by a handful of type II transposable elements: *Hermes*, *mariner*, *piggyBac*, and *Minos* (reviewed in Atkinson et al. 2001; Handler 2001; see also Sumitani et al. 2003).

*Minos* was originally identified in *D. hydei* and belongs to the *Tc1*/*mariner* superfamily of transposable elements (Franz and Savakis 1991). It is 1.8 kb long, with two 255-bp inverted terminal repeats flanking a two-exon transposase gene (Franz et al. 1994). Recombinant purified transposases encoded by members of this superfamily are able to catalyze transposition *in vitro* (reviewed in Plasterk et al. 1999). This independence from species-specific factors has been proposed to account for their widespread occurrence among metazoa and for their usefulness as DNA delivery vectors (Vos et al. 1996). In agreement with this, *Minos*-based vectors have been used for the genetic transformation of the dipteran species *D. melanogaster* (Loukeris et al. 1995a), *Ceratitis capitata* (Loukeris et al. 1995b), and *Anopheles stephensi* (Catteruccia et al. 2000) and most recently for the germline transformation of the ascidian *Ciona intestinalis* (Sasakura et al. 2003). Furthermore, *Minos* activity has been demonstrated in divergent groups of insects (Shimizu et al. 2000; Zhang et al. 2002) and in mammalian tissues and cell lines (Klinakis et al. 2000b; Zagoraiou et al. 2001; Drabek et al. 2003). In this article we report the transformation of the red flour beetle *Tribolium castaneum* using the *Minos* element as a vector.

The spectrum of insect species amenable to transformation has also been significantly broadened by the development of new marker genes that allow the straightforward identification of transgenic animals. Earlier selection systems were based on the rescue of mutants affecting eye pigmentation (reviewed in Ashburner et al. 1998). These genetic markers were easy to score, but required a serious investment of time when applied to new species,
since an eye-color gene had to be molecularly characterized and a corresponding loss-of-function mutant isolated each time. This laborious procedure is circumvented by the use of fluorescent proteins as dominant markers, which enable scoring of transgenic progeny in wild-type backgrounds (Tsien 1998). Until recently, however, the use of these markers was restricted by the lack of functional regulatory sequences able to drive their expression in diverse species. A breakthrough toward general transformation markers active in multiple species was achieved by the utilization of artificial promoters responsive to well-conserved transcription factors. An artificial Pax6-dependent promoter, known as 3xP3 (Sheng et al. 1997), combined with an enhanced version of the green fluorescent protein (EGFP), was first used successfully in Tribolium and Drosophila (Berghammer et al. 1999b). This marker has subsequently allowed the rapid identification of transgenic individuals, at various developmental stages, in at least three different orders of insects (reviewed in Horn et al. 2002).

A further level of improvement can be achieved by engineering new sources of transposase that are not dependent on the existence of characterized active promoters in the particular species of interest. During the transformation procedure, eggs are usually injected with two plasmids, one carrying the marked transposon vector (referred to as the donor) and the other expressing the transposase (referred to as the helper). The transposase is usually under the control of a Drosophila promoter (e.g., hsp70). However, these promoters may not be sufficiently active in all species (Zhang et al. 2002). Substitution of the helper plasmid by in vitro synthesized capped mRNA alleviates the need for testing already established promoters or characterizing new ones to drive expression of the transposase gene in the targeted species. In the case of Minos-mediated transgenesis, this “ready-to-use” transposase has been shown to increase transformation rates significantly in both D. melanogaster and C. capitata (Kapetanaki et al. 2002). Here we show that this improvement is more generally applicable and extends to T. castaneum.

We report here an effective method for the stable genetic transformation of the red flour beetle T. castaneum, using the Minos transposable element. We present a molecular analysis of Minos integration events into the host genome and provide quantitative measures for several parameters that determine the usefulness of this element as an effective tool for genetic manipulation in Tribolium. Given the increasing attention that Tribolium receives as an experimental organism for developmental studies, evolutionary comparisons, and pest control, the availability of a second highly active transformation vector, in addition to piggyBac (Berghammer et al. 1999b; Lorenzen et al. 2003), is an important step toward the introduction of sophisticated genetic techniques (insertional mutagenesis, enhancer trapping, and gene trapping) in this organism.

**MATERIALS AND METHODS**

**T. castaneum rearing and micro-injections:** A nonisogenized strain of T. castaneum, homozygous for the recessive eye-color mutation pearl (p; Lorenzen et al. 2002), was used throughout these experiments. The lack of eye pigments in this strain facilitates the detection of fluorescence in the eyes of transformed individuals.

Beetles were reared under standard laboratory conditions and processed as previously described (Berghammer et al. 1999a; Lorenzen et al. 2003; and http://www.zi.biologie.uni-muenchen.de/science/tribolium/klingler/frames.html). For injections, beetles were allowed to lay eggs for 3 hr at 24°C. Eggs were treated with 0.2% bleach for 1 min; transferred to coverslips; and injected with a mixture containing 500 ng/µl of the donor plasmid pMi(3xP3-EGFP), 375 ng/µl of the helper plasmid pHSS6hsILMi20 or 375 ng/µl of transposase-capped mRNA, and 0.05% of the inert dye phenol red (Sigma, St. Louis) in water. Coverslips with injected embryos were then transferred to apple juice agar plates at 33°C, within sealed plastic containers, to avoid desiccation. Male and female survivors (G0’s) were backcrossed individually to three female or two male pearl beetles, respectively. A detailed protocol is available on request.

**Plasmids:** Throughout these experiments we used the pMi(3xP3-EGFP) donor plasmid (kindly provided by A. Klinakis, A. Babaratsas, and C. Savakis), containing a Minos transposase with the EGFP coding sequence under control of the 3xP3 promoter (Horn and Wimmer 2000; Figure 1C). The 3xP3-EGFP gene cassette produces green fluorescence in the eyes of individuals carrying this construct (Berghammer et al. 1999b).

The helper plasmid pHSS6hsILMi20, containing the Minos transposase-coding sequence under the control of the Drosophila hsp70 promoter, has been described previously (Klinakis et al. 2000a). The plasmid pBlueSKMimRNA, used for the *in vitro* synthesis of Minos transposase mRNA, is a derivative of pNB40ILTMI (Kapetanaki et al. 2002). A Pdi (blunt)/Not fragment of pNB40ILTMI, containing the Minos transposase transcription unit, was cloned into KpnI (blunt)/Not-cut pBlueScriptSK II+ (Stratagene, La Jolla, CA), placing the Minos transposase under the T7 promoter.

**In vitro synthesis of Minos transposase mRNA:** Capped Minos transposase mRNA was prepared from the pBlueSKMimRNA vector (linearized with NotI), using the mMESSAGE mMACHINE kit (Ambion, Austin, TX). The transcription reaction was carried out using T7 RNA polymerase, according to the manufacturer’s instructions, followed by phenol-chloroform extraction and isopropanol precipitation of the mRNA. Small aliquots of the mRNA were stored in isopropanol at −20°C. Before micro-injection, the mRNA was precipitated, washed with ethanol, resuspended in water, and quantitated using a spectrophotometer.

**Southern blot analysis and inverse PCR:** Genomic DNA was prepared from pools of adult beetles using the Puregene DNA isolation kit (Gentra Systems, Research Triangle Park, NC). About 2 µg of genomic DNA was digested with either PstI or AflI, size separated by agarose gel electrophoresis, and blotted onto PROTRAN nitrocellulose membranes (Schleicher & Schuell, Keene, NH). Radiolabeled probes and hybridizations were carried out using standard techniques (Sambrook et al. 1989). Inverse PCR was carried out with AflI-digested genomic DNA and Minos-specific primers, as described previously (Klinakis et al. 2000b). Amplified DNA fragments were cycle sequenced and subjected to BLAST analysis in the EMBL/GenBank databases.

**Epiﬂuorescence microscopy:** EGFP fluorescence was observed using a Leica MZ12 fluorescence stereomicroscope, equipped with Plan apo 1.6× objective, a 100-W Hg lamp,
Minos-Mediated Transformation of Tribolium

Figure 1.—Molecular analysis of Minos insertions. (A) Southern analysis of PvuII-digested genomic DNA probed with sequences corresponding to the EGFP coding region (E probe). Each lane represents genomic DNA isolated from a pool of 12 transgenic beetles that derive from a single transformed parent. A single band is expected per Minos insertion. (B) Southern analysis of AluI-digested genomic DNA from the same pools of beetles as in A, probed with sequences corresponding to the Minos inverted repeats (M probe). Two bands per Minos insertion are expected. (C) Map of the Mi/3xP3-EGFP transposon construct used in this work (not to scale). LIR and RIR correspond to the left and right inverted repeats of Minos, respectively. The EGFP coding sequence is placed under the control of an artificial promoter containing three Pax-6 binding sites and the Drosophila hsp70 basal promoter. (D) Flanking sequences of three independent Minos insertions in the Tribolium genome (from RNA lines 3.2, 4.2, and 6.1), recovered by inverse PCR. The characteristic duplicated TA dinucleotide (in boldface type) is found on either side of the integration site.

RESULTS

High-efficiency of transformation using DNA or mRNA helpers: The ability of the Minos element to transpose into the germline of T. castaneum was tested by co-injecting a plasmid carrying the Mi/3xP3-EGFP} transposon (Figure 1C) with either of two sources of Minos transposase: a helper plasmid or capped mRNA encoding the Minos transposase (see MATERIALS AND METHODS). Approximately 600 preblastoderm embryos of a white-eyed pearl strain were injected in each case. The vast majority of surviving G₀ individuals were fertile and their offspring (G₁’s) were scored for fluorescence in their eyes to identify transgenic animals.

Among the 167 fertile G₀’s injected with the helper plasmid, 19 produced transgenic offspring (“DNA lines”), while 67 of 207 fertile G₀’s injected with the helper mRNA produced transgenic offspring (“RNA lines”). Thus, the deduced transformation efficiencies are 11.4% for the DNA helper and 32.4% for the RNA helper (Table 1). Chi-square analysis shows that this 2.8-fold difference in transformation rates is highly significant (P < 0.001). No difference in transformation efficiency was observed between male and female G₀’s.

Transposition of Minos into the host genome: Integration of the Minos element into the host genome was confirmed by Southern hybridization. Genomic DNA was prepared from pools, each composed of ~12 fluorescent sibling G₁ beetles (derived from the same injected G₀). Twelve such samples were prepared from G₀’s injected with the helper plasmid and 24 from G₀’s injected with helper mRNA. Each pool was tested for the number of integrated copies of Minos, using two different restriction enzyme/probe combinations. PvuII-digested DNA was probed for the EGFP coding sequence (E probe), resulting in one band per insertion (Figure 1, A and C), while AluI-digested DNA was probed for the Minos inverted repeats (M probe), resulting in two bands per insertion (Figure 1, B and C). The M probe used did not hybridize to genomic DNA from untransformed pearl beetles (nor do specific primers for the Minos terminal repeats amplify any product), suggesting that no Minos-related transposable elements were already present in the coleopteran genome.

Sixty-three insertions were detected in total among the transformed DNA and RNA lines subjected to Southern analysis (Tables 2 and 3). All detected insertions are characterized by bands >1.45 kb with the E probe and bands >0.26 and 0.38 kb with the M probe (these are the minimum sizes expected for integral copies of the Mi/3xP3-EGFP} element), suggesting that these represent insertions of the entire Mi/3xP3-EGFP} element into the host genome.

Insertions carrying sequences of the donor plasmid (external to the transposon) are expected to produce a 1.58-kb band with the E probe and 0.27- and 0.4-kb bands with the M probe, due to the presence of additional PvuII and AluI sites in the donor plasmid. These fragment sizes were detected on just three occasions, exclusively in lines carrying multiple Minos insertions (Tables 2 and 3). The presence of external sequences

and a GFP filter set (excitation filter 480/40 nm, emission filter 510 nm). Photography was performed using a Wild MPS 51S camera or a ProgRes C14 digital camera.
was confirmed by probing with the plasmid backbone of pMi[3xP3-EGFP] (data not shown). The vast majority of insertions did not show these bands and are thus thought to derive from genuine transposition events of the Minos element into the Tribolium genome.

To confirm this, we carried out inverse PCR and sequenced the DNA flanking the Minos element in three independent RNA lines. Like other members of the Tc1/mariner superfamily, Minos is known to insert in a TA dinucleotide, which is duplicated upon insertion (Arca et al. 1997). In the three lines that we sequenced, the inverted terminal repeats of the Mi[3xP3-EGFP] element were flanked by this characteristic TA dinucleotide, followed by sequences that were unrelated to those of the donor plasmid (Figure 1D). Database searches revealed that in one of these lines (line 4.2), the Minos element was inserted within a previously identified 360-bp satellite DNA element present in the Tribolium genome (Ugarkovic et al. 1996).

**Number of transformed progeny per G₀ (cluster size):** The percentage of fluorescent progeny (transformed G₁’s) obtained per G₀, referred to as cluster size, was determined for beetles injected with the DNA and RNA helpers, respectively (Figure 2). The distributions of these values are broad (Figure 2), with the mean cluster size per transformant-producing G₀ being 10.3% for the DNA helper (ranging between 1 and 35%) and 15.2% for the RNA helper (ranging between 1 and 96%). Thus,

### TABLE 1

**Summary of transformation experiments**

<table>
<thead>
<tr>
<th>Donor</th>
<th>Helper</th>
<th>No. of embryos injected</th>
<th>No. of surviving G₀’s</th>
<th>No. of fertile G₀’s</th>
<th>No. of transformants producing G₁’s</th>
<th>% transformation efficiency*</th>
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</thead>
<tbody>
<tr>
<td>pMi[3xP3-EGFP]</td>
<td>DNA</td>
<td>~600</td>
<td>171</td>
<td>167</td>
<td>19</td>
<td>11.4</td>
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<tr>
<td>pMi[3xP3-EGFP]</td>
<td>RNA</td>
<td>~600</td>
<td>210</td>
<td>207</td>
<td>67</td>
<td>32.4</td>
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</tbody>
</table>

*Percentage of fertile G₀’s producing transformed G₁ progeny.

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### TABLE 2

**DNA lines subjected to Southern and segregation analysis**

<table>
<thead>
<tr>
<th>G₀ cross</th>
<th>Gender</th>
<th>Fluorescent-eyed G₁ progeny</th>
<th>Total G₁ progeny</th>
<th>Cluster size (%)</th>
<th>G₀ insertions³</th>
<th>G₁ cross</th>
<th>G₁ segregations</th>
<th>G₁ insertions¶</th>
<th>Nature of insertions¶</th>
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<td>228</td>
<td>14.47</td>
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<td>1.1</td>
<td>1</td>
<td></td>
<td></td>
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<tr>
<td>2</td>
<td>M</td>
<td>5</td>
<td>352</td>
<td>1.42</td>
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<td>2.1</td>
<td>1</td>
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<td></td>
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<tr>
<td>3</td>
<td>M</td>
<td>23</td>
<td>344</td>
<td>6.69</td>
<td>1</td>
<td>3.1</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>60</td>
<td>297</td>
<td>20.20</td>
<td>2</td>
<td>4.1</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>13</td>
<td>297</td>
<td>4.38</td>
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<td>5.1</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>13</td>
<td>283</td>
<td>4.59</td>
<td>1</td>
<td>6.1</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>39</td>
<td>310</td>
<td>12.58</td>
<td>1</td>
<td>7.1</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>M</td>
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<td>337</td>
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<td>3</td>
<td>Two linked, plasmid sequence</td>
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<td>9</td>
<td>F</td>
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<td>9.1</td>
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<tr>
<td>10</td>
<td>F</td>
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<tr>
<td>12</td>
<td>F</td>
<td>20</td>
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<td>12.1</td>
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</tbody>
</table>

*Cluster size is the percentage of transformed G₁’s produced by individual G₀’s backcrossed to pearl beetles.
³Number of Minos insertions in the germline of each G₀, as inferred from transformed G₁ progeny subjected to Southern analysis.
¶Fluorescent G₁ siblings backcrossed individually to pearl beetles.
¶¶Number of Minos insertions in the germline of each G₁, as inferred from the segregation ratios of the 3xP3-EGFP marker in the G₂ generation.
¶Number of Minos insertions in the germline of each G₁, as inferred from transformed G₂ progeny subjected to Southern analysis.
†The majority of integration events are single unlinked Minos insertions. Only lines displaying either linked insertions (deduced from a difference between the number of actual and independently segregating insertions) or insertions carrying plasmid sequences (deduced by Southern analysis) are shown.
### TABLE 3

RNA lines subjected to Southern and segregation analysis

<table>
<thead>
<tr>
<th>G₀ cross</th>
<th>Gender</th>
<th>Fluorescent-eyed G₁ progeny</th>
<th>Total G₁ progeny</th>
<th>Cluster size (%)</th>
<th>G₀ insertions</th>
<th>G₁ cross</th>
<th>Segregation analysis</th>
<th>G₁ insertions</th>
<th>Nature of insertions</th>
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<td></td>
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<td>M</td>
<td>74</td>
<td>249</td>
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<tr>
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<td>M</td>
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<td>12.09</td>
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<td>1</td>
<td></td>
</tr>
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<td>M</td>
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<td>1</td>
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<td>Two linked, plasmid sequence</td>
</tr>
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</table>

\(a\) Cluster size is the percentage of transformed G₁’s produced by individual G₀’s backcrossed to pearl beetles.

\(b\) Number of Minos insertions in the germline of each G₀, as inferred from transformed G₁ progeny subjected to Southern analysis.

\(c\) Fluorescent G₁ siblings backcrossed individually to pearl beetles.

\(d\) Number of Minos insertions in the germline of each G₁, as inferred from the segregation ratios of the \(3\times P3-EGFP\) marker in the G₂ generation.

\(e\) Number of Minos insertions in the germline of each G₁, as inferred from transformed G₂ progeny subjected to Southern analysis.

\(f\) The majority of integration events are single unlinked Minos insertions. Only lines displaying either linked insertions (deduced from a difference between the number of actual and independently segregating insertions) or insertions carrying plasmid sequences (deduced by Southern analysis) are shown.

while the RNA helper almost triples the transformation frequency (see above), cluster size increases by only \(\sim 50\%\). Consequently, the number of G₁ progeny that need to be screened per G₀ to detect a transformant is similar for RNA and DNA helpers. The number of independent insertions obtained per G₀: We used Southern analysis in pools of 12 transgenic G₁ progeny per G₀ (described above) to measure the number of independent Minos insertions that can be obtained from the germline of a single injected G₀; i.e., sampling 12 transformed gametes from each germline. Pilot experiments indicated that the hybridization conditions used were sensitive enough to detect insertion(s) present even in only 1 of the 12 beetles constituting each sample (data not shown).

Among the 12 DNA lines subjected to this analysis, 9 lines carried a single insertion, 1 carried two insertions, and 2 carried three insertions (Table 2). Among the 24
RNA lines that were analyzed, 8 carried a single insertion, 11 carried two insertions, and 5 carried three or more insertions (Table 3). It is evident that the RNA helper is capable not only of transforming a higher proportion of injected animals, but also of generating a larger number of independent insertions per germ-line, in comparison to the DNA helper (multiple insertions in 67% vs. 25% of the lines tested).

To address whether there is a direct relation between the number of insertions per G₀ germ-line and the proportion of transformed G₁ progeny produced per G₀ (cluster size), we examined whether there is a correlation between these values for individual DNA and RNA lines. The correlation coefficient is high for the DNA lines (Figure 3A, \( r = 0.92 \)), supporting the expectation that these values should be directly related to each other and to the level of activity of the transposase in individual injected G₀'s. The correlation coefficient for the RNA lines, however, was found to be significantly lower (Figure 3B, \( r = 0.50 \)). This is illustrated by RNA-injected G₀'s carrying multiple insertions but giving rise to <5% transformed progeny and, conversely, by G₀'s carrying a single insertion but giving rise to >20% transformed progeny. The latter may be explained if the RNA helper can drive transposition at earlier stages, giving rise to larger clones of germ cells marked by the same insertion.

**Most transformed (G₁) individuals carry single Minos insertions:** We have shown that individual G₀ beetles can carry multiple independent insertions of a Minos element in their germ-line. Depending on whether these insertions have hit the same or independent germ cells, these can be recovered as multiple insertions in a single G₁ or as single insertions in several different G₁ progeny. To resolve this, we performed Southern analysis in pools of 12 G₂ transformants produced by individual G₁ beetles (backcrossed to pearl beetles), to deduce the number of insertions present in the genome of these G₁'s. We also used the segregation ratio of transformed to non-transformed progeny of individual G₁ beetles, to estimate the number of unlinked insertions present in these G₁'s. In most cases, the number of insertions determined by Southern hybridization was consistent with that deduced from segregation ratios, with three exceptions, which presumably correspond to linked insertions (Tables 2 and 3).

As expected, for G₀'s giving rise to single inserts, all G₁'s were found to contain the same single insertion of the transposon. However, also in the case of G₀'s giving rise to multiple insertions, a significant proportion of their G₁ offspring were found to each carry only a single copy of the transposon (Tables 2 and 3), suggesting that in many cases different germ cells had been targeted in one G₀ animal. Overall, the majority of transformed G₁'s are inferred to carry single insertions of the transposon.

**Eye fluorescence phenotypes:** It is well known that the expression of transformation markers can be influenced by the particular locus where a mobile element is inserted (Loukeris et al. 1995a; Horn et al. 2000). In our experiments, significant differences were observed in the expression of the 3xP3-EGFP marker among different lines of transformants, ranging from strong expression in the entire eye to weak expression in a small number of ommatidia. A significant number of DNA and RNA lines (about one-quarter of the lines examined) produced G₁’s with distinct eye fluorescence phenotypes. The majority of these lines contained different Minos insertions. Similarly, distinct eye phenotypes were observed segregating in the progeny of G₁’s carrying more than one Minos insertion. This behavior is similar to that observed with other transformation markers (e.g., white in Drosophila) and may help to distinguish individuals that carry different Minos insertions.

**Enhancer trapping by Mi(3xP3-EGFP):** The 3xP3-EGFP marker used in these experiments is regulated by a minimal promoter and a number of upstream Pax-6 binding sites that activate expression specifically in the eyes and in parts of the central nervous system in
Tribolium (Berghammer et al. 1999b; Lorenzen et al. 2003). Depending on the site of integration of the transposon in the host genome, it is conceivable that EGFP expression could also be activated by nearby enhancers, an effect that is widely known as “enhancer trapping” (O’Kane and Gehring 1987; Bellen et al. 1989; Bier et al. 1989). Enhancer trapping has been reported recently, using the piggyBac element in Drosophila and Tribolium (Horn et al. 2003; Lorenzen et al. 2003). To determine whether this occurs at an appreciable frequency with the Mi/3xP3-EGFP element, we looked at the patterns of EGFP fluorescence in larvae, pupae, and adults of all 86 transformed lines. Enhancer traps were identified as novel EGFP expression patterns in at least 10 lines (Figure 4). This suggests that enhancer trap screens could be carried out in Tribolium using Minos-based vectors.

**DISCUSSION**

**High-efficiency transformation of Tribolium using Minos:** We have tested the ability of the Minos element to transpose in the beetle T. castaneum and examined a number of quantitative and qualitative parameters that bear on its utility as a tool for genetic manipulations in this species.

First, we determined the frequency at which the surviving G0 (injected) beetles give rise to transformed progeny. Transformation frequencies were 11.4% using plasmid DNA as a helper and 32.4% using mRNA as helper. These frequencies are sufficiently high for routine transgenic experiments and, in the case of the RNA helper, the transformation rate is among the highest reported in insects (Atkinson et al. 2001; Handler 2001). Equally impressive transformation rates in Tribolium have been achieved using piggyBac-based vectors as well (Berghammer et al. 1999b; Lorenzen et al. 2003).

The second parameter we examined is the frequency at which transformed G1 progeny are recovered from transformant-producing G0’s, the so-called cluster size. This gives an estimate of the number of G1 progeny that need to be screened per G0 to recover a transformation event. Cluster sizes have a broad distribution (Figure 2) with mean values of 10.3% for the DNA helper and 15.2% for the RNA helper. Using the RNA helper, it seems possible to recover ~55% of transformed lines.

**FIGURE 3.—**Correlation between the number of insertions recovered per G0 germline and the proportion of transformed G1 progeny produced per G0 (cluster size). (A) Plot of cluster size vs. number of insertions for DNA lines, showing a high positive correlation between these values ($r = 0.92$). (B) Plot of cluster size vs. number of insertions for RNA lines, showing a lower correlation between these values ($r = 0.50$). Note difference in scale in the y-axis for DNA vs. RNA lines.
Figure 4.—Enhancer trap lines recovered from Mi(3xP3-EGFP) insertions. Lines show new patterns of EGFP fluorescence, in addition to those driven by the 3xP3 element. (A) Expression in adult abdominal muscles (arrowhead marks the expected 3xP3-driven expression in the eye), (B) expression in adult thoracic muscles (ventral view), (C) expression in ventro-lateral stripes in the larva, (D) expression in the dorsal hemo-lymph vessel of the larva.

by screening just 10 individuals (G1’s) per G0 and ~75% of transformed lines by screening ~20 individuals per G0. These numbers are sufficiently high to allow for comfortable screening of large numbers of injected beetles and are comparable to those obtained with the piggyBac element (LORENZEN et al. 2003).

A third parameter of interest is the number of independent Minos insertions that can be recovered per injected individual. Multiple insertions recovered per G0 may be an advantage in screens where each independent insertion has the potential to reveal new information (e.g., enhancer trap screens), but they may be a disadvantage in screens where single insertional events need to be recovered (e.g., insertional mutagenesis screens). The most useful situation is when independent insertions can be recovered in different G1 progeny arising from a single G0. In our experiments, 25% of the DNA lines and 67% of the RNA lines tested contained more than one Minos insertion, but in a significant proportion of these cases single insertions were recovered in individual G1 beetles. These could often be recognized by different eye fluorescence phenotypes. Overall, the majority of transformed G1’s that were recovered carried single Minos insertions within their genome.

Other important parameters that influence the use of transposon vectors are the stability of insertions, the tendency of the element to insert as single or tandem copies within the genome, and the specificity of transposition events, i.e., whether the mobile element is cleanly excised from its flanking sequences and whether it has a preference for particular target sequences. In all studies that have been carried out to date Minos insertions appear to be extremely stable in the absence of a source of transposase (LOUKERIS et al. 1995a; KAPETANAKI et al. 2002). In our experiments this was seen most clearly in the Southern analysis, where we consistently recovered the same bands in G1 individuals and in their G2 progeny (data not shown). We have also confirmed that the great majority of Minos insertions are single unlinked insertions, that most of them do not carry the flanking plasmid sequences, and that the only obvious preference for target sites is the presence of the TA dinucleotide at the site of insertion (ARCA et al. 1997).

Interestingly, two of the three lines carrying linked insertions correspond to cases where plasmid sequences were also found incorporated into the genome. A two-step mechanism, involving the integration of a second Mi(3xP3-EGFP) transposon into the donor plasmid, followed by transposition of the resulting compound transposon into the genome, could explain these results (LOUKERIS et al. 1995a).

Finally, an important parameter for many transformation experiments is the ability of the vector to carry large inserts. While we have not tested the effect of insert size on transformation efficiency in Tribolium (all our experiments were carried out with the 2-kb Mi(3xP3-EGFP) element), a number of relevant observations are available from Drosophila: using the same RNA helper, transformation frequencies of 32% were obtained with a 5.8-kb transposon, 12% with a 7-kb transposon, and 25% with a 9.1-kb transposon (A. METAXAKIS and C. SAVAKIS, personal communication; A. PAVLOPOULOS, unpublished observations). These results suggest that the transposition activity of Minos in Drosophila is not severely affected by large insert sizes. Given that Minos activity is unlikely to depend on host-specific factors (as deduced from the broad host specificity of this element), it is likely that these vectors will be able to carry relatively large inserts also in Tribolium and in other species of interest. A similar ability to carry large insert sizes (up to 9.5 kb) has also been demonstrated for piggyBac-based transposons (LORENZEN et al. 2003).

Overall, these results demonstrate that Minos has the ability to transpose very efficiently in T. castaneum and can be used as a vector for routine transformation experiments in this species. Although particular quantitative
parameters may vary in different experiments (depending on variations in the injection protocol, amount and quality of the helper, overall size of the transposable element, etc.), the performance measured in these pilot experiments suggests that Minos will be an excellent tool for most applications (see below).

**DNA vs. RNA helper:** Comparing the results obtained using DNA and RNA helpers suggests that the RNA helper is a more efficient source of transposase: it gives an almost three-fold increase in the frequency of transformation (32.4% vs. 11.4%), increases somewhat the cluster size (15.2% vs. 10.3%), and increases the number of independent insertions obtained per G0. All this is achieved without lowering the survival or fertility rates of injected G0’s (Table 1), indicating that the RNA helper could be more useful for generating transfectants in large-scale projects. The main drawbacks of using the RNA helper are the additional steps required to synthesize the mRNA, the extra precautions that must be taken to prevent RNA degradation, and in some cases the need to deal with multiple insertions that are obtained in a larger proportion of the G1’s.

**Prospects for genetic manipulation in Tribolium:** Efficient transformation in Tribolium allows powerful new tools and techniques to be used in this species. These include the use of insertional mutagenesis, enhancer trapping, and gene trapping for the identification of new genes (O’Kane and Gehring 1987; Cooley et al. 1988; Bellen et al. 1989; Bier et al. 1989; Spradling et al. 1995); the use of the UAS/GALA and FLP/FRT systems for targeted misexpression and mosaic analysis (Golic and Lindquist 1989; Brand and Perrimon 1993); and the use of reporter constructs for studying cis-regulatory elements (Ludwig et al. 2000), techniques that have so far been available only in model organisms like Drosophila, Caenorhabditis elegans, and the mouse.

Besides Minos, another transposable element, piggyBac, has been shown to mediate transformation and enhancer detection with high efficiency in Tribolium (Berghammer et al. 1999b; Lorenzen et al. 2003). Having two different vectors for transformation offers several advantages and greatly expands the possibilities for genetic manipulation in a species: it helps overcome problems of insertional biases of individual elements (Spradling et al. 1999), allows efficient dual transposon systems to be used for insertional screens (with separate helper and mobile/mutator elements integrated into the genome; Cooley et al. 1988; Horn et al. 2003), allows the use of compound transposons to facilitate the detection of excision events and to generate chromosomal deletions (Huet et al. 2002), and allows the generation of new insertions in genetic backgrounds where other transposable elements remain stable (Hacker et al. 2003). Thus, powerful genetics and reverse-genetic technologies that were once available only in Drosophila can now be applied to Tribolium.

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**LITERATURE CITED**


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