



Pergamon

Insect Biochemistry and Molecular Biology 28 (1998) 819–825

*Insect
Biochemistry
and
Molecular
Biology*

Rapid Communication

Infection of lepidoptera with a pseudotyped retroviral vector

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Received 5 June 1997; received in revised form 29 May 1998; accepted 29 May 1998

Abstract

Studies requiring the introduction and expression of manipulated gene constructs have been technically difficult in non-drosophilid insects. Retroviruses can be engineered to be replication defective and to serve as vectors for gene constructs of interest. In this study, pseudotyped MoMLV(VSV-G) retroviral vectors are shown to successfully infect lepidopteran cells in vitro and in vivo. In *Spodoptera frugiperda* cells in vitro and in *Manduca sexta* in vivo, infection and conversion to proviral DNA were confirmed by PCR amplification and Southern blot hybridization of vector-specific sequences. Gene expression and integration of proviral DNA were also documented in vitro. This is the first report of retroviral infection in lepidoptera and suggests that pseudotyped retroviral vectors could be powerful tools in gene manipulation studies of non-drosophilid insects. © 1998 Elsevier Science Ltd. All rights reserved.

Keywords: *Manduca sexta*; Pseudotyped retrovirus; Insect; Vector; Antenna

1. Introduction

The ability to experimentally manipulate the expression of a gene in vivo remains the definitive assay in studies of temporal and spatial regulation of gene expression and the functions of expressed proteins in living organisms. P-element based transformation methods in *Drosophila melanogaster* have been instrumental in studying such processes; however, similar technologies are not widely available for other insects. Studies in non-drosophilid systems have necessitated the use of in vitro cell culture systems, introducing gene constructs by transfection (Morris et al., 1995; Lan and Riddiford, 1997) or by baculoviral infection (Hammock et al., 1990). The baculoviral system has also been successful in introducing gene constructs in vivo into larval and pupal tissues of the silkworm *Bombyx mori* (e.g. Iatrou and Meidinger, 1990), and into larval tissue of the sphinx moth *Manduca sexta* (Gretch et al., 1991). However, with respect to long term studies, the baculovirus life cycle results in host cell death and the baculoviral gen-

ome does not integrate into host DNA (Iatrou and Meidinger, 1990; Iatrou, 1995). Transposable elements that are not specific to *Drosophila* show promise as gene transfer vectors in diverse insect species, including lepidoptera and mosquito (e.g. Erlick et al., 1997; Coates et al., 1998).

Alternatively, retroviral vectors derived from the Moloney murine leukemia virus (MoMLV) have proven to be efficient vectors for gene transfer in mammalian systems (Leber et al., 1996). Pseudotyped MoMLV retroviral vectors in which the envelope protein of the MoMLV has been replaced by the envelope glycoprotein of vesicular stomatitis virus (VSV-G) have been shown to infect cells from a broad range of species, including fish, newt, clam and mosquito (Lin et al., 1994; Burns et al., 1993, 1994; Lu et al., 1996; Matsubara et al., 1996). These retroviral vectors can stably integrate into the host cell genome and are not toxic to the host cell.

In the current study, we show that pseudotyped retroviral vectors successfully infect the lepidopteran cell line *Spodoptera frugiperda* (Sf-9) and antennal cells of *M. sexta* when injected into early developing male pupae. This suggests that pseudotyped retroviral vectors should be useful in introducing gene constructs into cells in vivo for such purposes as the analysis of promoter function

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or for the marking of specific cells for developmental lineage analysis.

2. Materials and methods

The lepidopteran *Sf-9* cell line (ATCC# CRL 1711, kind gift of Jean Wang, UCSD, La Jolla, CA) was maintained in Grace's Insect medium supplemented with 10% fetal calf serum and antibiotics. The 293-gag-pol, PA317, and 208F cell lines were as previously described (Burns et al., 1993). *M. sexta* were obtained as fertilized eggs from Ms. Linda Liska (United States Department of Agriculture, Beltsville, MD). Animals were reared at 27°C on a 16:8 light/dark cycle. All studies were performed on 2 day old pupae.

The pseudotyped retroviral vectors, LSRNL(VSV-G), LN-IE-1-lucL(VSV-G), and LLRNL(VSV-G), and the amphotropic vector, LSRNL-A, were prepared and titered as previously described (Miyanojara et al., 1992; Xu et al., 1989; Yee et al., 1994; Burns et al., 1994). In these constructs, the Moloney murine leukemia virus long terminal repeat (L) drives expression of the following genes: hepatitis B surface antigen (S), firefly luciferase (L), or neomycin phosphotransferase (N). The Rous sarcoma virus long terminal repeat (R) drives expression of (N) or (L) (Fig. 1(A)). Negative control vector particles were harvested from the 293-gag-pol/LSRNL producer cell line without transfection of the plasmid encoding the envelope protein, VSV-G. Such non-infectious,

'bald' particles contain the viral nucleocapsid surrounded by producer cell membrane that lacks the viral envelope glycoprotein.

The plasmid pLN-IE-1-luciferase-L, in which the gene for firefly luciferase is expressed from the baculovirus immediate early promoter (IE-1) (Jarvis et al., 1990), was constructed by subcloning the IE-1 promoter (including the hr5 enhancer region) from pIE1HE3 (kind gift of Donald A. Jarvis, Texas A&M University) into XhoI-Hind III sites in pLNhsp70lucL (Jordan et al., 1988).

For in vitro infections, 80% confluent monolayers of *Sf-9* cells grown on inserts that fit into a 6-well plate (Costar) were infected by the flow-through method (Palsson and Andreadis, 1997) with virus-containing supernatant plus the polycation, polybrene (2 µg/ml). After exposure to virus-containing supernatant, cells were removed from the insert and placed in 6-well plates. After 48 h, DNA was extracted from virus-exposed and control cells as previously described (Matsubara et al., 1996). Primers specific to the Hepatitis B surface antigen (HBsAg) gene were used in the polymerase chain reaction (PCR) to amplify proviral DNA from 1 µg of cellular DNA as previously described (Burns et al., 1994). Control primers for cellular β-actin that yield a 250 bp PCR product (Ben-Ezra et al., 1991) were used in the same reaction tube to confirm the presence of amplifiable DNA in all samples. Southern transfer and labeling of 15 ng of HBsAg PCR product labeled with ³²P by the random primer method were as described (Burns et al., 1994). For luciferase assays, cells were lysed and analyzed for enzyme activity in a Turner Design 20/20 luminometer as described (Jordan et al., 1988).

To obtain the DNA sequence at the site of proviral integration by inverse PCR, DNA was extracted from approximately 5 × 10⁵ virus-exposed *Sf-9* cells 48 h post-infection and 2 µg were digested with PstI at 37°C overnight, phenol-chloroform extracted, ethanol precipitated, and resuspended in 20 µl of ddH₂O (Fig. 1(B)). Resulting restriction fragments were then circularized by ligation; 375 ng of DNA solution was incubated with T4 ligase (New England Biolabs) in the manufacturer's buffer for 4 h at 16°C. A subset of the circularized DNA was presumed to contain retroviral inserts. The circular ligation product was divided into thirds and each fraction linearized within the presumptive remaining retroviral sequence by restriction enzyme digestion with either KpnI, SmaI, or EcoRV under recommended conditions. Each of these enzymes has a single recognition sequence in the MoMLV LTR and the region 3' to the LTR but before the binding site for the sense inverse PCR primer (Fig. 1(B)). Three different enzymes were tested with the hope that at least one would not cleave the unknown sequence between the inverse primers. Digested DNA was resuspended in 10 µl of ddH₂O and 3 µl used for

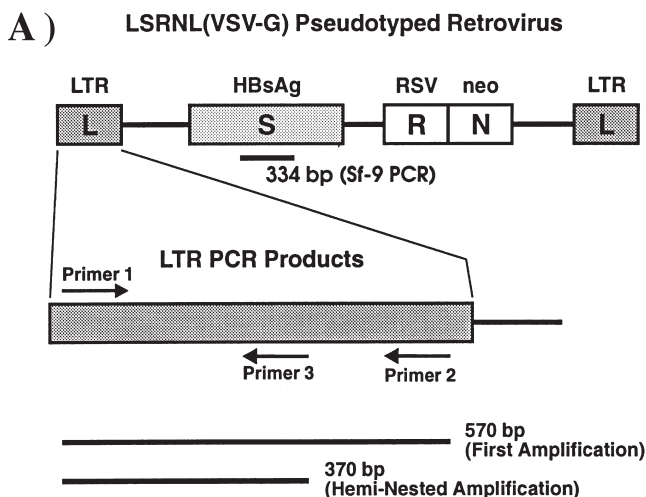


Fig. 1. Genetic organization of LSRNL(VSV-G) retrovirus and PCR scheme for detecting vector sequences and integration. (A) PCR strategy for detecting proviral sequences. See text for details; drawing is not to scale. (B) Inverse PCR strategy for amplification and sequencing of *Sf-9* DNA at the proviral integration site. (C) Partial sequence of inverse PCR product. The 5' end of the MoMLV LTR has a two-base pair deletion (AG substituted for AA) characteristic of integrase-mediated insertion, followed by sequence (arrow) identified as *Sf-9* DNA by dot blot hybridization (data not shown). 'A*A' = base identity, 'A:' = base substitution or gap.

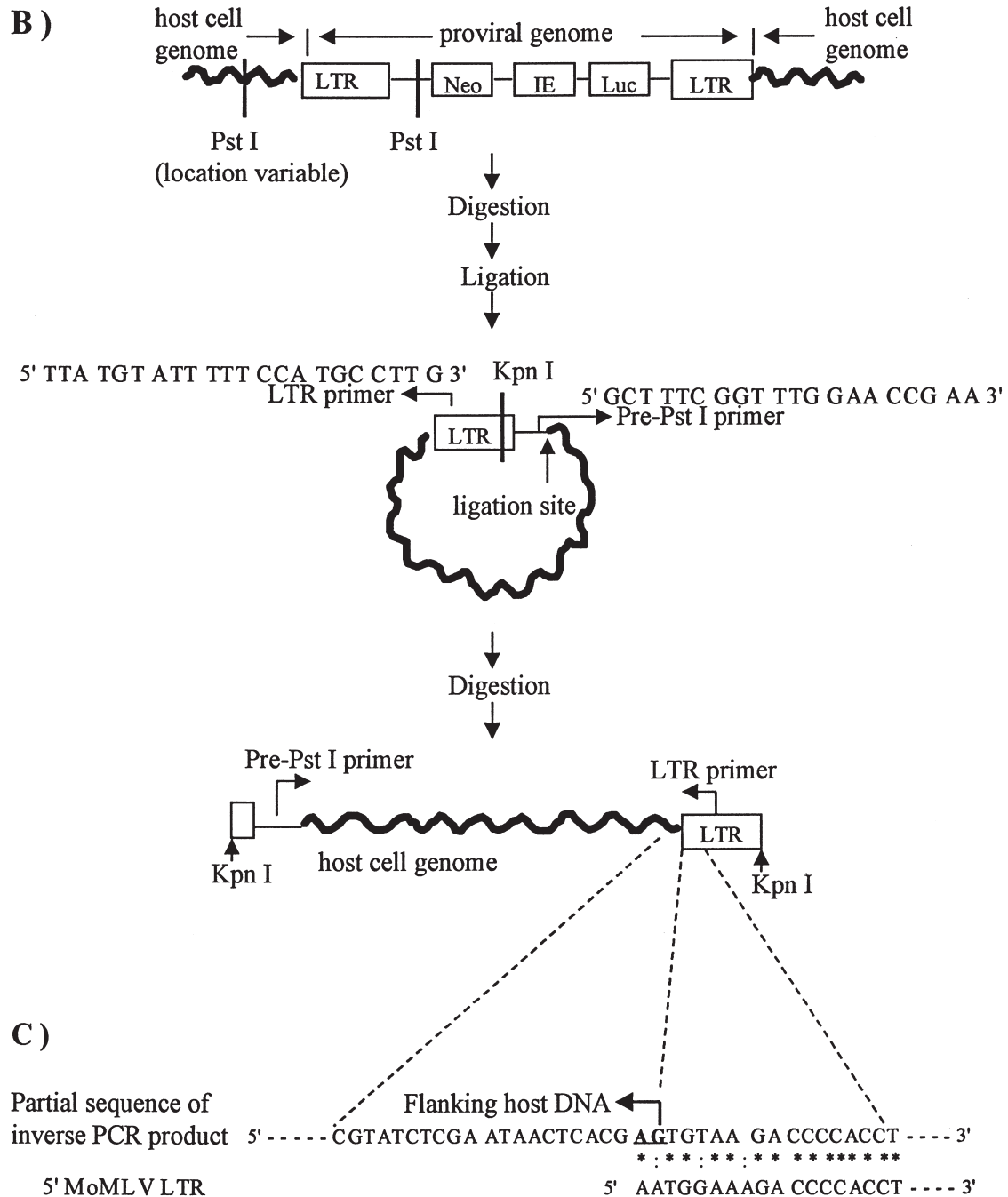


Fig. 1. Continued.

the inverse PCR amplification of DNA situated between the presumably digested retroviral sequences. Primers were designed to hybridize to the 5' end of the MoMLV LTR (antisense primer) and the region of the vector 17 base pairs upstream of the PstI site (sense primer) (Fig. 1). PCR reactions were performed using the GeneAmp 9600 thermal cycler (Perkin Elmer) and the GeneAmp XL PCR kit (0.5 μ M each primer, 1.5 mM Mg(OAc)₂; reaction conditions were 40 cycles of 93°C (1 min), 55°C (3 min), 72°C (5 min) followed by 72°C (10 min). The PCR reaction product was diluted 1:5 in ddH₂O and 1

μ l reamplified as described above; products were analyzed on a 0.7% agarose gel. Bands were isolated from the agarose using the Qiaex II gel extraction kit (Qiagen). Approximately 200 ng of PCR product was directly sequenced by cycle sequencing (373 Automated DNA Sequencer, Applied Biosystems, Perkin Elmer; 6% polyacrylamide gel).

To confirm that the inverse PCR sequence was from *Sf-9* cells, the PCR product was labeled with ³²P with the Prime-It II Kit (Stratagene) and hybridized to 1, 2, and 10 μ g of *Sf-9* and control human producer cell line

DNA on a nylon membrane. The membrane was exposed to film with an enhancing screen at $-70^{\circ}\text{C} \times 5$ days.

For in vivo infection, the LSRNL(VSV-G) vector was diluted to 5×10^5 cfu/ml in Grace's medium containing polybrene ($5\text{--}20 \mu\text{g/ml}$). Vector solution was applied through holes in the cuticle; the holes were sealed afterward with melted myristic acid (Sigma). Infected pupae were allowed to develop for 5 days at 27°C ; antennae were removed, and DNA isolated and analyzed for the presence of vector. Control animals were treated with non-infectious ('bald') retroviral vector, or with vector-free medium ('mock').

For in vivo analysis, antennal DNA was isolated from individual animals using the QIAamp Tissue Kit (Qiagen) with overnight tissue lysis with proteinase K at 55°C followed by RNase A treatment (0.66 mg/ml), yielding $2\text{--}6 \mu\text{g}$ of DNA per antenna. Primary PCR amplifications used 300 ng DNA per reaction (sense Primer 1: TGAAAGACCCACCCGT and antisense Primer 2: ACGGGTAGTCAATCACTCAG); secondary reamplifications used $0.01 \mu\text{l}$ of the primary reaction per reaction (Primer 1 and nested antisense primer, Primer 3: ACTGATTGGTTAGTTC AAT). For a positive control of template DNA quality, a 433 bp fragment of *M. sexta* lysozyme gene (Genbank accession number, S71028) was amplified under identical conditions using the primers TCCTGAGAATTTGATGAG (sense) and ACTTTGTCCGTGTATCTG (antisense). All PCR reactions ($10 \mu\text{l}$) were performed in glass capillary tubes using a 1605 Air Thermo-Cycler (Idaho Technology); reaction buffer contained $250 \mu\text{g/ml}$ BSA, 2 mM MgCl_2 , and $5 \mu\text{M}$ each primer; reaction conditions were 94°C (3 min) followed by 30 cycles of 94°C (1 s), 50°C (15 s), 74°C (15 s). Products were examined on 1.5% agarose gels (TBE), stained with EtBr, photographed, and processed for Southern blot hybridization.

For hybridization, an LTR probe was amplified from genomic DNA isolated from 293-gag-pol/LSRNL producer cells using Primers 1 and 2 (Fig. 1(A)); 120 ng PCR product was labeled using the Multiprime DNA labeling system (Amersham) and digoxigenin-dUTP (Boehringer-Mannheim). PCR products were transferred from agarose gels to nylon membranes, hybridized with 2.5 ng/ml probe for 16 h at 42°C in 50% formamide, and washed to 0.5X SSC at 60°C . Hybridized probes were visualized by luminous detection (Boehringer-Mannheim; Lumiphos-530) on X-ray film.

Nuclear ecdysteroid receptor (EcR) expression was visualized using the 9B9 monoclonal antibody (L. M. Riddiford, Department of Zoology, University of Washington). Outer antennal cuticle with attached sensory epidermis (pre-apolysis) was removed and fixed in 4% paraformaldehyde, permeabilized for 30 min (0.3% Triton X-100, 2N HCl in PBS), blocked for 1 h (PBS containing 1% BSA, 1% non-fat dry milk, 1% Triton

X-100, 0.05% sodium azide), and incubated with 9B9 antibody ($1:1000$ in blocking solution; 48 h , 4°C). Following overnight incubation with secondary antibody (goat anti-mouse IgG-alkaline phosphatase, Sigma, $1:1000$), tissue was stained using nitroblue tetrazolium (NTB) and X-phosphate (Boehringer-Mannheim Biochemica).

Mitotic activity was visualized by incorporation of 5-bromo-2'-deoxyuridine (BrdU). Animals were injected in the head with $1 \mu\text{l}$ of 10 mM BrdU stock solution (Boehringer-Mannheim) per gram of body weight and grown an additional 18 h . Dissection and processing were as described above for the 9B9 antibody; primary and secondary (AP) antibodies were supplied with the kit.

3. Results and discussion

To initially test the infection of lepidopteran cells in vitro with the pseudotyped vector, LSRNL(VSV-G), we incubated 60% confluent monolayers of *Sf-9* cells with $5 \mu\text{g/ml}$ of the polycation polybrene, and 3×10^5 cfu/ml of either amphotropic LSRNL virus (LSRNL-A) or pseudotyped virus LSRNL(VSV-G), or media alone. After 48 h , DNA was extracted from the cells and analyzed by PCR with primers specific for the HBsAg gene (Fig. 2) (Burns et al., 1994). Control amplification with cross-hybridizing human β -actin primers confirmed the

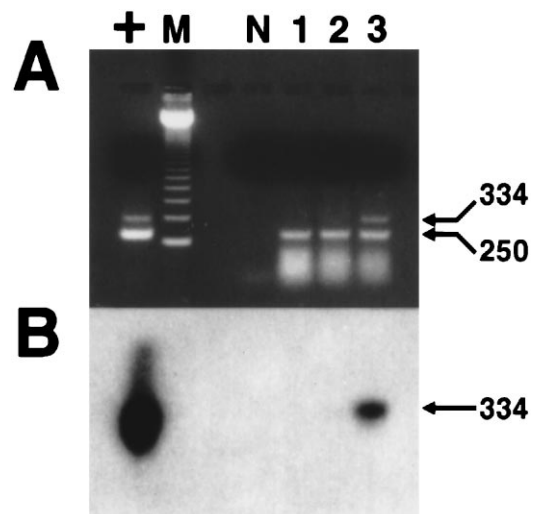


Fig. 2. Infection of lepidopteran *Sf-9* cell line with amphotropic and pseudotyped retroviral vectors. Lane 1–3: Amplification using both β -actin and HBsAg primers of $1 \mu\text{g}$ DNA extracted from *Sf-9* cells infected with (1) LSRNL-A (amphotropic vector, containing the *env* of MoMLV), (2) media alone, and (3) LSRNL(VSV-G) (pseudotyped vector, containing the *env* of VSV). '+', positive control amplification of $1 \mu\text{g}$ DNA from 293-gag-pol/LSRNL producer cells. 'M', molecular size marker (123 bp ladder). 'N', no DNA template negative control. Arrows on the EtBr stained gel indicate the expected 250 bp β -actin and 334 bp HBsAg PCR products (A). Arrow on the Southern blot indicates the expected 334 bp HBsAg PCR product (B).

presence of amplifiable DNA in all samples (Fig. 2(A)). Viral DNA was detected only in extracts from pseudotyped vector-infected cells. As expected, the amphotropic vector, which requires a specific cell surface receptor protein, was not infectious in the lepidopteran cells. A PCR product of 334 bp predicted size could be visualized by Southern blot hybridization of amplified DNA from *Sf-9* cells infected with LSRNL(VSV-G) (Fig. 2(B)).

To confirm that proviral integration into the host cell genome had occurred, inverse PCR followed by sequencing of the flanking DNA region was performed (Fig. 1(B, C)). Amplification of the *Sma*I- and *Kpn*I-digested DNA from infected cells yielded a product of approximately 1.5 kb. This product was re-amplified, and approximately 330 base pairs were sequenced. The sequence included approximately 50 bases from the 5' end of the MoMLV LTR with a two-base pair deletion, followed by 280 bases with no match in the Genbank database (Fig. 1(C)). The two-base pair deletion at the junction of the proviral and genomic sequences is characteristic of retroviral integrase-mediated integration. To confirm that the flanking sequence was of lepidopteran origin, the fragment was labeled with 32 P and hybridized under stringent conditions to dot blots of *Sf-9* and control human DNA. Hybridization was seen only with the lepidopteran DNA, thus confirming that the flanking sequence was from the infected *Sf-9* cells (data not shown).

To test the function of different promoters for gene expression, *Sf-9* cells were infected with 3×10^5 cfu/well of either LLRNL(VSV-G) (luciferase expressed from the MoMLV LTR) or LN-IE-1-lucL(VSV-G) (luciferase expressed from the baculovirus IE-1 promoter). After 48 h, the cells were lysed and luciferase activity determined. The MoMLV promoter yielded higher levels of gene expression (mean 5.5 ± 0.3 light units) than the baculovirus promoter (mean 0.2 ± 0.2 light units) (negative control cells = 0.008 light units). Taken together, these data suggest that attachment, uncoating, reverse transcription, and integration of the pseudotyped retroviral vector occurs in lepidopteran cells and that gene expression can be mediated by both internal promoters and the MoMLV LTR.

Our general interest in regulated gene expression in insect olfaction (Vogt, 1995) prompted us to test the infectability of LSRNL(VSV-G) on epithelial cells of developing adult antenna of male *Manduca sexta*, 2–3 days after pupation. Under laboratory conditions, *M. sexta* adult development lasts about 18 days. Each adult antenna derives from an imaginal disc that elongates at pupation into a tubular structure approximately 25 mm long by 2 mm diameter (Fig. 3(A)). The antennal epidermis is initially a monolayer of cells attached to the pupal cuticle for about 3 days, and then detaches by apolysis. At the onset of development, the antennal epithelium

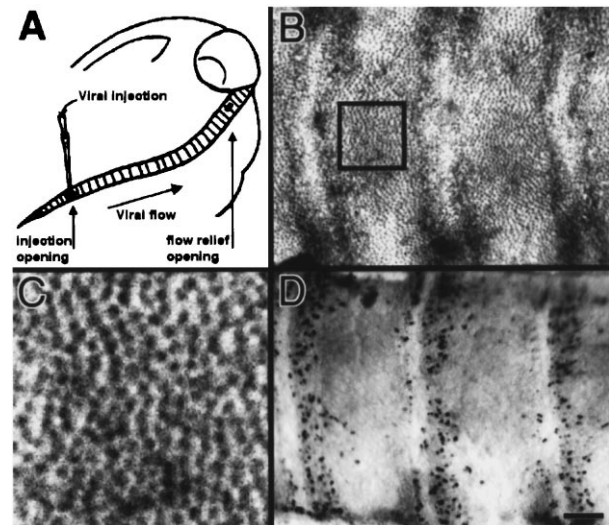


Fig. 3. Schematic of *M. sexta* pupal head illustrating the application of retroviral vector and control solutions (A) and immunohistological characterization of antennal tissue at time of infection (B–D). (A) Solutions were applied through holes in cuticle of pupal antenna. (B) Cell density of sensory epithelium of two annuli (stage 2), visualized by nuclear staining using ecdysteroid receptor (EcR) antibody 9B9; boxed region is the area ($35 \mu\text{m}^2$) used to count nuclei for cell number estimation. (C) Enlargement of boxed area in (B) showing 215 EcR-immunoreactive nuclei. (D) Mitotic activity in sensory epithelium of two annuli (stage 2), visualized by incorporation of BrdU. Tissue shown in panels B–D was taken from the same individual. Scale bar (lower right) represents $120 \mu\text{m}$ (B, D) or $30 \mu\text{m}$ (C).

contains an estimated 215 cells/ $35 \mu\text{m}^2$ (based on counting immunostained nuclei, Fig. 3(B, C)), or approximately 9.6×10^6 cells per antenna based on the surface area of a cylinder with the above dimensions. Extensive mitotic cell divisions occur in the antennal epithelium during the initial 3 days, giving rise to the neurons and support cells of the olfactory sensilla (Franco and Vogt, unpublished); these mitoses can be visualized by incorporation of BrdU (Fig. 3(D)).

To infect antennal tissue, openings were made through the pupal cuticle at the proximal and distal ends of one of the pair of antennae; $20 \mu\text{l}$ of either infectious virus (10^4 cfu) or control solutions were introduced into the lumen of the developing antenna through the distal opening (Fig. 3(A)). Animals were allowed to develop an additional 5 days at which time antennae were removed for analysis. Based on our above estimates of cell number, the application of 10^4 cfu suggests a ratio of about 1:95 infectious particles per host cell (multiplicity of infection = 0.01). The true value was likely lower due to ongoing mitotic activity increasing the number of cells and the likelihood that some of the viral material is diluted into the circulating hemolymph, thus reducing the number of viral particles in the antenna. At the time of dissection, some tissue deterioration was evident at the site of viral application, but the remainder of the

antenna appeared structurally and developmentally normal.

PCR amplification of individual antennal DNA extractions from 34 animals demonstrated the presence of viral DNA; a representative experiment with 10 animals is shown in Fig. 4. DNA from each individual showed a positive control amplification of *M. sexta* lysozyme (433 bp) DNA, confirming the quality of the DNA isolation (Fig. 4(A)). Primary PCR products using LTR primers 1 and 2 were undetectable by EtBr staining (Fig. 4(A)); however, products were detected in 7 of 10 individuals following Southern blot hybridization with digoxigenin-labeled LTR probe (570 bp product, Fig. 4(B)). Reamplification of the primary products using an internal LTR antisense primer (Primer 3 and Primer 1) generated EtBr-detectable products in 7 of 10 samples (370 bp, Fig. 4(C)), further confirming the identity of the PCR products as amplified vector DNA. Negative controls showed no hybridization. Reamplification of DNA from 'bald' and 'mock' individuals also produced products,

but these were not of the appropriate size. Hybridization using the digoxigenin-labeled LTR probe confirmed the identity of the seven positive products; no hybridization was observed to amplification products derived from the negative control tissues (Fig. 4(D)).

As in the *in vitro* experiments, the amplification of LTR sequence from antennal-derived DNA using a *Taq* DNA polymerase indicates that the pseudotyped retroviral vector had successfully infected antennal cells *in vivo*. Viral RNA had entered the cell cytoplasm and had been reverse transcribed into double stranded viral DNA. This is supported by our negative controls; no LTR template was present in animals treated with non-infectious viral supernatant or Grace's medium alone. Non-infectious particles contain viral RNA and associated enzymes, but lack an envelope gene and are thus unable to infect host cells.

These experiments show that the pseudotyped LSRNL(VSV-G) retrovirus can infect lepidopteran cells both *in vitro* and *in vivo*, and that the reverse transcribed

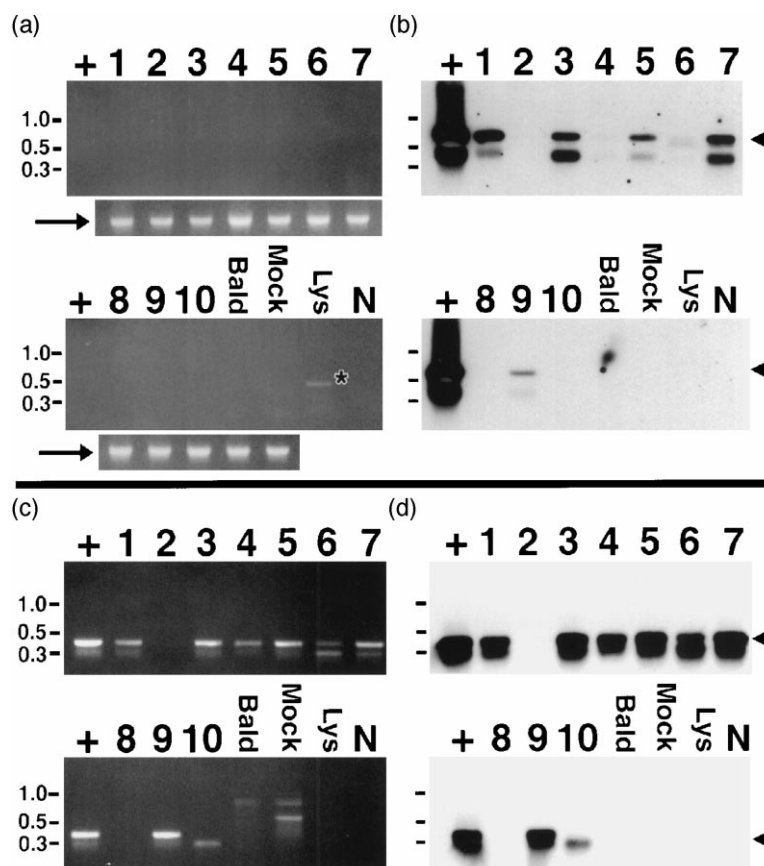


Fig. 4. PCR amplification and Southern blot analysis of *M. sexta* antennal DNA. (A) Primary PCR amplification of antennal DNA (LTR Primers 1 and 2). (B) Southern blot hybridization of primary PCR products. (C) Secondary PCR amplification of primary PCR products (LTR Primers 1 and 3). (D) Southern blot hybridization of secondary PCR products. Arrow heads indicate the expected 570 bp primary LTR PCR product (B) and 370 bp secondary product in (D). Lysozyme PCR product (433 bp) amplified from samples 1–10 was run on a separate gel (A, Lys). Doublets may represent double-stranded and single-stranded PCR product. Lane labels are as follows: '+', genomic DNA from 293-gag-pol/LSRNL producer cells; '1–10', DNA derived from 10 individual virus-treated antennae. The corresponding number in each panel identifies DNA products derived from the same antenna. Negative controls: 'Bald', non-infectious retrovirus-treated antenna; 'Mock', antenna treated with Grace's medium alone; 'Lys', lysozyme gene PCR product; 'N', no template control. Size markers are in kilobase units.

viral DNA can integrate into the genome of a lepidopteran host cell. Previously, similar pseudotyped retroviral vectors were shown to stably integrate into the germ line of other invertebrates, including mosquito (Matsubara et al., 1996) and the mollusc, *Mulinia lateralis* (Lu et al., 1996), thus demonstrating the phylogenetic diversity of target cells for these vectors. The ability of pseudotyped retroviruses to infect insect cells and integrate into the insect genome suggest that such retroviruses should prove powerful tools in studies of non-drosophilid insects where it is desirable to analyze cell-type specific promoters, to ectopically express or over-express specific genes, to analyze fates of cells emerging from mitotic events, or to establish stable transgenic lines.

Acknowledgements

We thank Barbara Sullivan for production of retroviral vectors and Atsushi Miyahara for the 293-gag-pol/LSRNL producer cell line. This project was supported by grants from the National Institutes of Health (NICDC RO1-DC00588, RGV; NIAID R01-AI37671, JCB) and the United States Department of Agriculture (CRGP 94-37302-0615, RGV).

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