piggyBac-mediated Germline Transformation of the Malaria Mosquito Anopheles stephensi Using the Red Fluorescent Protein dsRED as a Selectable Marker*

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It is estimated that every year malaria infects ~300 million people and accounts for the death of 2 million individuals. The Plasmodium parasites that cause malaria in humans are transmitted exclusively by mosquito species belonging to the Anopheles genus. The recent development of a gene transfer technology for Anopheles stephensi mosquitoes, using the Minos transposable element marked with the enhanced green fluorescent protein EGFP (Catteruccia, F., Nolan, T., Loukeris, T. G., Blass, C., Savakis, C., Kafatos, F. C., and Crisanti, A. (2000) Nature 405, 959–962), provides now a powerful tool to investigate the role of mosquito molecules involved in the interaction with the malaria parasite. Such technology, when further developed with additional markers and transposable elements, will be invaluable for analyzing the biology of the vector and for developing malaria-resistant mosquitoes to be used as a tool to control malaria transmission in the field. We report here the germline transformation of A. stephensi mosquitoes using a piggyBac-based transposon to drive integration of the gene encoding for the red fluorescent protein dsRED. A. stephensi embryos were injected with transformation vector pPBRED containing the dsRED marker cloned within the arms of piggyBac. Microscopic analysis of G1 larvae revealed the presence of seven fluorescent phenotypes whose different molecular origins were confirmed by Southern blotting analysis. Sequencing of the insertion sites in two lines demonstrated that integrations had occurred at TATA nucleotides in accordance with piggyBac-mediated transpositions.

The recent development of an efficient gene transfer technology for Anopheles stephensi mosquitoes, achieved by using Minos-based transposon (4) loaded with the EGFP selective marker (1), has expanded the possibility of studying the genetics of human malaria vectors at the functional level. Although EGFP has proven to be an invaluable visible marker for identifying transformed individuals in different insect species (1, 5–7), the availability of only this selectable marker limits the range of applications of a gene transfer technology in malaria vectors. New molecular tools are now needed to exploit fully the potential of germline transformation for Anopheles mosquitoes, with the aim of unraveling the interactions between host molecules and Plasmodium parasites, as well as performing functional studies such as transposon tagging and enhancer trapping. Ultimately this technology could be used to develop transgenic mosquitoes with a nonpermissive phenotype for parasite development. These mosquitoes could be used in malaria control programs with the aim to replace the permissive wild type vectors.

As shown in Drosophila melanogaster, the availability of various molecular and genetic tools to achieve germline transformation has contributed tremendously to our understanding of the fruit fly biology, leading to the identification of hundreds of genes involved in development, immunity, tissue modeling, and embryogenesis. The transposable elements P, Minos, piggyBac, and herness have, among others, all been successfully used to drive integration of exogenous DNA into the fruit fly genome (8–11). At the same time, the use of an array of visible selectable markers, such as white, rosy, cinnabar, and more recently EGFP (12–15), has greatly facilitated the screening procedure and allowed complex functional studies of genes and their promoter regions. Germline transformation of A. stephensi mosquitoes is anticipated to open a wide range of applications to explore the genome of these important disease vectors when sustained by a parallel development of appropriate molecular tools. So far the availability of a system for germline transformation based on the combination of a single transposable element with a single selectable marker has limited the array of applications of gene transfer technology in Anopheles. In this study, we have achieved germline transformation of A. stephensi mosquitoes by using the piggyBac transposable element, initially isolated in the cabbage looper Trichoplusia ni (2), to drive integration of foreign genes. Moreover, we have validated the use of the red fluorescent protein dsRED (3) as a visible selectable marker in germline transformation in the same mosquito species.

EXPERIMENTAL PROCEDURES

Plasmid Construction—The pMiRED transformation vector (Fig. 1a) was derived from plasmid pMiNot (11) by inserting a DNA fragment containing the dsRED gene (CLONTECH) under the control of the actin 5C promoter from D. melanogaster. The helper plasmids pHSS6hslM20 and phsp-pBac, respectively providing the Minos and the piggyBac transposase genes, have been described previously (10, 16). The pPBRED transformation vector (Fig. 1a) was derived from plasmid pHKOΔ (17) by cloning a BgII linker, containing an internal NotI site, into the unique BgII site within the piggyBac gene. A DNA cassette including the dsRED gene under the control of the actin 5C promoter and additional sequences (stuffer) was then cloned as a single NotI fragment into the inserted linker.

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1 The abbreviation used is: EGFP, enhanced green fluorescent protein.
Embryo Microinjection—Blood-fed A. stephensi mosquitoes (strain sd 500) were allowed to lay eggs in a solution of 0.1 mM p-nitrophenyl p'-guanidino-benzoxae 48–72 h after a blood meal. Embryos were then transferred onto glass slides and microinjected in injection buffer essentially as described previously (1). Embryos were microinjected with a mixture of helper plasmid pHSS6sILM20 (100 µg ml⁻¹) and pMiRED (400 µg ml⁻¹) in the case of Minos-mediated integrations and with plasmid phsp-pBac (100 µg ml⁻¹) and pPBRED (400 µg ml⁻¹) in the case of piggyBac-mediated integrations. Hatched larvae were analyzed on an inverted microscope using a Texas Red filter to detect dsRED expression.

Southern Blot Analyses and Sequencing of Integration Sites—Genomic DNA from transgenic A. stephensi adults (G0 generation) and from wild type mosquitoes was digested with the restriction endonuclease Hincll or EcoRI and blotted as described previously (18). Digested genomic DNA (~4 µg/lane) was separated on a 0.8% agarose gel and transferred onto a nylon membrane. The membranes were hybridized overnight at 65°C with different 32P-labeled probes. In the case of the Minos-mediated integration, genomic DNA was hybridized with the previously described probe M (1) (Fig. 1a), encompassing both the left and the right arms of Minos. In the case of piggyBac-mediated integrations, probe P, a PCR product encompassing sequences of the left and right arms of the piggyBac transposon, was used. To sequence the piggyBac integration sites, genomic Lambda Zap EcoRI libraries (Stratagene) were constructed from the DNA extracted from G2 larvae of A. stephensi transgenic lines 101 and A3 and hybridized with probe P (Fig. 1a). The cloned insertion sites were sequenced using primers annealing internally to the piggyBac inverted repeats (pPB-R, 5'-CGTACCTACTGACTTACCTGC-3'; pPB-L, 5'-CATCGCCTTGCAGAAGCCC-3') (Table I).

RESULTS

We tested whether the red fluorescent protein dsRED could be used as a visible selectable marker for germline transformation of A. stephensi mosquitoes. In a pilot experiment, A. stephensi embryos were microinjected with vector pMiRED, which contained the dsRED gene from Discosoma sp. under the transcriptional control of the actin 5C promoter from D. melanogaster inserted between the Minos inverted repeats (Fig. 1a). pMiRED was microinjected together with plasmid pHSS6sILM20, which provided the enzymatic activity necessary for Minos transposition (16). The 23 adults obtained from 102 injected embryos were outcrossed to wild type A. stephensi mosquitoes, and their progeny was analyzed for fluorescence using a Texas Red excitation filter (Table II). Fluorescent G1 larvae were detected in the progeny of G0 female No. 7. The segregation of the red allele in following generations was in agreement with the occurrence of a single insertion (data not shown). To assess the nature of the integration event, we performed Southern blotting analysis of the transgenic population (line MinRED1) on genomic DNA extracted from G2 larvae and digested with the EcoRI or Hincll restriction endonuclease. Two bands could be detected after hybridization with probe M, which encompassed both arms of Minos, demonstrating that a single insertion of the transposon had occurred (Fig. 1b) (1). Interestingly the pattern of expression of fluorescence changed throughout larval development. In newly hatched larvae, a faint fluorescence could be detected in nerve cells around the larval body (Fig. 2a). The levels of fluorescence increased in second and third instar larvae, which displayed the gut expression pattern previously described in transgenic A. stephensi transformed with an actin 5C-EGFP transposon (Fig. 2b) (1). Importantly fluorescence was more easily detected in the gut of adult mosquitoes than in the case of transgenic lines expressing the EGFP marker (Fig. 2c).

We then tested the ability of the piggyBac transposable element to drive integration of exogenous DNA into the germline of A. stephensi mosquitoes. A total of 303 embryos was injected with a mixture of transformation vector pPBRED, containing the actin-dsRED cassette inserted within the inverted repeats of piggyBac, and plasmid phsp-pBac, providing the piggyBac transposase gene (Fig. 1a) (10). The 76 adults surviving injections were divided into three groups of males and one group of females, and each group was outcrossed separately (Table II). G1 females were group-mated and allowed to lay eggs in isolation, while G0 males were tested collectively. A minimum of three G0 adults (female No. 10 and individuals from male groups A and C, see Table II) produced fluorescent progeny, with a minimum integration frequency of 4%, comparable to that obtained using Minos to transform the same mosquito species (1). Due to the testing procedure used for G0 males, it was not possible to assess whether the fluorescent G1 larvae in groups A and C were derived from one or more progenitors. Among the 34 G1 females, 12 did not lay eggs after multiple feedings, accounting for a total estimated sterility of 35%. Microscopic analysis of G1 larvae revealed the presence of as many as seven different fluorescent phenotypes: three from female No. 10 (101-13), three from male group A (A1-3), and one from male group C (C1), which were
piggyBac-mediated Transformation of A. stephensi Using dsRED

TABLE II
Outcome of injection experiments

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Embryos injected</th>
<th>Larvae</th>
<th>Adults</th>
<th>WT outcross</th>
<th>Group/ founder</th>
<th>fluorescent/ total G1s</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMiRED</td>
<td>102</td>
<td>32 (31%)</td>
<td>23 (22%)</td>
<td>13 males</td>
<td>No. 7</td>
<td>0 / 7,252</td>
</tr>
<tr>
<td>pPBRED</td>
<td>303</td>
<td>117 (39%)</td>
<td>76 (25%)</td>
<td>15 males</td>
<td>A</td>
<td>19 / 2,340***</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>20 males</td>
<td>B</td>
<td>0 / 4,793</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>7 males</td>
<td>C</td>
<td>25 / 214</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>34 females</td>
<td>No. 10</td>
<td>38 / 2,797***</td>
</tr>
</tbody>
</table>

Fig. 2. A. stephensi mosquitoes expressing dsRED. a and b, confocal fluorescence microphotographs of transgenic first and third instar larvae showing differential dsRED expression. c, adult female mosquito showing strong dsRED expression in the gut.

We have demonstrated the ability of the piggyBac transposable element to drive integration of exogenous DNA in A. stephensi mosquitoes. The availability of a second functional transposable element in A. stephensi will introduce important improvements to the transgenic system recently developed in these mosquitoes by using Minos as a delivery vector (1). Minos-transformed mosquito lines can now be further manipulated by introducing additional genes with the use of piggyBac transposons. Furthermore, mobilization studies can be performed to assess the capability of nonautonomous Minos and piggyBac transposons to be remobilized in crosses with transgenic lines producing their specific transposase genes. Since the Minos and piggyBac elements do not seem to cross-mobilize each other, they are well suited for such studies. Remobilization experiments will be fundamental in understanding the feasibility of using autonomous transposable elements to spread malaria-refractory genes throughout a wild population.

Our results in A. stephensi mosquitoes, which have been confirmed by the work performed in collaborating laboratories, testify to the broad host range of piggyBac, which makes it a very attractive transposon for germline transformation experiments. An EGFP-loaded piggyBac transposon has also recently been validated as DNA delivery vector in Anopheles gambiae mosquitoes, although at frequencies much lower than those obtained in this study (6). However, when comparing piggyBac to Minos, the fact that the latter element generally creates single integrations may represent an advantage in terms of studying the effects of introducing foreign DNA into the genome of heterologous organisms (1). The presence of piggyBac-like transposons in different species seems to indicate the occurrence of horizontal transfer of this element (19). Horizontal transfer has also been postulated for Minos (20).

More data are needed on these two transposons before population replacement schemes can be implemented.

We have also validated the use of dsRED as a selectable marker in germline transformation of A. stephensi mosquitoes. dsRED, isolated from Discosoma sp. (3), has been used as a reporter gene in Saccharomyces cerevisiae and in zebrafish and very recently as a selectable marker in D. melanogaster (21–23). Previous Minos-mediated integrations in A. stephensi were achieved using the green fluorescent protein EGFP as a selectable marker (1). Interestingly, in A. stephensi mosquitoes, dsRED showed slower kinetics of formation than EGFP as demonstrated by the weak fluorescent phenotype observed in first instar larvae of all dsRED transgenic lines. However, dsRED is visible in the gut of adult A. stephensi individuals, a feature that would allow the easy identification of transgenic insects in recapture studies after release in the wild. Importantly the emission profiles of the dsRED and EGFP markers do not overlap in the progeny of mixed transgenic populations (data not shown). The availability of an additional selectable marker will extend the range of applications of germline transformation in Anopheles mosquitoes, allowing functional studies such as transposon remobilization, enhancer trapping, and transposon tagging, which are based on crossing of lines expressing different markers. It will also have an important impact on the manipulation of other insect species for which mutation-based visible markers are not available. In conclusion, the results achieved in this study are anticipated to have an essential impact upon the development of an array of genetic tools for studying the biology of Anopheles mosquitoes.
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REFERENCES