Research article

Gene targeting in mosquito cells: a demonstration of 'knockout' technology in extrachromosomal gene arrays

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Abstract

Background: Gene targeting would offer a number of advantages over current transposon-based strategies for insect transformation. These include freedom from both position effects associated with quasi-random integration and concerns over transgene instability mediated by endogenous transposases, independence from phylogenetic restrictions on transposon mobility and the ability to generate gene knockouts.

Results: We describe here our initial investigations of gene targeting in the mosquito. The target site was a hygromycin resistance gene, stably maintained as part of an extrachromosomal array. Using a promoter-trap strategy to enrich for targeted events, a neomycin resistance gene was integrated into the target site. This resulted in knockout of hygromycin resistance concurrent with the expression of high levels of neomycin resistance from the resident promoter. PCR amplification of the targeted site generated a product that was specific to the targeted cell line and consistent with precise integration of the neomycin resistance gene into the 5' end of the hygromycin resistance gene. Sequencing of the PCR product and Southern analysis of cellular DNA subsequently confirmed this molecular structure.

Conclusions: These experiments provide the first demonstration of gene targeting in mosquito tissue and show that mosquito cells possess the necessary machinery to bring about precise integration of exogenous sequences through homologous recombination. Further development of these procedures and their extension to chromosomally located targets hold much promise for the exploitation of gene targeting in a wide range of medically and economically important insect species.

Background

The genetic manipulation of insect genomes may herald novel strategies for the control of insect-borne disease and could provide the means both to limit economic damage by crop pests and increase productivity in commercially important insects. Such manipulation is now considered routine in the fruit fly, *Drosophila melanogaster* and is based on the exploitation of transposable genetic elements such as *P*. Current attempts at the transformation of non-drosophilid insects have also focused on this approach but phylogenetic restriction in mobility of the *P*-element has necessitated a search for alternative functional transposons [1]. As a result, there are now four transposable elements, derived from different eukaryotic transposable element families, that have been successfully deployed across dipteran, lepidopteran and coleopteran insects. First, the *Mosl* element, derived from *D. mauritiana* and belonging to the *Mariner* family

[2], which has been used to transform *D. melanogaster*[3], *D. virilis*[4], *Aedes aegypti*[5] and *Musca domestica*[6]. Secondly, the *Hermes* element, derived from the house fly *M. domestica* and a member of the *hAT* family [7], which has been used to transform *D. melanogaster*[8], *Ae. aegypti*[9], *Anopheles gambiae* cells [10], *Tribolium castaneum*[11], *Stomoxys calcitrans*[12], *Ceratitis capitata*[13] and *Culex quinquefasciatus* [P. W. Atkinson, *personal comm.*]. Thirdly, the *Minos* element, derived from *D. hydei* and a member of the *Tc*1 family [14], which has been used to transform *D. melanogaster*[15], *C. capitata*[16] and *An. stephensi*[17]. Finally, the *piggyBac* element, derived from *Trichoplusia ni* and a member of the TTAA family [18,19], which has been used to transform *C. capitata*[20], *D. melanogaster*[21], *T. castaneum*[11], *Bombyx mori*[22], *Pectinophora gossypiella*[23], *Bactrocera dorsalis*[24], *Anastrepha suspensa*[25], *M. domestica*[26], *Lucilia cuprina* [M. J. *Scott, personal comm.*], *Ae. aegypti* [M. J. Fraser, *personal comm.*], *An. gambiae* [M. Q. Benedict, *personal. comm.*], *An. stephensi* [M. Jacobs-Lorena, *personal comm.*] and *An. albimanus* [A. M. Handler, *personal comm.*].

Apart from this focus on transposable elements, other approaches to the transformation of non-drosophilid insects include the use of viral vectors. The Sindbis Alphavirus [27] is proving to be particularly effective at transducing genes into mosquito tissue [28] but does have certain limitations and has not been used successfully to generate transgenic insects. Similarly, pantropic retroviruses have been used to mediate stable gene transfer and gene expression in somatic cells from a variety of insect species [29–31] but have not proved effective at generating transgenic insects.

Despite these recent successes, both transposon and viral-mediated strategies are constrained to some extent by the quasi-random nature of the integration sites. This can give rise to insertional inactivation of essential genes and all transgenes introduced in this way can suffer dramatically from position effects on expression. For example, the transgene may not be expressed (or may be expressed sub-optimally) if integration occurs in a transcriptionally inactive region of the genome. As an alternative approach to insect transformation, we have been investigating the potential of gene targeting through homologous recombination. Such a mechanism would be independent of phylogenetic restrictions on transposon mobility and free of concerns over transgene instability mediated by non-specific transposases from endogenous mobile elements. Gene targeting would facilitate the precise introduction of transgenes into predetermined chromosomal sites of demonstrated transcriptional activity. It could also be used to 'knock out' both alleles of an endogenous gene in order to achieve specific phenotypic modifications. In addition, with appropriate construction of the gene-targeting vector, it would be possible to introduce specific mutations into a target gene and study the resulting phenotype [32] or revert mutant to wild-type alleles [33].

Gene targeting through homologous recombination has been exploited in yeasts [34], mammalian cells [35–38], protozoans [39–43], slime mould [44], plant cells [45], intact plants such as the moss, *Physcomitrella patens*[46] and *Arabidopsis*[47], fungal pathogens [48,49] and chicken cells [50]. Although little information is available for insects, the investigation and optimisation of targeting strategies has been greatly facilitated by using cultured somatic cells as a model system. Through such approaches, the machinery of homologous recombination has been demonstrated in both mosquitoes [51] and *Drosophila*[52]. More recently, gene targeting has been demonstrated *in vivo* in *Drosophila* through an elegant combination of transposon-mediated transformation and site-specific recombination. In these experiments, a construct carrying part of the target gene was integrated by means of a transposable element vector. Subsequently, a site-specific recombinase (FLP) and a site-specific endonuclease (I-*Sce*I) were used to generate extrachromosomal DNA molecules with a double-strand break in the region of homology. Such molecules would be present in every nucleus, providing an efficient substrate for gene targeting [53].

The most significant progress in optimising experimental parameters for gene targeting has involved the use of mouse embryonic stem cells and these studies have revealed the importance of vector design [54]. In particular, factors such as overall length of homology, isogenicity between donor and target sequences and vector topology may play an important role. Moreover, it has been found necessary to incorporate positive selectable markers to identify transformants as well as some mechanism for the enrichment of targeted integrations, which are likely to occur at a lower frequency than random (non-homologous) events. Such enrichment might include the use of negative selectable markers, such as the HSV-*tk* gene, which is cytotoxic in the presence of nucleoside analogues [55]. Similarly, it may involve promoter-trap strategies where a positive selectable marker, such as a neomycin resistance gene, is only expressed from an endogenous promoter in the event of targeted integration [56].

As part of our attempts to define the potential of gene targeting in the mosquito, we describe here the successful targeting of a hygromycin resistance transgene, previously transformed into the *Ae. aegypti* Mos20 cell line

and stably maintained as one or more multi-copy, extrachromosomal tandem arrays. The targeting replacement vector we employed carried a region of homology (the hygromycin resistance gene and SV40 terminator) disrupted by a promoterless neomycin resistance gene (*neo*). In this design, the *neo* gene serves as a promoter-trap for the enrichment of targeted integration since neomycin resistance can only be expressed from the promoter of the hygromycin resistance gene in the event of precise homologous recombination. Targeted integration events would therefore be detectable both by efficient expression of neomycin resistance and by inactivation (knockout) of hygromycin resistance.

Results and Discussion

Stable transformation of Mos20 cells to hygromycin resistance

Ae. aegypti Mos20 cells [57] were transfected with pACT-HYG (Fig. 1A) and selected with hygromycin. Twelve resistant colonies were established and subsequently maintained under intense hygromycin selection. High molecular weight DNA from one of these clones (AH-4) was analysed to determine the molecular basis of the transformation event. DNA was isolated and digested separately with *Bam*HI, *Eco*RI, *Nco*I, and *Sph*I. *Eco*RI cleaves once within pACT-HYG whereas all the others cut the transformation vector twice (Fig. 1A).

Following electrophoresis of the digestion products, a Southern blot was probed with the hygromycin resistance gene. The resulting autoradiograph revealed that the probe did not hybridise to untransformed (control) Mos20 genomic DNA (Fig. 2A; Lane C). Only high molecular weight signals (>23 Kb) were evident in undigested DNA (Fig. 2A; Lane U) indicating that the hygromycin resistance transgenes were not simply being expressed from free monomer plasmids within the cells. Digestion of AH-4 DNA with *Bam*HI gave rise to a signal of 1.1 Kb (Fig. 2A; Lane B) whereas *Eco*RI digestion yielded a major signal of 7.3 Kb (Fig. 2A; Lane E). *Nco*I digestion resulted in signals of 6.7 Kb and 0.6 Kb (Fig. 2A; Lane N) whereas the major signal in DNA digested with *Sph*I appeared at 3.5 Kb (Fig. 2A; Lane S). Reference to the structure of pACT-HYG (Fig. 1A) reveals that these signals correspond to digests of the free plasmid. This situation arises because cell lines transformed in this way frequently maintain the transgenes as multicopy tandem arrays, sometimes integrated into the chromosomes and sometimes as episomal arrays, or minute chromosomes [58]. It follows that Southern blots will give rise to banding patterns consistent with digests of the circular transformation vector. Similar banding patterns have been observed elsewhere following the stable transformation of mosquito cell lines [58–60], *Drosophila* cell lines [61–63] and mammalian cell lines [64]. It is believed that the

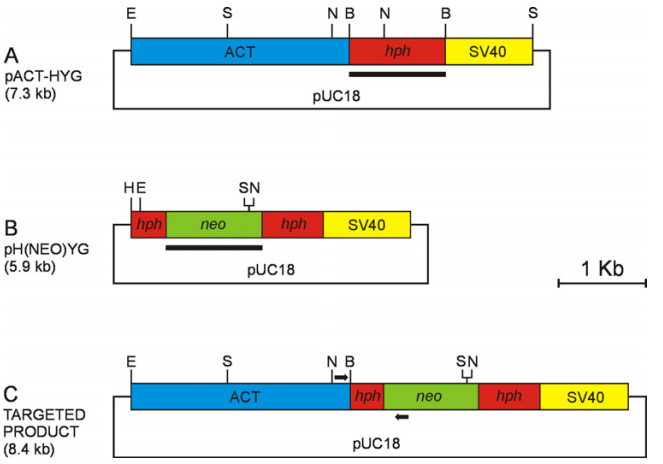

Figure 1
Structural maps of the input plasmids and predicted targeted product (A): The target site plasmid, pACT-HYG, carries the hygromycin B phosphotransferase gene (*hph*), driven by the *actin5C* promoter from *Drosophila* (ACT) with transcription terminated by the SV40 polyadenylation signal (SV40). The *hph* fragment used as a probe for Southern analysis is indicated by a solid bar and all relevant restriction sites are shown (B, *Bam*HI; E, *Eco*RI; N, *Nco*I; S, *Sph*I). (B): The targeting vector plasmid, pH(NEO)YG, carries a copy of the hygromycin B phosphotransferase gene (*hph*), disrupted by insertion of a promoterless neomycin phosphotransferase coding sequence (*neo*). Following precise integration into the target site, transcription of the *neo* gene would be terminated by the SV40 polyadenylation signal (SV40). The *neo* fragment used as a probe for Southern analysis is indicated by a solid bar and all relevant restriction sites are shown (H, *Hin*dIII; E, *Eco*RI; S, *Sph*I; N, *Nco*I). (C): Structural map of the predicted targeted product with the neomycin resistance coding sequence (*neo*) inserted precisely into the hygromycin phosphotransferase target gene (*hph*) and expressed from the *Drosophila actin5C* promoter (ACT). The locations of the forward and reverse primers used in the PCR analysis are indicated by arrows and all relevant restriction sites are shown (E, *Eco*RI; S, *Sph*I; N, *Nco*I; B, *Bam*HI).

tandem arrangement occurs independently of the replicative capacity of the transforming DNA and is facilitated by the use of large quantities of DNA during transfection [64].

Subsequent investigation showed that the hygromycin resistance phenotype was a stable component of the AH-4 cell clone, even in the absence of selection pressure for over six months. Fluorescence in-situ hybridisation (FISH) did not detect hygromycin resistance transgenes in metaphase chromosome spreads (data not shown) but hygromycin resistance plasmids could readily be rescued from 2 μg aliquots of high molecular weight DNA, digested with *Xho*I (which cuts once within pACT-HYG) and recircularised at low DNA concentrations (10 ng/ml). In this case, 18 ampicillin resistant colonies were rescued from digested AH-4 DNA but none from similarly digest-

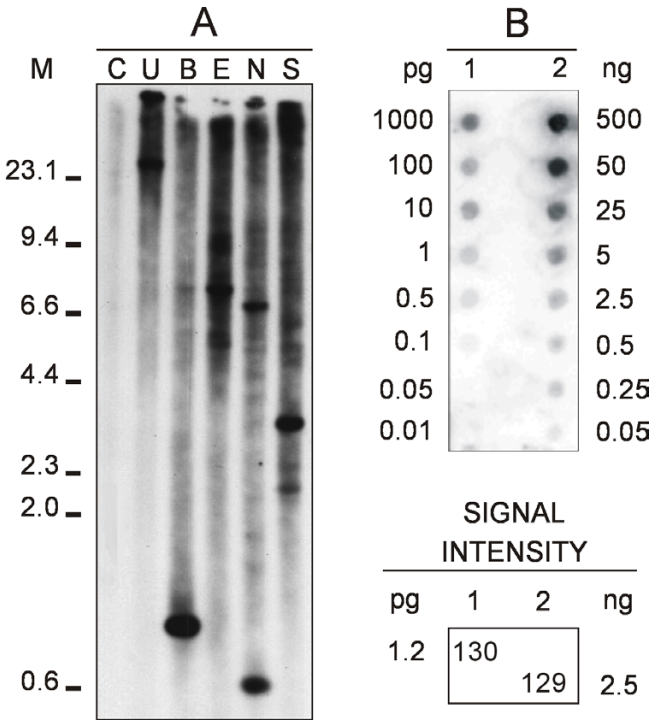

Figure 2
Southern analysis of AH-4 cell line and determination of target site copy number (A): High molecular weight DNA from the AH-4 cell line (5 μg per lane) was digested separately with *Bam*HI (Lane B), *Eco*RI (Lane E), *Nco*I (Lane N) or *Sph*I (Lane S) and fractionated on 0.75% agarose alongside undigested DNA (Lane U) and untransformed (control) Mos20 DNA (Lane C). The DNA was transferred to a nitrocellulose membrane and hybridised overnight at 42°C with the hygromycin resistance gene. The membrane was washed at high stringency (0.1 × SSC; 0.1 % SDS; 60°C) and exposed to X-ray film against an intensifying screen for 5 hours at -70°C. (B): The copy number of pACT-HYG target sites within the AH-4 cell line was determined by quantitative dot-blotting. The upper panel shows hybridisation of the hygromycin resistance gene to serial dilutions (1000 to 0.01 pg; left hand scale) of supercoiled pACT-HYG (Lane 1) and serial dilutions (500 to 0.05 ng; right hand scale) of high molecular weight DNA from AH-4 cells (Lane 2). The membrane was hybridised overnight at 42°C, washed at high stringency (0.1 × SSC; 0.1 % SDS; 60°C) and exposed to a phosphor imaging screen for 2 hours. This was scanned on a Molecular Dynamics Storm 860 phosphorimager with hybridisation intensity returned as a linear scale volume report. The lower panel shows signal intensity (average volume report) from either 1.2 pg plasmid DNA or 2.5 ng AH-4 DNA.

ed DNA of the untransfected Mos20 control. Interestingly, 3 colonies were also rescued from an aliquot of undigested AH-4 DNA, suggesting that monomer plasmids are occasionally shed from the arrays. All but two of the rescued plasmids analysed gave restriction patterns indistinguishable from those expected of the parent vector pACT-HYG. The two aberrant plasmids (RP8 and RP9) gave very different restriction patterns (Fig. 3A). In the case of RP8, novel DNA sequence was identified using the plasmid-based M13 primers (Fig. 3B) and BLAST searches indicated weak homology to filarial chitinase and bacterial NADH oxidase genes (data not shown). This suggests that the majority of the input plasmids retained the parental structure but that a minority, either individually or as part of arrays, had incorporated sections of bacterial or mosquito genomic DNA through recombinational processes.

The number of copies of pACT-HYG within the AH-4 cell line was determined by quantitative dot blotting of high molecular weight DNA against serial dilutions of supercoiled plasmid (Fig. 2B). The dot blot membrane was hybridised to the hygromycin resistance gene and signal intensity inferred on a linear scale using high-resolution phosphorimaging. Analysis of the data indicated that signal intensity from 2.5 ng AH-4 DNA was equivalent to that from 1.2 pg pACT-HYG plasmid (Fig. 2B; lower panel). This would suggest that around 0.05 % of the AH-4 DNA comprised pACT-HYG sequences. Taking the *Aedes* (Mos20) genome size to be 1.5×10^9 bp [65] this gives 7.2×10^5 bp of plasmid related sequence which, at a plasmid size of 7300 bp, equates to a copy number of around 100. However, because of potential rearrangements, not all of these might represent functional copies. These data, together with the functional and molecular analyses described below, indicated that around 100 copies of pACT-HYG were maintained extrachromosomally within the AH-4 cell line. Our data suggest that the majority exist as a primary tandem array that retains the parent plasmid structure. However, the presence of aberrant plasmid structures (Fig. 3) and the presence of fainter background signals on Southern blots of AH-4 DNA (Fig. 2A) suggest that there may also be secondary arrays comprising rearranged (and probably non-functional) copies of the hygromycin resistance plasmid, pACT-HYG.

Targeting the hygromycin resistance genes

The targeting vector pH(NEO)YG (Fig. 1B) was of the replacement type, where the region of homology is disrupted by an intervening sequence. In this case, the region of homology was a fragment of 2.1 Kb comprising the hygromycin resistance gene and SV40 termination sequence. The pUC 18 backbone did not form part of the region of homology since its orientation in the targeting vector was opposite to that of the target sequence within the AH-4 cell line. The intervening sequence was a promoterless bacterial neomycin resistance gene that, in the event of a targeted integration event would be driven from the *actin5C* promoter in the AH-4 cell line. With two exceptions, covering just a few base pairs, the region of homology between target and vector was isogenic. These exceptions were a base pair modification to the 5'

A

Plasmid	*Bam*HI	*Bgl*II	*Hind*III	*Pst*I	*Xho*I	*Eco*RI
pACT-HYG	6.1 1.1	3.7 3.5	7.2	7.2	7.2	7.2
RP8	4.7	3.2 1.5	4.7	3.4 1.3	4.7	4.7
RP9	9.4 2.9	8.5 3.8	7.6 2.5 2.2	3.9 3.6 2.5 1.2 1.1	10.8 1.5	5.5 3.2 2.6 2.0

B

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  1 ttgtaatgct actcactata gggccaattg ggccctctag atgcatgCTC
 51 GAGcggccgc cagtgtgatg gatatctgca gaattcggct tgggtatgtt
101 cgtggatgtt actacaattg gttttgtaat ggacggagat cctacagtgc
151 caggtgtaat gttgagactt acggagtttt cttcatgtta ggaattgtgt
201 tcctaattct gtccgttttt tatttcggaa gtatgtttgt tagaagcagt
251 tttcgaaaat taatcaagtg aaactgaaac atacatcaac aaaaagccaa
301 ctgcaagagt tggctttttt cttaaggttt cgctctttca tattgatgtg
351 ggaaaaagca atggtcctta tttttaaatg aaccccgaac cggaaggtaa
401 ggatttctta ataattttcg cgggcgacaa aaatgagatc tgcttgcatt
451 gattttagta aaatgtcctc agcatgtttg ccgatttatc aatccaacag
501 cgccggtagc aatttcggca tctttttttag cagcttccgc taatggaacc
551 tgatagccat caaacaaatt gatcttcgcg cctgaatgtt tcctccactt
601 gaaacatcga ccgagacatc atcaagccga attccagcac actggcggcc
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Figure 3
Restriction fragment and sequence analysis of parental and rescued plasmids (A): The progenitor plasmid (pACT-HYG) and two aberrant rescued plasmids (RP8, RP9) were digested with the restriction enzymes indicated and the products visualised by agarose gel electrophoresis. The resulting fragment sizes from each reaction are given in Kb. (B): The rescued plasmid RP8 was sequenced using the M13 forward and reverse primer sites. Data shown in yellow (1–86; 629–650) represent cloning vector sequences with the *Xho*I site used to linearise the DNA prior to plasmid rescue underlined in bold uppercase. Data shown in blue (87–628) represent novel sequence not found in the progenitor plasmid pACT-HYG and derived either from the host cell bacterial genome or the Mos20 mosquito cell line genome.

end of the *hph* gene in pACT-HYG that removed an *Eco*RI site to facilitate cloning [66] and the destruction of the *Nco*I site in the *hph* gene within pH(NEO)YG following insertion of the intervening *neo* coding sequence.

It is known that homologous recombination efficiency is maximised by the introduction of a double strand break near the region of homology within the targeting vector [67]. To this end, the targeting vector (pH(NEO)YG) was linearized at the 5' end of the *hph* gene by digestion with *Hind*III (Fig. 1B) and transfected into both AH-4 and Mos20 cells. After two days of selection with G418 at a concentration of 500 μg/ml, almost all AH-4 and Mos20 cells had died. The remaining cells were maintained in reduced volumes of serum rich medium to facilitate low-density growth. After 4 weeks, *neo* resistant colonies were evident among those cells derived from the AH-4 cell line. In three separate transfections of Mos20 cells (twelve 25 cm^2 flasks in total) there was no evidence of any cell survival under G418 selection. This strongly supports the hypothesis that neomycin resistance is only likely to be expressed from the *actin5C* promoter in the AH-4 cell line in the event of targeted integration.

Two neomycin resistant colonies were retrieved from four flasks of transfected AH-4 cells but only one of them (designated HT-1) was successfully expanded through 96 and 24 well plates into 25 cm^2 flasks for more detailed functional and molecular analyses. Determination of the overall efficiency of this approach in cultured cells is not straightforward since it is dependent on primary transfection efficiency and is also confounded by the loss of successfully targeted cells at low density under selection. In fact, most cell lines display poor viability when grown at low cell densities. The primary transfection efficiency for Mos20 cells was determined by introducing a construct expressing the enhanced green fluorescent protein reporter gene from the *Drosophila actin5C* promoter. Cells were examined under a Zeiss Axioplan fluorescence microscope using a standard FITC filter set 48 hours post-transfection. The primary transfection efficiency determined in this way was $7.6 \pm 3.3 \times 10^{-3}$ and given that approximately 1×10^7 cells were transfected this would suggest a targeting efficiency of around 1.25×10^{-5}. This, however, is likely to be a considerable underestimate because of the loss of targeted cells at very low densities under selection. Such problems may be partly alleviated by strategies that incorporate feeder cells if these can be developed for mosquitoes.

Functional and molecular analysis of the targeted cell clone HT-1

Analysis of the gene targeted cell clone HT-1 showed that all hygromycin resistance had been lost. Whereas AH-4 cells were resistant to hygromycin at concentrations up to 300 μg/ml, targeted HT-1 cells were killed at hygromycin concentrations of 25 μg/ml, the same as that required to kill control Mos20 cells. At the same time, HT-1 cells had acquired high levels of G418 resistance, with cells growing well at a concentration of 1.5 mg/ml whereas control cells were killed at 50 μg/ml. Furthermore, the newly acquired neomycin resistance was stable even when the HT-1 cells were maintained for over 6 months in the absence of selection pressure.

PCR analysis and sequencing were used to confirm the targeted integration of the *neo* coding sequence into the hygromycin resistance target site within the HT-1 cell line (Fig. 4). The PCR design incorporated a forward primer located at the 3' end of the *actin5C* promoter and a reverse primer located at the 5' end of the *neo* coding sequence. These were used in a reaction against HT-1 cellular DNA with the targeting vector plasmid pH(NEO)YG and AH-4 cellular DNA serving as negative controls. As expected, no products were amplified from either control template (Fig. 4; Lanes 1 and 2) since each of them carried only one of the primer sites. However, the HT-1 template gave rise to a single band of 850 bp corresponding to the size expected from a targeted inser-

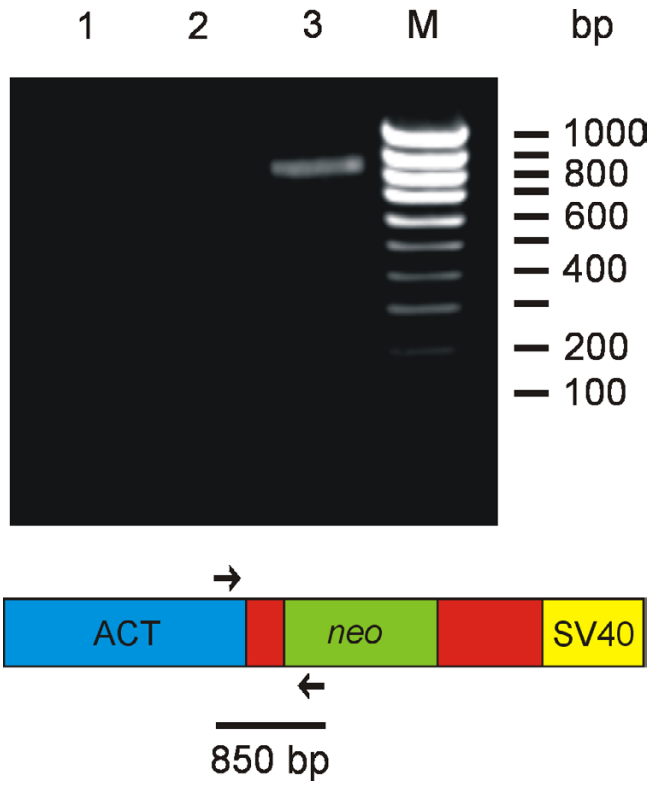

Figure 4
PCR analysis of the targeted site in the HT-1 cell line The forward primer was sited at the 3' end of the *Drosophila actin5C* promoter and the reverse primer was located at the 5' end of the neomycin resistance coding sequence. Amplification took place over 30 cycles (95°C, 30 seconds; 50°C, 30 seconds; 72°C, 1 minute) in a total volume of 50 µl containing 1 × PCR buffer, 2 mM $MgCl_2$, 200 µM each dNTP, 0.2 µM each primer and either 50 ng high molecular weight DNA or 1 ng plasmid DNA template. The upper panel shows an agarose gel analysis of 20 µl reaction products from AH-4 cellular DNA template (Lane 1); targeting vector plasmid (pH(NEO)YG) template (Lane 2) and HT-1 cellular DNA template (Lane 3). Lane M carries molecular size markers (MBI 100 bp ladder). The lower panel shows a structural map of the amplified region with the neomycin resistance coding sequence (*neo*) inserted into the hygromycin resistance gene (shown in red) downstream of the *Drosophila actin5C* promoter (ACT) with transcription terminated by the SV40 polyadenylation signal (SV40). The PCR primers (arrowed) can only amplify a product of 850 bp in the event of targeted integration of *neo* into the *hph* gene.

tion of *neo* into the *hph* gene (Fig. 4; Lane 3). The PCR product was cloned and sequenced and the sequence data confirmed the targeted integration of the *neo* gene into the 5' end of the *hph* gene (data not shown).

The molecular basis of the targeted integration was investigated further by Southern analysis of cellular DNA from the AH-4 and HT-1 cell lines. High molecular weight DNA was digested with four restriction enzymes (*Bam*HI, *Eco*RI, *Nco*I and *Sph*I) chosen for their ability to distinguish the DNA organisation in recipient and targeted cells. Digested and undigested DNA samples were fractionated by electrophoresis and transferred to nitrocellulose. The membrane was sequentially probed with the *hph* gene (Fig. 5A) and the *neo* gene (Fig. 5B) ensuring that the membrane was completely stripped between hybridisations. Two general points arose from these autoradiographs. First, undigested DNA gave only high molecular weight signals with each of the probes, indicating that both the target and the targeted product were maintained as tandem arrays. Secondly, there was a clear difference in banding pattern between AH-4 and HT-1 DNA, indicating either modification or loss of the primary AH-4 array structure following uptake of sequences from the targeting vector. To facilitate interpretation of the Southern blots, a map was constructed showing the predicted structure of the gene-targeted product within the HT-1 cell clone (Fig. 1C).

Southern analysis with the *neo* probe revealed no signals from AH-4 DNA (Fig. 5B; AH-4) thus indicating an absence of *neo* homology in this cell line prior to the introduction of the targeting vector. However, the *neo* probe gave rise to banding patterns in HT-1 DNA (Fig. 5B; HT-1) that were entirely consistent with those expected following digestion of the targeted product (Fig. 1C). Both *Bam*HI and *Eco*RI cut the targeted product once, giving rise to 8.4 Kb bands (Lanes B and E). *Nco*I cuts the targeted product twice resulting in two bands of 6.8 Kb and 1.6 Kb (Lane N) and *Sph*I also cuts the targeted product twice resulting in two bands of 5.65 Kb and 2.75 Kb (Lane S). Southern analysis of AH-4 DNA with the *hph* probe (Fig. 5A; AH-4) gave rise to banding patterns consistent with digestions of circular pACT-HYG, as described previously. With two exceptions, probing HT-1 DNA with the *hph* gene (Fig. 5A; HT-1) gave rise to banding patterns identical to those described for the *neo* probe and consistent with the proposed targeted product (Fig. 1C). The exceptions were additional bands of around 1.8 Kb and 1.9 Kb in the *Nco*I and *Sph*I digests respectively (Fig. 5A; HT-1; Lanes N and S). Since these bands were additional to the size of the predicted targeted product and were not detected by the *neo* probe, the most likely interpretation is that they represent further rearrangements outside of the primary pACT-HYG array, perhaps involving recombination with the targeting vector but not incorporation of the *neo* sequences. One possibility is that additional re-arrangements could have resulted from the plasmid vector backbones, which were in opposite orientations in the hygromycin resistance targets and the targeting vector.

Taken together, our data clearly show a knock-out of hygromycin resistance in the HT-1 cell line concurrent with

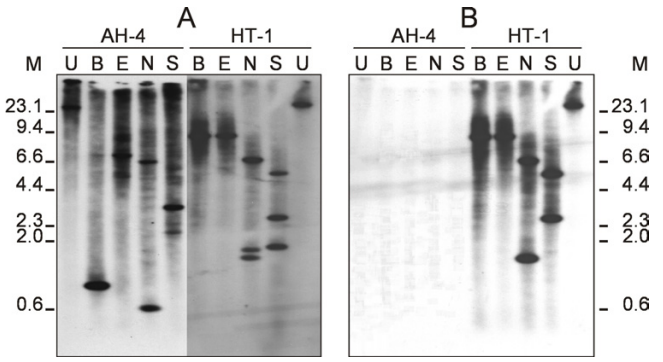

Figure 5
Southern analysis of DNA from the AH-4 and HT-1 cell lines High molecular weight DNA from the AH-4 and HT-1 cell lines (5 μg per lane) was digested separately with *Bam*HI (Lanes B); *Eco*RI (Lanes E); *Nco*I (Lanes N) or *Sph*I (Lanes S) and fractionated on 0.75% agarose alongside undigested DNA (Lanes U). The DNA was transferred to a nitrocellulose membrane and hybridised sequentially with the hygromycin resistance gene (A) and the neomycin resistance gene (B). Hybridisation took place overnight at 42°C with membranes washed at high stringency (0.1 × SSC; 0.1% SDS; 60°C) and exposed to X-ray film against an intensifying screen for 5 hours at -70°C.

the establishment of high levels of neomycin resistance. Molecular analysis revealed that both the target site and the targeted product existed as multi-copy arrays and confirmed the targeted integration of *neo* coding sequences into the *hph* gene. We estimate that there were around 100 functional copies of the *hph* gene in the primary AH4 array and the anticipated frequency of homologous recombination makes it unlikely that each of these could have been individually targeted. We therefore speculate that there are two possible mechanisms through which the observed modification might have arisen. First, a single copy of pACT-HYG within the primary AH-4 array may have been correctly targeted, with the subsequent loss of non-targeted *hph* genes and amplification of the targeted product under G418 selection. Alternatively, a monomer copy of pACT-HYG (such as those shed from the primary AH-4 array and detected by plasmid rescue) may have been correctly targeted and then amplified into an array under G418 selection, with concomitant loss of the primary pACT-HYG array. Either of these outcomes would have been facilitated by the intense neomycin selection and absence of hygromycin selection during cloning and expansion of the HT-1 cell line.

We now hope to extend this approach, initially to target single chromosomal genes within mosquito cells and, eventually, to bring about targeted modifications to the genome of intact insects. We speculate that this latter aim might be accomplished by techniques similar to those exploited in mouse embryonic stem (ES) cells [68,69]. ES cells are derived from the inner cell mass of the mouse blastocyst and remain undifferentiated under suitable tissue culture conditions. When these *in vitro* cultured cells are introduced into a blastocyst for continuation of their development *in utero* they can yield mouse chimeras able to transmit the ES cell genome to their offspring. Genetic changes introduced into ES cells in tissue culture can therefore be transferred into the mouse germline by breeding the chimeras and screening offspring for the ES genotype. Currently, ES cells are routinely derived from certain strains of mice, but similar cells have also been derived from the rat [70] and the pig [71]. In insects, there is potential to use germline precursor (pole) cells as an equivalent of the mouse ES cell system. *Drosophila* pole cells removed from one embryo can be successfully transplanted into another, where the cells become incorporated into the germ line [72,73]. Large quantities of *Drosophila* pole cells can be isolated from density gradients [74] and short-term *in vitro* cultured *D. melanogaster* pole cells have normal metabolism and ultrastructure and are able to give rise to functional germ cells *in vivo*[75]. Thus the possibility exists that pole cells could be genetically manipulated before they are reintroduced into embryos. Recent attempts at transfection of primary cultured pole cells followed by reintroduction into host embryos showed some success in the medfly [76] and it would be worthwhile to pursue similar techniques in mosquitoes.

There is also the potential to develop gene targeting in insects directly through embryo microinjection, provided that the efficiency of homologous recombination could be optimised to facilitate targeted modifications *in vivo*. There are reports in mammals of efficient introduction of functional transgenes by homologous recombination through microinjection [77]. Moreover, microinjection is accepted as the standard technique for the introduction of exogenous DNA into living insects and suitable protocols have been developed for a variety of species. The problems of targeting efficiency for such *in vivo* experiments could perhaps be addressed by combining homologous recombination with recent advances in transposon and viral-mediated transformation. Double strand breaks induced by *P* element transposition in the *Drosophila* genome have been used to generate high targeting efficiencies [78–80]. The potential therefore exists to use double strand breaks introduced by compatible transposable elements in other insects to facilitate gene targeting. It may also be possible to exploit more widely the combined use of transposition and site-specific recombination as described by Rong and Golic [see background; [53]]. Another novel strategy, developed recently in the silkworm, *B. mori*, uses a modified baculovirus to exploit the highly efficient viral-mediated

delivery of the targeting sequence [81]. These experiments provided an encouraging *in vivo* targeting efficiency of around 0.16%. Whether viral-mediated gene targeting can be more universally applied in insect transgenesis remains to be seen but experiments such as these clearly support the exploration of such technology.

Further efficiency gains could perhaps be achieved by exploiting gene silencing through double-stranded RNA interference (RNAi) to downregulate nuclear enzymes such as PARP (poly [ADP-ribose]polymerase), which is known to inhibit homologous recombination in higher eukaryotes [82]. The involvement of PARP in modulation of homologous recombination could explain a number of recent findings. It has been shown that homologous recombination frequencies in *Xenopus* oocytes are much higher than those in mature eggs or embryos [83]. Interestingly, this transition stage from oocyte to egg is also the first developmental stage when PARP activity can be detected [84]. Thus, homologous recombination appears to be the preferential pathway for DNA repair in early *Xenopus* oocytes. If this turns out to be generally true of germ cells then it could be argued that gene targeting might be much more efficient if it were directed at gametes. Whether this tissue difference reflects PARP activity, the haploid status of the gametes *per se* or some other germline specific environmental factor is not clear. However, a similar conclusion was reached in a recent study of gene targeting in the moss, *Physcomitrella patens*, where unusually high frequencies of homologous recombination were attributed to the predominantly haploid gametophytic life-cycle [46]. Thus, gene targeting vectors directed at insect oocytes (or perhaps more easily, sperm) may be able to generate high frequencies of homologous recombination and targeted transgene integration. In principle at least, such gene targeted gametes (whether modified *in vivo* or *in vitro*) could subsequently be fused to generate transgenic insects. This is an intriguing prospect but one that might require considerable preliminary work to facilitate the isolation, modification and survival of the targeted gametes.

Conclusions

These experiments provide the first demonstration of precise gene targeting in mosquito tissue. The targeted gene was a hygromycin resistance determinant, previously introduced into the Mos20 cell line and stably maintained in the resulting AH-4 cell line as part of an extrachromosomal tandem array. Using a promoter-trap strategy for the enrichment of targeted events, a promoterless neomycin resistance gene within the targeting vector was integrated precisely into the target site to generate the HT-1 cell line. This event resulted in both knockout of the hygromycin resistance gene and functional expression of the newly introduced neomycin resistance gene from the resident *actin5C* promoter. These experiments show that mosquito cells possess the necessary machinery to bring about precise gene targeting mediated by homologous recombination. Further development of these procedures and their extension to chromosomally located targets hold much promise for the exploitation of gene targeting in a wide range of medically and economically important insect species.

Materials and Methods

Construction of plasmid vectors

pACT-HYG (Fig. 1A) is a pUC18 based plasmid carrying the hygromycin B phosphotransferase (*hph*) gene as a positive selectable marker expressed from the *Drosophila actin5C* promoter. The *hph* gene was isolated as a 1.1 Kb *Bam*HI fragment from the plasmid pTRA151 [66] and ligated into the *Bam*HI site located between the *Drosophila actin5C* promoter and the SV40 polyadenylation signal in pACT-SV (unpublished data). The correct orientation of the *hph* gene was determined by restriction mapping. pH(NEO)YG (Fig. 1B) is a gene targeting replacement vector, which carries the hygromycin B phosphotransferase gene (*hph*) disrupted at the 5' end by insertion of a promoterless neomycin phosphotransferase gene (*neo*). A 2.2 Kb *Bam*HI-*Hin*dIII fragment containing the *hph* gene and the SV40 polyadenylation signal was isolated from pUChshyg [58] and cloned into pUC18 to form pHYG. This was cut with *Nco*I at the 5' end of the *hph* gene, end-filled to generate a 5' dCTP overhang and ligated to the promoterless *neo* coding sequence (isolated as a 1.1 Kb *Bam*HI fragment and end-filled to leave a 5' dGTP overhang) to create pH(NEO)YG. The correct orientation of the *neo* gene was confirmed by restriction mapping and by sequencing around the *hph:neo* junctions.

Cell transfection

Adherent cells were seeded at a concentration of 5×10^5/ml into 25 cm^2 flasks and grown to 75% confluence at 27 ± 0.5°C in 5 ml Medium 199 supplemented with foetal bovine serum (10%), yeastolate (1 μg/ml), lactalbumin hydrolysate (4 μg/ml) and L-glutamine (2 mM). Transfection mixtures were prepared by mixing 5 μg of plasmid DNA with 30 μl DOTAP (N-[l-(2,3-dioleoyloxy)propy]-N,N,N-trimethylammonium methylsulphate) in serum free Medium 199 for 15 minutes at room temperature. Cells were washed twice in Hanks Buffered Saline and incubated with the transfection mixture at 27 ± 0.5°C for 12 hours. Normal growth medium was restored and the cells allowed to recover for 48 hours at 27 ± 0.5°C before selection.

Generation of the hygromycin resistant clone (AH-4)

Replicate 25 cm^2 flasks of *Ae. aegypti* Mos20 cells [57] were each transfected with 5 μg supercoiled pACT-HYG and selected with hygromycin at a concentration of 100 μg/ml. The majority of the cells had died by first passage and, at this stage, the hygromycin concentration was reduced to 20 μg/ml to facilitate low-density survival. Surviving colonies (around 30–50 per flask) became apparent after 2–4 weeks and the hygromycin concentration was increased to 50 μg/ml as the density of these cells increased. Resistant clones were picked and expanded through 96 and 24 well plates into 25 cm^2 flasks. Twelve clones were established under intense hygromycin selection at a concentration of 200 μg/ml and one such clone (AH-4) was subjected to detailed analysis.

Generation of the gene targeted clone HT-1

The targeting vector pH(NEO)YG (Fig. 1B) was linearized at the 5' end of the *hph* gene by digestion with *Hin*dIII and 5 μg aliquots were transfected into replicate 25 cm^2 flasks of AH-4 cells. Normal Mos20 cells were transfected with the same construct as a negative control. Cells were selected with G418 at a concentration of 500 μg/ml and the majority of AH-4 and Mos20 cells died within two days. Flasks were maintained with reduced volumes (2 ml) of serum-rich (20%) medium to facilitate low-density survival. After two weeks, neomycin resistant colonies were evident among the AH-4 cells but none resulted from the control Mos20 cells. Surviving clones were picked and expanded through 96 and 24 well plates prior to establishment under G418 selection (500 μg/ml) in standard 25 cm^2 flasks.

PCR analysis

The forward primer (5'-GCATTGCGGCTGATAAGGTTTT-3') was located at the 3' end of the *Drosophila actin5C* promoter sequence and the reverse primer (5'-TCGGCAGGAGCAAGGTGAGAT-3') was located at the 5' end of the neomycin resistance coding sequence. These were used in a reaction against high molecular weight DNA from the HT-1 cell clone with DNA from the targeting vector plasmid pH(NEO)YG and high molecular weight DNA from the AH-4 cell line serving as negative controls. Amplification took place over 30 cycles (95°C, 30 seconds; 50°C, 30 seconds; 72°C, 1 minute) in a total volume of 50 μl containing 1 × PCR buffer, 2 mM $MgCl_2$, 200 μM each dNTP, 0.2 μM each primer and either 50 ng genomic DNA or 1 ng plasmid DNA template. 20 μl samples of PCR reaction product were subsequently visualised by agarose gel electrophoresis. PCR products were cloned into pGEM-T (Promega) and DNA from recombinant clones was purified on Qiagen columns prior to automated sequencing.

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