the heparin-antithrombin system is important in the balance between procoagulant and anticoagulant pathways on the vessel wall. The results of the present in vitro study suggest, however, that under certain conditions, the binding of antithrombin by heparin could lead to a potentially opposing outcome. The participation of heparin in the inactivation of antithrombin by elastase represents an unexpected action for this anticoagulant. A similar occurrence on in vivo surface-bound heparin could lead to a localized reversal of the nonthrombogenic character of the vascular endothelial lining.

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11. Functional activity levels of heparin were assayed by a chromogenic substrate assay with factor Xa as described by A. N. Teien and M. Lie [*Thromb. Res.* **10**, 399 (1977)]. Platelet factor 4 levels were determined with a commercial competitive radioimmunoassay kit (Abbott Laboratories).
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26 February 1987; accepted 8 June 1987

Stable Integration and Expression of a Bacterial Gene in the Mosquito *Anopheles gambiae*

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Foreign DNA was successfully introduced into the germline of the African mosquito vector of malaria *Anopheles gambiae*. Stable integration of genes into the germlines of insects had been achieved previously only in *Drosophila melanogaster* and related species and required the use of the P element transposon. In these experiments with *Anopheles gambiae*, the plasmid pUChsneo was used, which contains the selectable marker *neo* gene flanked by P element inverted repeats. Mosquitoes injected with this plasmid were screened for resistance to the neomycin analog G-418. A single event of plasmid insertion was recovered. Integration appears to be stable and, thus far, resistance to G-418 has been expressed for eight generations. The transformation event appears to be independent of P.

THE WORLDWIDE RESURGENCE OF malaria is due in part to drug resistance in the malaria parasite and insecticide resistance in the anopheline mosquito vectors. The development of control schemes based on genetic modification of the capacity of vector populations to transmit malaria (*1*) may eventually provide alternatives to traditional control methods. To achieve this goal, it will be necessary to develop methods for the physical introduction of genes into mosquito lines. This will make it possible to evaluate the effects of potentially parasiticidal genes (for example, diphenoloxidase) on the development of the parasite in vivo. Foreign DNA has been integrated into the germline of *Drosophila melanogaster* and related *Drosophila* species (*2*) through the use of the P element, a germline-specific transposable element of *D. melanogaster* (*3*). Similar transposons have not been found, nor has stable integration been achieved, in other insects. In this study, we developed a method for introducing DNA into the germline of the African malaria vector *Anopheles gambiae*.

To inject DNA into mosquito embryos, we initially followed the techniques described for injection of DNA into *D. melanogaster* embryos (*4*). However, a number of modifications were necessary because of the unique properties of mosquito eggs. The most important difference was the inability to remove the chorion, necessitating a needle that could penetrate the egg shell without killing the embryo.

Mosquito oviposition was controlled to provide eggs of precise age for injection (*5*). Eggs were maintained at 21° to 22°C to slow development of pole cells, the embryonic precursor of the germline. The pole cells of *A. gambiae* appear ultrastructurally similar to those of *D. melanogaster* (*6*) and form about 3 hours after oviposition at 21°C. Between 1.5 and 3 hours after oviposition, eggs floating on a drop of water were aligned and attached to double-stick cellophane tape; the other surface of the tape was attached to a slide. The water was removed from around the eggs and the eggs were dehydrated for 1 to 1.5 minutes, depending on the age of the eggs, in a desiccator until they were slightly bowed inward. Slight dehydration was required so that the eggs would not leak during injection. Excessive dehydration decreased viability and was avoided. After desiccation, prehydrated

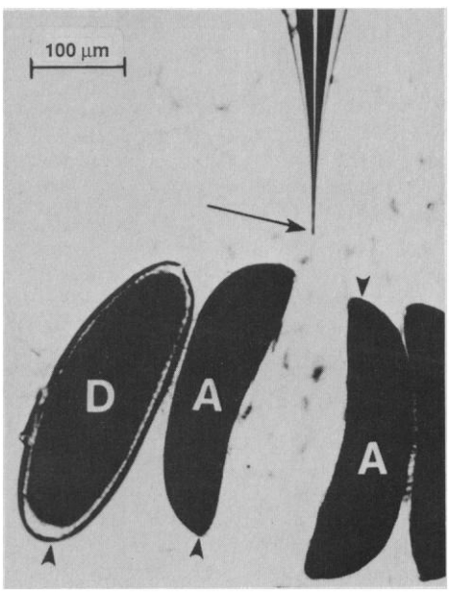

Fig. 1. Comparison of *A. gambiae* (A) and *D. melanogaster* (D) eggs. Arrowheads identify the posterior end of each egg. The end of the injection needle (arrow) is shown.

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halocarbon oil (Series 700, Halocarbon Products Corporation, 82 Burlews Court, Hackensack, New Jersey) was placed over the eggs and the slide was placed in a humid chamber. Because of the rapid movement of water across anopheline egg membranes compared to *Drosophila* egg membranes, the oil must be hydrated before use, and the oil-covered eggs must be kept in a humid chamber except during injection. The chorion of anopheline eggs rigidly maintains integrity of the embryo by limiting expansion during water uptake. Whereas the *Drosophila* chorion is easily removed, removal of the mosquito chorion results in embryonic death.

The glass microneedle used for injection of DNA into anopheline embryos must penetrate the chorion and vitelline membrane without killing the embryo. The design differs from needles used to penetrate less rigid dechorionated *Drosophila* eggs in that the narrow portion of the needle must be short (~100 μm) (Fig. 1), and the tip must be narrow (≤3 μm) and have a sharp piercing tip or acrosome (*7*). The needle was held at a 15° angle and penetrated the posterior pole of the egg just in front of the posterior tip (arrowhead in Fig. 1).

Whereas DNA injection can be observed directly in *Drosophila* eggs, the anopheline egg is opaque. Therefore, an automatic injection device (Eppendorf microinjector 5242) that delivers a reproducible quantum of liquid was used. The DNA concentration was 250 to 500 μg/ml of the plasmid pUChsneo (*8*) and 50 to 100 μg/ml of the plasmid pUChsπ(Δ2-3)wc (*9*) in 5 m*M* KCl, 0.1 m*M* sodium phosphate, *p*H 6.8.

After injection, water was added over the oil to facilitate rehydration. Within 30 minutes after injection, the eggs were lifted off the tape and placed on moist filter paper, which extended up the edge of a petri dish. The eggs were rehydrated on the moist filter paper and transferred to an insectary at 27°C where the larvae emerged 2 days later to swim into the water at the bottom of the dish.

As shown in Table 1, 24% of the injected eggs hatched whereas 58 to 78% of the noninjected eggs collected on the same days hatched. A high percentage of larvae survived to adulthood. The overall results on survival compare favorably with those obtained with *Drosophila* (*2*). Because a large number of adult male mosquitoes must be placed with females for mating to occur, the percentage of fertile male adults is unknown. The number of fertile females was not determined in the study.

Table 1. Survival of injected eggs to larval stage and adulthood.

Date (June 1986)	Number of		Adults		Percentage of		
	Eggs injected	Larvae emerged	♀	♂	Eggs to larvae	Larvae to adults	Eggs to adults
16	433	99	23	27	22.9	50.5	11.5
18	393	109	33	36	27.7	63.3	17.6
20	173	55	13	17	31.8	54.5	17.3
23	297	77			25.9		
24	354	91	76	95	25.7	59.4	13.4
25	441	84			19.0		
30	188	36			19.1		
Total	2279	551	145	175	24.2	58.1	14.0

The 145 female and 175 male adults (G_0 generation) resulting from injected embryos (Table 1) were mated with each other to produce a G_1 generation. First-instar G_1 larvae and control larvae were treated with 200 ng/ml of the neomycin analog G-418 (*10*). When over 99% of the control larvae had died, usually by the third or fourth day of G-418 treatment, surviving G_1 larvae were transferred to water without G-418. Of 9283 larvae selected with G-418, 28 G_1 males and 27 G_1 females survived to adulthood. After survival of the next generation was ensured, DNA was prepared separately from the 23 males and 13 females that were still alive at that time (*11*). This DNA was digested either with Pst I (males) or Hind III (females) and analyzed by Southern blotting for the presence of sequences homologous to pUChsneo. Ten of 23 males and 3 of 13 females contained the integrated plas-

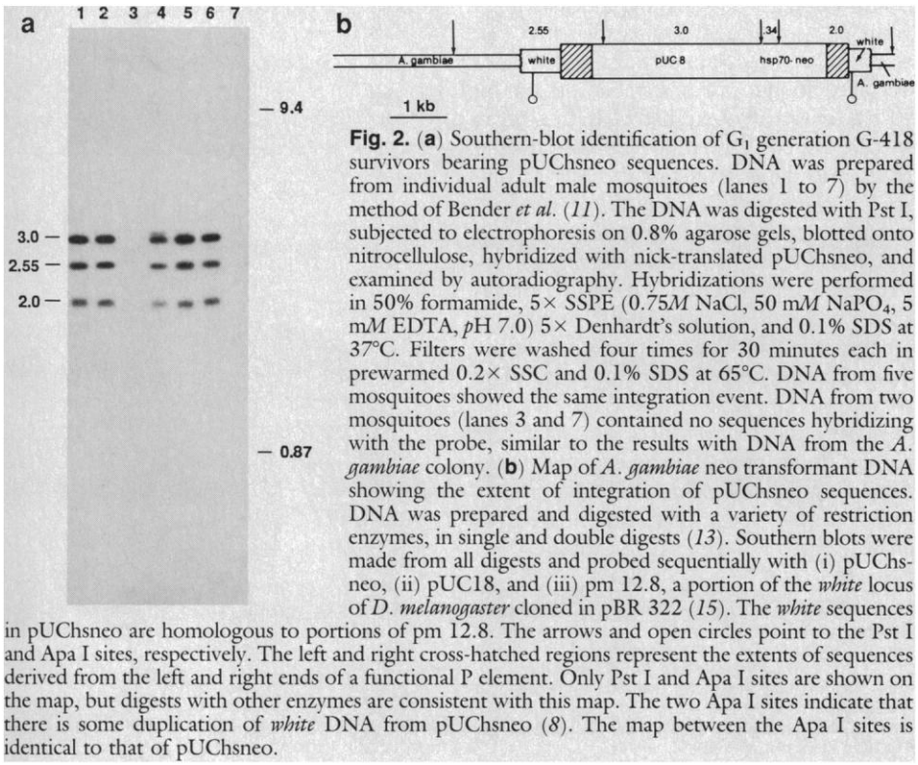

Fig. 2. (**a**) Southern-blot identification of G_1 generation G-418 survivors bearing pUChsneo sequences. DNA was prepared from individual adult male mosquitoes (lanes 1 to 7) by the method of Bender *et al.* (*11*). The DNA was digested with Pst I, subjected to electrophoresis on 0.8% agarose gels, blotted onto nitrocellulose, hybridized with nick-translated pUChsneo, and examined by autoradiography. Hybridizations were performed in 50% formamide, 5× SSPE (0.75*M* NaCl, 50 m*M* $NaPO_4$, 5 m*M* EDTA, *p*H 7.0) 5× Denhardt's solution, and 0.1% SDS at 37°C. Filters were washed four times for 30 minutes each in prewarmed 0.2× SSC and 0.1% SDS at 65°C. DNA from five mosquitoes showed the same integration event. DNA from two mosquitoes (lanes 3 and 7) contained no sequences hybridizing with the probe, similar to the results with DNA from the *A. gambiae* colony. (**b**) Map of *A. gambiae* neo transformant DNA showing the extent of integration of pUChsneo sequences. DNA was prepared and digested with a variety of restriction enzymes, in single and double digests (*13*). Southern blots were made from all digests and probed sequentially with (i) pUChsneo, (ii) pUC18, and (iii) pm 12.8, a portion of the *white* locus of *D. melanogaster* cloned in pBR 322 (*15*). The *white* sequences in pUChsneo are homologous to portions of pm 12.8. The arrows and open circles point to the Pst I and Apa I sites, respectively. The left and right cross-hatched regions represent the extents of sequences derived from the left and right ends of a functional P element. Only Pst I and Apa I sites are shown on the map, but digests with other enzymes are consistent with this map. The two Apa I sites indicate that there is some duplication of *white* DNA from pUChsneo (*8*). The map between the Apa I sites is identical to that of pUChsneo.

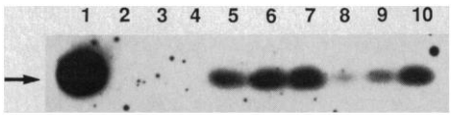

Fig. 3. Assay for neomycin phosphotransferase (NPT). Transformed adult female (lanes 5, 6, and 7) and male (lanes 8, 9, and 10) mosquitoes and control, untransformed adult mosquitoes (lanes 2, 3, and 4) were left at insectary temperature of 27°C (lanes 2, 5, and 8) or heat shocked at 37°C for 15 minutes (lanes 3, 6, and 9) or for 1 hour (lanes 4, 7, and 10). Ninety minutes after exposure to heat shock, individual mosquitoes in 50 μl of phosphate-buffered saline were ground with a pestle in an Eppendorf tube, freeze-thawed three times, and centrifuged (Eppendorf). The supernatant was mixed with an equal volume of loading buffer. The supernatants were subjected to electrophoresis (15 hours, 50 V) on a nondenaturing polyacrylamide (Pharmacia) gel system, and transfer of ^{32}P from $\gamma^{32}P$-ATP (>5000 Ci/mmol, Amersham) to a kanamycin (Sigma) substrate was achieved by using the method of Reiss *et al.* (*16*) to detect NPT activity. The 29-kD NPT protein from *neo*-transformed cells was run as a control in lane 1. Note the increased expression of NPT after exposure to heat shock.

mid DNA (Fig. 2a) (*12*). The sizes of the Pst I fragments identified by the probe were essentially identical for all ten G_1 males showing hybridization. This indicates that a single event of integration had occurred (Fig. 2), presumably in a primordial germ cell that gave rise to many identical gametes. Analysis of Pst I–digested DNA from G-418 survivors through at least the G_5 generation shows the same pattern of fragments (*13*). Thus, this integration event is stable. The integrated *neo* was expressed constitutively at greater levels in adult male and female mosquitoes than in larvae. Heat shock increased the level of expression in both larvae and G_8 adults (Fig. 3), indicating that the hsp 70 on the integrated *neo* gene is functional.

To determine the nature of the integration event, we analyzed DNA from adult G-418 survivors with the use of several restriction enzymes (Fig. 2b) (*12*). Previous studies of DNA sequences at 18 P element integration sites in *Drosophila* showed that integration is a precise event extending through the 31-bp terminal repeats of P and transposing no flanking DNA (*14*). If the integration event in *A. gambiae* had occurred by the same mechanism as in *Drosophila*, then no DNA from *white* [see (*8*)] should flank the P element terminal repeats. When the Southern blots used for mapping the integration event were reprobed with the relevant *white* sequence (*15*), *white* DNA was found flanking both sequences of P. For example, the probe of the Pst I digest shown in Fig. 2 with a fragment of the *white* locus in pBR322 (pm 12.8) showed hybridization with the same three DNA fragments as with pUChsneo. If integration had been precise at the P terminal repeats and had not included flanking *white* sequences, then pm 12.8 would have only hybridized with the 3.0-kb fragment and not the 2.55-kb and 2.0-kb fragments. Thus, it appears that integration was independent of the P element. More information about the nature of the transformation event in *A. gambiae* will be derived from plasmid rescue of the inserted sequences and flanking mosquito DNA and from sequencing regions about the two integration junctions. We also need to analyze more transformants to see whether P will have any relevance to integration of foreign DNA in *A. gambiae*.

We have demonstrated that DNA can be stably integrated into insects other than *Drosophila* species. This required novel solutions to the problems of dehydration, needle construction for penetration of intact eggs, injection of DNA into opaque eggs, and antibiotic selection. Although we have observed only one integration event, it should be possible to improve the efficiency of integration with a high-frequency integration vector or other molecular manipulations. This work extends the use of genetic transformation to medically important insects and, in anopheline mosquitoes, opens the possibility of studying the effects of novel promoter-gene constructs on the development of malaria parasites.

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5. Four days after a blood meal, female mosquitoes were anesthetized with ether and their wings were removed. During the recovery from anesthesia (1 hour or more), the mosquitoes were kept in the humid insectary. When the mosquitoes were placed on water at 21° to 22°C in a petri dish that had a strip of filter paper around the edge, they began to lay eggs. Ten minutes later the mosquitoes were removed.
6. R. K. Sakai, W. Daniel, L. H. Miller, unpublished data.
7. Needles were made by using a de Fonbrune-style microforge fitted with a compound microscope providing working magnification of ×250. Glass tubing (1.0 mm outer diameter and 0.8 mm inner diameter) was first pulled by hand and then on a microforge with a 1-g weight. The tip was broken until it was approximately 100 μm in length and 2.5 to 3.0 μm in diameter at the tip (Fig. 1). One side of the tip was gently brought into contact with a hot glass ball on the microforge and immediately pulled away to form a sharp acrosome. The needle was unable to penetrate the egg without an acrosome.
8. The plasmid pUChsneo, used for transformation, was constructed and sent to us by H. Steller and V. Pirrotta [*EMBO J.* **4**, 167 (1985)]. It contains the gene for neomycin resistance (*neo*) from the transposon Tn5 downstream from a *Drosophila* hsp 70 heat shock promoter. The construct includes pUC8 and hsp 70 neo flanked by regions of P element sequence ending in the 31-bp terminal repeats required for P-mediated integration. Sequences from the *Drosophila white* locus from which this P element was cloned flank both ends of P and were ligated together to circularize the plasmid.
9. The plasmid pUChsπ(Δ2-3)wc was used as helper and was the gift of F. Laski and G. Rubin. The design of this plasmid was based on the discovery that one control of P element transposition is at the level of messenger RNA splicing [F. A. Laski, D. C. Rio, G. M. Rubin, *Cell* **44**, 7 (1986)]. The intron between exons 2 and 3 of the P element has been removed; the P element is on an hsp 70 promoter. One of the inverted repeats of the P element has been removed (winged clipped, wc) to prevent P-driven integration of this element. The modified element is inserted into pUC18.
10. To establish the conditions for selection with G-418, we reared newly emerged wild-type mosquito larvae of the G3 strain of *A. gambiae* (obtained from the London School of Hygiene and Tropical Medicine and originating in the Gambia, West Africa) in 16 by 27.5 cm enamel-coated pans in 300 ml of water with various concentrations of G-418. The concentrations of G-418 (Gibco) are the concentrations of active ingredient. Death of some larvae occurred at 25 ng/ml; >99% died at 200 ng/ml. Infrequently (<1%), a pan at 200 ng/ml had many surviving larvae (breakthrough in selection). Despite heating the pans at 300°C overnight to kill microorganisms that may carry neomycin resistance genes and despite keeping rearing conditions constant, we continued to find an occasional pan with high numbers of mosquito survivors. Larvae from these rare breakthrough pans were discarded.
11. DNA was prepared from etherized adult mosquitoes using a modification of the techniques of W. Bender, P. Spierer, D. S. Hogness [*J. Mol. Biol.* **168**, 17 (1983)]. Grinding was performed in 1.5-ml centrifuge tubes with the use of Kontes teflon pestles and was continued until no body parts were visually identifiable.
12. L. H. Miller *et al.*, unpublished data.
13. P. Romans, R. K. Sakai, L. H. Miller, unpublished data.
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15. Probe pm 12.8 was a gift of G. Rubin and contains a 1.3-kb Sal I fragment from the *white* locus of *D. melanogaster* cloned into pBR322.
16. B. Reiss *et al.*, *Gene* **30**, 211 (1984).

17. We thank F. Laski and G. Rubin for the pUChsπ(Δ2-3)wc prior to publication of the construct; H. Steller and V. Pirrotta for the pUChsneo; G. Rubin, F. Laski, H. Steller, A. Spradling, and A. Cockburn for discussions of the approaches and results; and I. Green and W. F. Anderson for manuscript review. P.R. was supported by a grant from the Rockefeller Foundation.

2 April 1987; accepted 23 June 1987