Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 6181–6185, June 1996
Genetics

Pantropic retroviral vectors integrate and express in cells of the malaria mosquito, *Anopheles gambiae*

TOMOYO MATSUBARA*, RICHARD W. BEEMAN†, HIROKO SHIKE*, NORA J. BESANSKY‡, ODETTE MUKABAYIRE‡, STEPHEN HIGGS§, ANTHONY A. JAMES¶, AND JANE C. BURNS*‖

*Department of Pediatrics, University of California, San Diego, School of Medicine, 9500 Gilman Drive, La Jolla, CA 92093; †U.S. Grain Marketing Research Laboratory, U.S. Department of Agriculture, Agricultural Research Service, 1515 College Avenue, Manhattan, KS 66502; ‡Centers for Disease Control and Prevention, Division of Parasitic Diseases, 4770 Buford Highway, Chamblee, GA 30341; §Arthropod-Borne and Infectious Diseases Laboratory, Colorado State University, Fort Collins, CO 80523; and ¶Department of Molecular Biology and Biochemistry, University of California, Irvine, CA 92717

Communicated by John H. Law, University of Arizona, Tucson, AZ, February 7, 1996 (received for review October 30, 1995)

ABSTRACT **The lack of efficient mechanisms for stable genetic transformation of medically important insects, such as anopheline mosquitoes, is the single most important impediment to progress in identifying novel control strategies. Currently available techniques for foreign gene expression in insect cells in culture lack the benefit of stable inheritance conferred by integration. To overcome this problem, a new class of pantropic retroviral vectors has been developed in which the amphotropic envelope is completely replaced by the G glycoprotein of vesicular stomatitis virus. The broadened host cell range of these particles allowed successful entry, integration, and expression of heterologous genes in cultured cells of *Anopheles gambiae*, the principle mosquito vector responsible for the transmission of over 100 million cases of malaria each year. Mosquito cells in culture infected with a pantropic vector expressing hygromycin phosphotransferase from the *Drosophila* hsp70 promoter were resistant to the antibiotic hygromycin B. Integrated provirus was detected in infected mosquito cell clones grown in selective media. Thus, pantropic retroviral vectors hold promise as a transformation system for mosquitoes *in vivo*.**

Current methods for introduction of heterologous DNA sequences into mosquito cells in culture include transfection of plasmid DNA and infection with Sindbis virus vectors that express high levels of foreign protein in the cytoplasm of infected cells (1–3). However, neither of these systems permits heritable, stable gene expression in whole organisms. Genetic transformation of adult mosquitoes has been achieved by microinjection of plasmid DNA sequences into the fertilized mosquito egg (4–6). However, application of this approach has been limited by the inefficiency with which stable, transgenic lines are produced. As a first step to developing a transformation system, we tested whether a new class of retroviral vector could mediate foreign gene expression in mosquito cells in culture.

Retroviral vectors in which foreign DNA sequences are expressed from the viral long terminal repeat (LTR) or internal promoters are valuable tools for heterologous gene expression in a number of vertebrate species (7). These vectors can contain up to 10–13 kb of heterologous DNA and can integrate stably into the genome of dividing cells. The range of host cells that can be infected by such vectors is limited to cells expressing a specific protein receptor that is the ligand for the retroviral envelope protein and mediates attachment to the cell surface and endocytosis of the viral particle. Retroviral pseudotypes adopt the host cell range of the virus donating the envelope protein (8, 9). Pantropic vectors with a broad host range have been constructed using the envelope glycoprotein of vesicular stomatitis virus (VSV-G), which binds to phospholipid components in the cell membrane (10). In addition, these pantropic vectors have increased membrane stability and can be concentrated to high titer by ultracentrifugation (11).

Previous experiments have documented the ability of wild-type vesicular stomatitis virus to productively infect mosquito and *Drosophila* cell lines (12, 13). To determine whether VSV-G pseudotyped vectors could attach, uncoat, and reverse-transcribe in mosquito cells in culture, we incubated pantropic vectors with MOS-55 cells from *Anopheles gambiae* (14). These retroviral vectors integrated into the mosquito genome and conferred a selectable phenotype to the infected cells.

METHODS

Cell Lines. The MOS-55 cell line, derived from larvae of *A. gambiae* (gift of C. J. Leake, London School of Hygiene and Tropical Medicine, United Kingdom), was maintained in L-15 medium supplemented with 10% fetal calf serum, 100 units of penicillin G per ml, and 100 μg of streptomycin sulfate per ml at room temperature. These cells are diploid (2N = 6) and have a doubling time of ≈16 h (14).

Vector Constructs. The plasmid pLSRNL has been previously described (ref. 15; see Fig. 1). To construct pLNhsp70ZL and pLNhsp70hygL, we digested pLNpoZL (16) with *Xho*1 and *Bam*HI to remove the polio ribosomal entry site (po) and the β-galactosidase cDNA (Z) and ligated the resulting 5.6-kb fragment with either the 3.5-kb *Xho*I–*Bam*HI fragment of p194.ZT (17), which contains the *Drosophila* hsp70 promoter controlling the expression of β-galactosidase, or the 1.9-kb *Xho*1–*Bgl*II fragment of pUChsp70-hyg (ref. 4; see Fig. 1).

Packaging Cell Lines and Pseudotyped Virus Production. The 293 gag-pol/LSRNL packaging cell line has been previously described (15). The 293 gag-pol/LNhsp70ZL and/LNhsp70hygL packaging cell lines were constructed according to the method of Yee *et al.* (18). Briefly, 20 μg of pLNhsp70ZL or pLNhsp70hygL were transfected by standard calcium phosphate coprecipitation into the PA317 cell line, which expresses the MoMLV *gag*, *pol*, and *env* gene products. Culture supernatants containing amphotropic virus were harvested at 48 h posttransfection, and 1 ml of viral supernatant used to infect the 293 gag-pol cell line, which expresses high levels of the MoMLV *gag* and *pol* gene products (11). Infected 293 gag-pol/LNhsp70ZL cells expressing the neomycin phosphotransferase (*neo*) gene product were selected in 400 μg of G418 per ml, and resistant colonies were screened for β-galactosidase

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: VSV-G, envelope glycoprotein of vesicular stomatitis virus; LTR, long terminal repeat.

Data deposition: The sequences reported in this paper have been deposited in the GenBank data base (accession nos. U31354 and U31415).

‖To whom reprint requests should be addressed at: Department of Pediatrics, 0609D, University of California, San Diego, School of Medicine, La Jolla, CA 92093. e-mail: jcburns@ucsd.edu.

expression by incubation of cell lysates with the substrate *o*-nitrophenol β-D-galactoside (ONPG) as described (19). For the 293 gag-pol/LNhsp70hygL cells expressing both the neomycin and hygromycin phosphotransferase gene products, G418-resistant cells were placed in hygromycin B (100 μg/ml), and clones resistant to both antibiotics were selected.

To produce pseudotyped virus, the clones, LNhsp70ZL.6 or LNhsp70hygL.3, were transfected by calcium phosphate coprecipitation with pHCMV-VSV-G (VSV-G expressed from the human cytomegalovirus promoter), and virus-containing supernatants were harvested at 48–96 h posttransfection, filtered (0.45-μm filter), and stored at −70°C as described (18). Virus titer was determined by infection of 208F rat fibroblasts followed by selection in G418 (400 μg/ml). Virus stocks for infection of MOS-55 cells contained between 1 and 5×10^5 infectious units per ml.

PCR Detection of Provirus. DNA was extracted from infected and control cells following overnight digestion at 37°C in a buffer containing 100 mM NaCl, 10 mM Tris (pH 7.4), 25 mM EDTA, 0.5% SDS, and 20 μg of proteinase K per ml. The aqueous phase was extracted with phenol:chloroform, precipitated with isopropanol, and resuspended in sterile water, and the concentration was determined by spectrophotometry at 260 nm. Primers specific for the hepatitis B surface antigen gene were used to amplify proviral DNA from 1 μg of cellular DNA as described (20). Control primers for β-actin were used to confirm the presence of amplifiable DNA in all samples (20).

Characterization of Proviral Integration Sites. Infected and control cellular DNA was subjected to hemi-nested, "one-sided" PCR (21) to amplify the sequence surrounding vector insertion sites. PCR primers were designed to amplify downstream insertion junctions containing the 3′-end of the 3′-LTR and flanking host sequences. For the first round of amplification, LTR primer 1 (5′-CCCAAGGACCTGAAATGAC-3′) was paired with the universal primer (5′-CTCCTCT-GAGTGATTGACTACCNNNNNNNNNCCTAC-3′) in the following reaction mix: 10 mM Tris (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 1 μM each primer, 200 μM each dNTP, and 0.2 μl of *Taq* polymerase (Perkin–Elmer). Following 30 amplification cycles (94°C for 30 sec, 50°C for 30 sec, and 72°C for 60 sec), 1 μl of PCR product was subjected to a second round of amplification (30 cycles) with LTR primer 2 (5′-GGGTTT-TCCCAGTCACGAC-3′) and linker primer 2 (5′-GGGTTT-TCCCAGTCACGAC-3′). The resulting products were separated on low melt agarose gels, eluted using the Promega Wizard PCR Prep kit, and sequenced using the Promega fmol cycle sequencing kit.

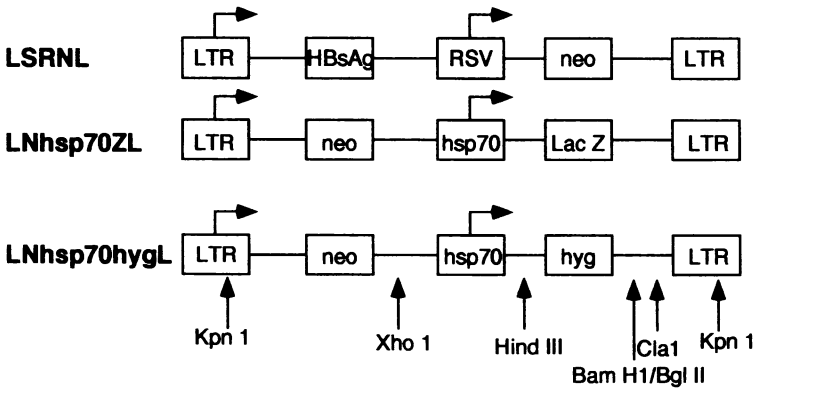
To confirm the mosquito origin of these flanking sequences, the *Eco*RI fragment of junction 1 (25 ng) was labeled by the random-priming method with [^{32}P]dATP and [^{32}P]dCTP and hybridized to 20 μg of uninfected, *Eco*RI-digested MOS-55 DNA resolved on an 0.8% agarose gel and transferred to a nylon membrane. *In situ* hybridization of biotinylated (Bionick Labeling System, GIBCO/BRL) junction 1 and junction 2 with the polytene chromosomes derived from the ovaries of female *A. gambiae* pink eye standard (PEST) strain was performed according to the method of Kumar and Collins (22).

To characterize the copy number and size of integrated provirus in unique clones of hygromycin-resistant MOS-55 cells, 20 μg of cellular DNA was digested with either *Hin*dIII or *Kpn*I (see Fig. 1) and hybridized to the ^{32}P-labeled *Hin*dIII–*Cla*I fragment from the plasmid pLNhsp70hygL.

Detection of β-Galactosidase Activity. Cell lysates were prepared from infected and control cells and incubated with the chemiluminescent substrate for β-galactosidase according to the manufacturer's instructions (Galacto Light, Tropix, Bedford, MA). Before assay, lysates were incubated at 50°C for 50 min to inactivate endogenous enzyme activity, which resulted in a 5% and 50% decrease in background activity for the MOS-55 and 208F cells, respectively. Enzyme activity was measured in a luminometer (Monolight 2001, Analytical Luminescence Laboratory, San Diego) calibrated to record light emission integrated over 10 sec. Protein content was determined with the Bio-Rad reagent. All assays were performed in triplicate, and the results of the β-galactosidase assay were expressed as light units per μg of protein.

RESULTS AND DISCUSSION

To determine whether VSV-G pseudotyped vectors infect mosquito cells in culture, MOS-55 cells (≈1×10^6 cells) were incubated with standard medium (10 ml per 10-cm plate), to which the polycation, polybrene (5 μg/ml), and 1 ml of 3×10^5 colony-forming units per ml of LSRNL(VSV-G) pseudotyped virus (Fig. 1) or 1 ml of media alone (mock-infected control) was added. After 48 h, DNA was extracted from the cells and analyzed by PCR with primers specific for the hepatitis B surface antigen cDNA in the provirus (Fig. 1). Control am-



Key to nomenclature:

L	LTR	MoMLV long terminal repeat
S	HBsAg	Hepatitis B surface antigen
R	RSV	Rous sarcoma virus long terminal repeat
N	neo	neomycin phosphotransferase
hsp70	hsp70	*Drosophila* heat shock 70 promoter
Z	Lac Z	*beta*-galactosidase
hyg	hyg	hygromycin phosphotransferase

FIG. 1. Genetic organization of retroviral vectors. Arrows indicate direction of transcription.

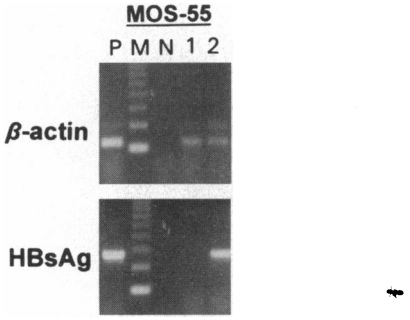


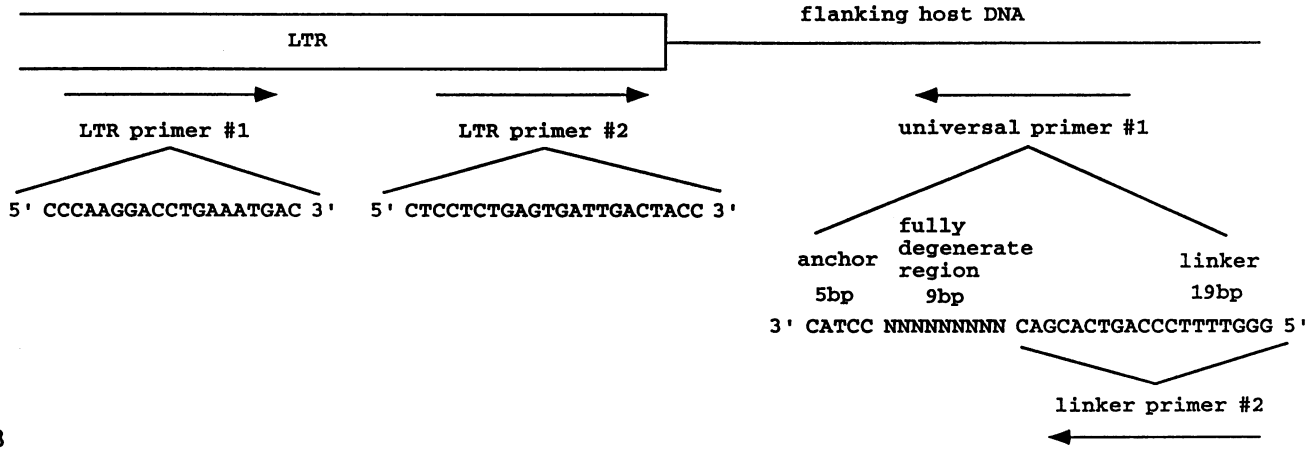
FIG. 2. Infection of MOS-55 cell line with pantropic retroviral vector. Lanes 1 and 2, amplification of 1 μg DNA from MOS-55 cells infected with (*i*) mock and (*ii*) LSRNL pantropic vector; lane P, positive control amplification of 1 μg of DNA from 293/LSRNL producer cells with one integrated copy of provirus per genome; lane M, molecular size marker (123-bp ladder); lane N, no DNA template negative control.

plification with crosshybridizing human β-actin primers (Fig. 2) and rRNA primers specific for *A. gambiae* (ref. 23 and data not shown) confirmed the presence of amplifiable mosquito DNA in all samples. Proviral DNA was detected only in extracts from pseudotyped vector-infected cells (Fig. 2). From these data, we infer that attachment, uncoating, and reverse transcription of the pseudotyped retroviral vector can be supported in mosquito cells in culture.

To determine if the provirus was integrated into the host cell genome, infected and control MOS-55 DNA prepared from pooled, unselected cells was subjected to hemi-nested PCR to amplify host–vector insertion junctions (17). Fig. 3 shows the primer sequences and the nucleotide sequence of two fragments amplified from infected mosquito cell DNA. The sequences contain the expected 2-bp deletion from the 3′-end of the LTR (characteristic of retroviral integration into mammalian genomes) followed by novel sequence presumed to originate from the host mosquito chromosome. To confirm the mosquito origin of this flanking DNA, the sequences were biotinylated and hybridized *in situ* to polytene chromosomes derived from the *A. gambiae* PEST strain. Fig. 4 *A* and *B* show the hybridization of junctions 1 and 2 to chromosome 2R, subdivision 7B, and interband 14E/15A, respectively. The specific hybridization signal was evident in repeat experiments on multiple nuclei for each probe. To further confirm the mosquito origin of the flanking DNA sequences, an *Eco*RI fragment of the plasmid containing clone 1 was gel-purified, labeled, and hybridized to *Eco*RI-digested genomic DNA prepared from uninfected MOS-55 cells. A single band of ≈3.5 kb on the genomic Southern analysis confirmed the mosquito origin of this flanking sequence (Fig. 4*C*).

Retroviral integration, which occurs at the time of cellular division and is mediated by the viral integrase brought into the cell with the infectious particle, can occur as single or multiple integration events per cell. To determine the number of independent integration events per infected cell, MOS-55 cells were infected with the pseudotyped vector LNhsp70hygL and selected cells in the antibiotic hygromycin B (20 μg/ml), and individual clones were subcultured. DNA extracted from four of these clones was digested with either *Kpn*I to yield unit-length provirus (4.4 kb) or with *Hin*dIII, which has a single recognition sequence between the hsp70 promoter and the *hyg* coding sequence (Fig. 1). Digested DNA was resolved on an 0.8% agarose gel, transferred to nylon, and hybridized to the *Hin*dIII–*Cla*I fragment of pLNhsp70hygL. Fig. 5 shows unit-length provirus of the predicted size (≈4.4 kb) from all four

A



B

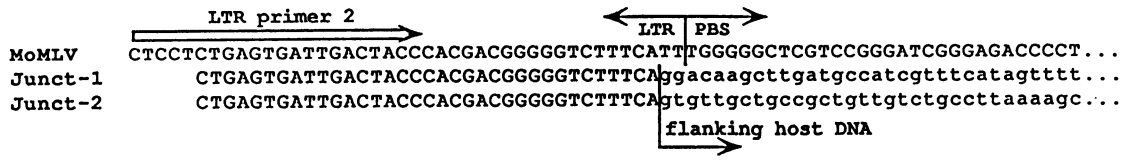


FIG. 3. Hemi-nested PCR amplification and sequence of retroviral integration junctions in *A. gambiae* cells. (*A*) Amplification strategy was as follows: round 1 (30 cycles), LTR primer 1 and universal primer; and round 2, 1 μl of PCR product from round 1 amplified with LTR primer 2 and linker primer 2. (*B*) Nucleotide sequence of cloned PCR products. MoMLV, internal Moloney murine leukemia virus sequences derived from *A. gambiae* by universal PCR beginning in the 5′-LTR and extending into the tRNA primer binding site (PBS); and Junct-1 and 2, apparent insertion junctions in the mosquito genome consisting of the 3′-end of the 3′-LTR with the terminal two nucleotides removed followed by flanking host DNA (lowercase letters). The cloned fragments contained ≈400 bp of flanking sequence, but only 57 bp are shown. The complete *A. gambiae* flanking sequences have been deposited with GenBank (junction 1, accession no. U31354; and junction 2, accession no. U31415).

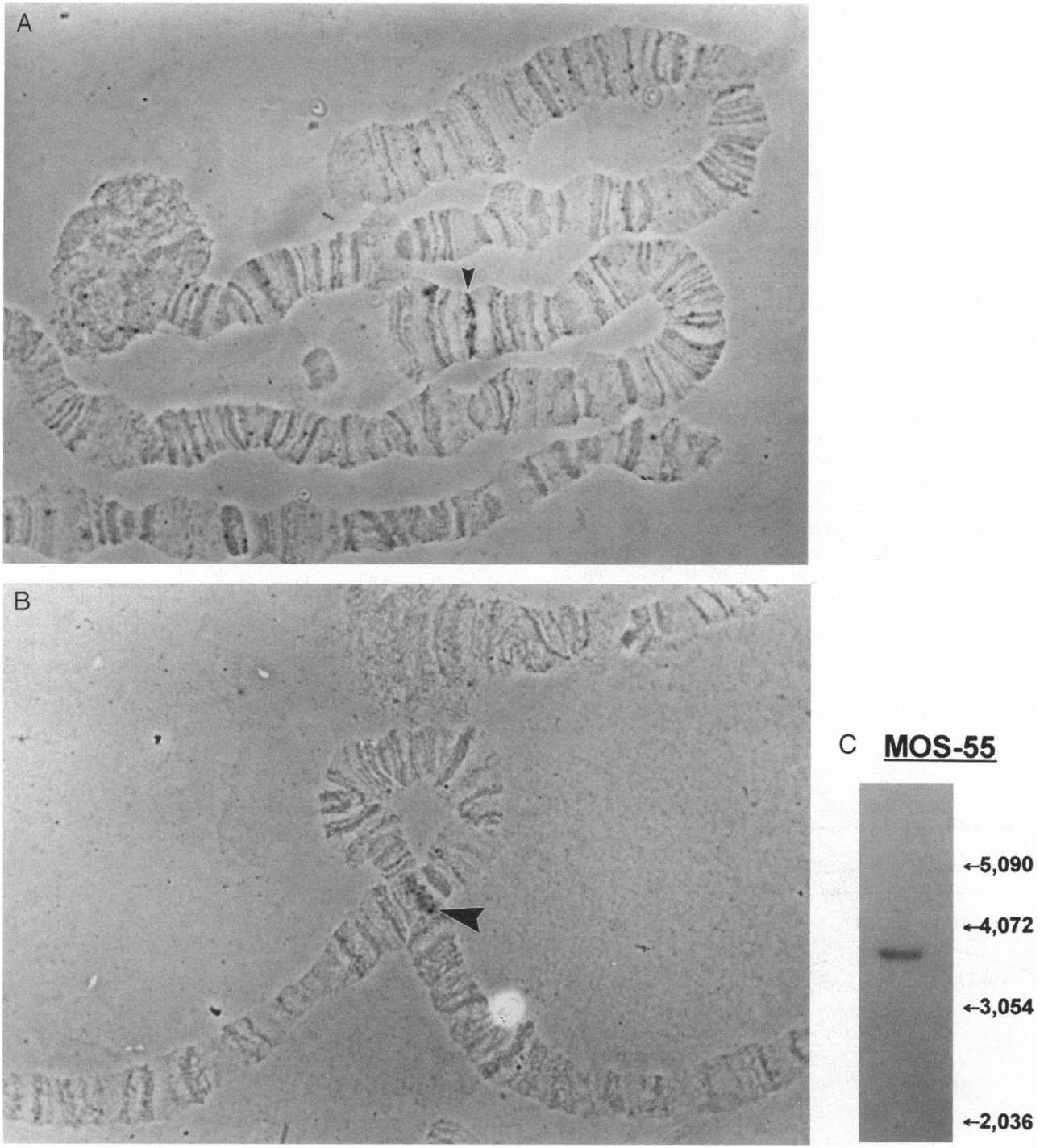


FIG. 4. (*A* and *B*) Retroviral integration demonstrated by *in situ* hybridization of cloned junctional DNA to *A. gambiae* ovarian polytene chromosomes, magnified ×1000 with phase contrast optics. Arrow shows hybridization of junction 1 (*A*) to chromosome 2R, subdivision 7B, and junction 2 (*B*) to chromosome 2R at the interband of subdivisions 14E and 15A. (*C*) Southern hybridization of the *Eco*RI fragment of junction 1 to 20 μg of *Eco*RI-digested uninfected MOS-55 DNA shows a single band at ≈3.5 kb.

clones. A single, unique proviral integration site was demonstrated with the *Hin*dIII-digested DNA containing the provirus and mosquito flanking sequence (Fig. 5). Thus, retroviral vectors can integrate at variable sites in the mosquito genome and confer a stable, selectable phenotype (hygromycin resistance) to infected cells.

To further characterize proviral gene expression, the retroviral construct, LNhsp70ZL, was constructed in which *neo* and β-galactosidase are expressed from the MoMLV LTR and the hsp70 promoter, respectively (Fig. 1). Cell lysates were tested in a chemiluminescent assay for β-galactosidase following heat inactivation of endogenous enzyme (Table 1). A 5-fold increase in enzyme activity was seen in infected, unselected MOS-55 cells as compared to mock-infected controls. Control infection of the rat fibroblast cell line, 208F, yielded a 9-fold increase in enzyme activity. Heat shock (41°C for 1 h, recovery at room temperature for 2 h) before preparation of cell lysates had no effect on enzyme activity in either the mammalian or mosquito cell lines (data not shown). We conclude that heterologous cDNA can be expressed in a mosquito cell line following infection with retroviral vectors containing insect-specific promoters.

Table 1. Expression of β-galactosidase activity in *A. gambiae* and mammalian cell lines infected with the pantropic retroviral vector LNhsp70ZL

Cell line	Treatment	Light units per μg of protein*
MOS-55	Mock-infected	510.1 ± 18.6
MOS-55	Infected	2824.8 ± 86.0
208F	Mock-infected	186.4 ± 10.3
208F	Infected	1723.8 ± 122.3

*Values expressed as means ± SD for triplicate determinations.

Pantropic retroviral vectors have been used to infect a wide variety of non-mammalian cells including fish (24), newt (20), and *Xenopus laevis* (19). The data presented here show for the first time that these vertebrate viruses can infect cultured insect cells, specifically those of *A. gambiae*, the major vector of human malaria. These results demonstrate the wide adaptability of pseudotyped retroviruses as general vectors for mediating transformation. In addition, it should be possible to adapt them for transformation of other insects that are targets of genetic control.

Pantropic retroviral vectors can be concentrated by ultracentrifugation to titers > 10^9 colony-forming units per ml with recovery of more than 70% of the initial virus (11). Concentrated, high titer viral vectors can be microinjected into fertilized mosquito eggs to create transgenic lines, and work is in progress to determine if they integrate and express *in vivo*. This same approach resulted in pseudotyped vector infection of zebrafish embryos with germ-line transmission and Mendelian segregation of the provirus in the F2 generation (24). Because no reliable system for germ-line transformation in mosquitoes has been developed, use of pantropic retroviral vectors for *in vivo* gene transfer may facilitate laboratory-based studies of transgenic mosquitoes.

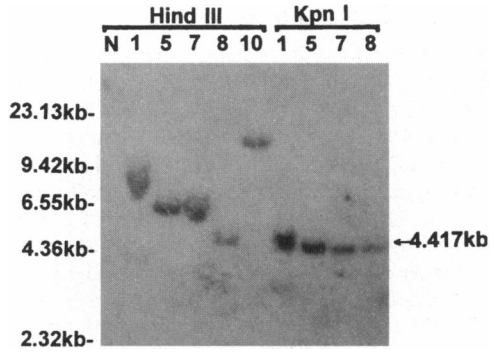


FIG. 5. Southern hybridization of LNhsp70hygL-infected MOS-55 clones. *Hin*dIII digestion of DNA extracted from uninfected MOS-55 cells (N) and LNhsp70hygL-infected clones 1, 5, 7, 8, and 10 shows single, unique bands indicating integration of single provirus at variable sites in the mosquito genome. *Kpn*I digestion of DNA extracted from clones 1, 5, 7, and 8 shows unit-length provirus (4.417 kb) from each clone.

The authors wish to thank Darren Stauth for technical assistance. This work was supported by grants from the John D. and Catherine T. MacArthur Foundation (awarded to A.A.J.), the University of California, San Diego Gene Therapy Program (Grant GTP-JB01, awarded to J.C.B.), the University-Wide Mosquito Research Program (Grant 95-004-1-2, awarded to J.C.B.), the U.S. Department of Agriculture (92-37302-7605, awarded to R.W.B.), and the National Institutes of Health (Grant R01AI37671, awarded to J.C.B., A.A.J., and S.H.).

1. Fallon, A. M. (1991) *Science* **352,** 828–829.
2. Higgs, S., Powers, A. M. & Olson, K. E. (1993) *Parasitol. Today* **9,** 444–452.
3. Monroe, T. J., Muhlmann-Diaz, M. C., Kovach, M. J., Carlson, J. O., Bedford, J. S. & Beaty, B. J. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 5725–5729.
4. McGrane, V., Carlson, J. O., Miller, B. R. & Beaty, B. J. (1988) *Am. J. Trop. Med. Hyg.* **39,** 502–510.
5. Mialhe, E. & Miller, L. H. (1994) *BioTechniques* **16,** 924–931.
6. Miller, L. H., Sakai, R. K., Romans, P., Gwadz, R. W., Kantoff, P. & Coon, H. G. (1987) *Science* **237,** 779–781.
7. Friedmann, T. (1992) *Nat. Genet.* **2,** 93–98.
8. Weiss, R. A., Boettiger, D. & Murphy, H. M. (1977) *Virology* **76,** 808–825.
9. Zavada, J. (1972) *J. Gen. Virol.* **15,** 183–191.
10. Emi, N., Friedmann, T. & Yee, J.-K. (1991) *J. Virol.* **65,** 1202–1207.
11. Burns, J. C., Friedmann, T., Driever, W., Burrascano, M. & Yee, J.-K. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 8033–8037.
12. Schloemer, R. H. & Wagner, R. R. (1975) *J. Virol.* **15,** 1029–1032.
13. Wyers, F., Blondel, D., Petitjean, A.-M. & Dezelee, S. (1989) *J. Gen. Virol.* **70,** 213–219.
14. Marhoul, Z. & Pudney, M. (1972) *Trans. R. Soc. Trop. Med. Hyg.* **66,** 183–184.
15. Miyanohara, A., Elam, R. L., Whitzum, J. L. & Friedmann, T. (1992) *New Biol.* **4,** 261–267.
16. Adam, M. A., Ramesh, N., Miller, A. D. & Osborne, W. R. A. (1991) *J. Virol.* **65,** 4985–4989.
17. Xiao, H. & Lis, J. T. (1988) *Science* **239,** 1139–1142.
18. Yee, J.-K., Friedmann, T. & Burns, J. C. (1994) *Methods Cell Biol.* **43,** 99–112.
19. Burns, J. C., Shimizu, C., Matsubara, T., Yee, J.-K., Kurdi-Haidar, B., Friedmann, T., Maliwat, E., McNeill, L. & Holt, C. (1996) *In Vitro* **32,** 78–84.
20. Burns, J. C., Matsubara, T., Lozinski, G., Yee, J.-K., Friedmann, T., Washabaugh, C. H. & Tsonis, P. A. (1994) *Dev. Biol.* **165,** 285–289.
21. Sarkar, G., Turner, R. T. & Bolander, M. E. (1993) *PCR Methods Applications* **2,** 318–322.
22. Kumar, V. & Collins, F. H. (1994) *Insect Mol. Biol.* **3,** 41–47.
23. Scott, J. A., Brogdon, W. G. & Collins, F. H. (1993) *Am. J. Trop. Med. Hyg.* **49,** 520–529.
24. Lin, S., Gaiano, N., Culp, P., Burns, J. C., Friedmann, T., Yee, J.-K. & Hopkins, N. (1994) *Science* **265,** 666–668.