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Efficient Generation of Infectious Recombinant Baculoviruses by Site-Specific Transposon-Mediated Insertion of Foreign Genes into a Baculovirus Genome Propagated in *Escherichia coli*

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The construction and purification of recombinant baculovirus vectors for the expression of foreign genes in insect cells by standard transfection and plaque assay methods can take as long as 4 to 6 weeks. This period can be reduced to several days by using a novel baculovirus shuttle vector (bacmid) that can replicate in *Escherichia coli* as a plasmid and can infect susceptible lepidopteran insect cells. The bacmid is a recombinant virus that contains a mini-F replicon, a kanamycin resistance marker, and *att*Tn*7*, the target site for the bacterial transposon Tn*7*. Expression cassettes comprising a baculovirus promoter driving expression of a foreign gene that is flanked by the left and right ends of Tn*7* can transpose to the target bacmid in *E. coli* when Tn*7* transposition functions are provided in *trans* by a helper plasmid. The foreign gene is expressed when the resulting composite bacmid is introduced into insect cells.

A wide variety of genes from viruses, fungi, plants, and animals have been expressed in insect cells infected with recombinant baculoviruses (26, 33, 35, 37, 39, 41, 43). Expression of the foreign gene is usually driven by the polyhedrin promoter of the *Autographa californica* nuclear polyhedrosis virus (AcNPV), which is highly transcribed during the late stages of infection. The recombinant proteins are often expressed at high levels in cultured insect cells or infected larvae and are functionally similar to their authentic counterparts (26, 33, 35, 37, 39, 41, 43).

AcNPV has a large (130 kb) circular double-stranded DNA genome with multiple recognition sites for many restriction endonucleases, and as a result, recombinant baculoviruses are traditionally constructed in a two-stage process. First, a foreign gene is cloned into a plasmid transfer vector downstream from a baculovirus promoter and flanked by baculovirus DNA derived from a nonessential locus, usually the polyhedrin gene. Second, this plasmid DNA is introduced into insect cells along with wild-type genomic viral DNA. Typically, 0.1 to 1% of the resulting progeny are recombinant, with the foreign gene inserted into the genome of the parent virus by homologous recombination in vivo. The recombinant virus is purified to homogeneity by sequential plaque assays, and recombinant viruses containing the foreign gene inserted into the polyhedrin locus can be identified by an altered plaque morphology, which is characterized by the absence of occluded virus in the nucleus of infected cells.

The construction of recombinant baculoviruses by standard transfection and plaque assay methods can take as long as 4 to 6 weeks, and many attempts to accelerate the identification and purification steps have been reported recently. Some of these methods include plaque lifts (50), serial limiting dilutions of virus (15), and cell affinity techniques (14). Each of these methods requires confirmation of the recombination event by visual screening of plaque morphology (42), DNA dot-blot hybridization (34), immunoblotting (7), or amplification of specific segments of the baculovirus genome by polymerase chain reaction (PCR) techniques (38, 55).

The identification of recombinant viruses can also be facilitated by using improved transfer vectors or through the use of improved parent viruses (reviewed in O'Reilly et al. [43]). Coexpression vectors are transfer vectors that contain another gene, such as the *lacZ* gene, under the control of a second viral or insect promoter (53, 57). In this case, recombinant viruses form blue plaques when the agarose overlay in a plaque assay contains X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) or Bluo-Gal (5-bromo-indolyl-β-D-galactopyranoside), which are chromogenic substrates for β-galactosidase. Although blue plaques can be identified after 3 to 4 days (compared with 5 to 6 days for optimal visualization of occlusion-minus plaques), multiple plaque assays are still required to purify the virus. It is also possible to screen for colorless plaques in a background of blue plaques if the parent virus contains the β-galactosidase gene at the same locus as the foreign gene in the transfer vector.

The fraction of recombinant progeny virus that results from homologous recombination between a transfer vector and a parent virus can be improved from 0.1 to 1.0% to nearly 30% by using parent virus that is linearized at one or more unique sites located near the target site for insertion of the foreign gene into the baculovirus genome (19, 29, 47). Linear viral DNA by itself is 15- to 150-fold less infectious than the circular viral DNA. A higher proportion of recombinant viruses (80% or higher) can be achieved by using linearized viral DNA that is missing an essential portion of the baculovirus genome downstream from the polyhedrin gene (27, 28).

Peakman et al. (45) described the use of the Cre-*lox* system of bacteriophage P1 to perform efficient site-specific recombination in vitro between a transfer vector and a

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TABLE 1. *E. coli* strains

Designation	Relevant genotype	Reference	Source
DH5αF'IQ	F' *proAB*$^+$ *lacI*^{q}ZΔM15 *zzf*::Tn*5* (Kan^r)/φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 endA1 recA1 hsdR17* (r_K^- m_K^+) *deoR thi-1 supE44* λ$^-$ *gyrA96 relA1*	24	GIBCO/BRL
DH10B	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) φ80d*lacZ*ΔM15 Δ*lacX74 endA1 recA1 deoR* Δ(*ara leu*)*7697 araD139 galU galK nupG rpsL*	18	GIBCO/BRL

modified parent virus that contain short oligonucleotides with recognition sequences (*lox*) for the Cre enzyme. Up to 50% of the viral progeny are recombinants. The disadvantages of this method include the occurrence of multiple insertions of the transfer vector into the parent virus and the requirement for multiple plaque assays to purify a recombinant virus.

Recently, Patel et al. (44) described a rapid method for generating recombinant baculoviruses which is based on homologous recombination between a baculovirus genome propagated in the yeast *Saccharomyces cerevisiae* and a baculovirus transfer vector that contains a segment of yeast DNA. The shuttle vector contains an autonomously replicating sequence (ARS) that permits replication in yeast cells, a CEN sequence that contains a mitotic centromere and ensures stable segregation of plasmid DNAs at a low copy number, and two selectable marker genes (*URA3* and *SUP4-0*) downstream from the polyhedrin promoter (P_{polh}) in the order P_{polh}, *SUP4-0*, ARS, *URA3*, and CEN. The transfer vector contains the foreign gene flanked on the 5' end by baculovirus sequences and on the 3' end by the yeast ARS. Recombinant shuttle vectors which lack the *SUP4-0* gene can grow in an appropriate yeast strain in the presence of a toxic arginine analog, canavanine. Direct selection of canavanine-resistant colonies is not efficient, however, because of the low frequency of transformation and a high background rate of reversion to canavanine resistance. Insect cells transfected with DNA isolated from selected yeast colonies produce virus and express the foreign gene under control of the polyhedrin promoter. Since all of the viral DNA isolated from yeast cells contains the foreign gene inserted into the baculovirus genome and there is no background of contaminating parent virus, the time-consuming step of plaque purification is eliminated. With this method, it is possible to obtain stocks of recombinant virus within 10 to 12 days. Two drawbacks, however, are the relatively low transformation efficiency of *S. cerevisiae* (4) and the extra labor required to purify the recombinant shuttle vector DNA over sucrose gradients before its introduction into insect cells (44).

In this report, we describe a novel strategy to efficiently generate recombinant baculoviruses that is based on the ability to rapidly generate the recombinant virus by site-specific transposition in *Escherichia coli*. We demonstrate that a recombinant baculovirus shuttle vector (bacmid) that will replicate in *E. coli* as a large plasmid and remain infectious when introduced into insect cells can be constructed. The baculovirus shuttle vector contains a mini-F replicon, which allows autonomous replication and stable segregation of plasmids at low copy number (20, 22, 25, 30, 31, 48), a selectable kanamycin resistance marker (42, 51, 54), and *att*Tn*7*, the target site for the bacterial transposon Tn*7* (5, 10), all inserted into the polyhedrin locus of AcNPV. Unlike most transposable elements, Tn*7* inserts at a high frequency into the single *att*Tn*7* site located on the *E. coli* chromosome and into DNA segments carrying *att*Tn*7* on a plasmid. Plasmids originally designed to deliver deletion derivatives of Tn*7* containing the *E. coli lacZY* genes to the *att*Tn*7* on the chromosome of soil bacteria (2, 3) were modified to contain a gentamicin resistance marker and a baculovirus promoter driving expression of a foreign gene and flanked by the left and right ends of Tn*7*. The mini-Tn*7* element on the donor plasmid transposes to the target plasmid (bacmid) in *E. coli* when Tn*7* transposition functions are provided by a helper plasmid. The foreign gene is expressed under the control of the baculovirus promoter when the resulting composite bacmid is introduced into insect cells. With this new method, it is possible to obtain pure stocks of recombinant virus within 7 to 10 days.

MATERIALS AND METHODS

Bacterial cells and plasmids. Brief descriptions of all the bacterial strains used in this work are shown in Table 1. *E. coli* DH10B (18) was used as the host for all bacterial plasmid manipulations. *E. coli* DH5αF'IQ (24) was used as the source of F' plasmid DNA. Both strains were obtained from GIBCO/BRL (Gaithersburg, Md.) as frozen competent cells. Brief descriptions of all the plasmids used or constructed for this work are given in Table 2.

Bacterial media. 2×YT broth and LB broth and agar were prepared as described previously (46). S.O.C. medium (46) was purchased from GIBCO/BRL. Supplements were incorporated into liquid and solid media at the following concentrations (micrograms per milliliter): ampicillin, 100; gentamicin, 7; kanamycin, 50; tetracycline, 10; X-Gal or Bluo-Gal, 100; and IPTG (isopropyl-β-D-thiogalactopyranoside), 40. Ampicillin, kanamycin, tetracycline, and IPTG were purchased from Sigma Chemical Co. (St. Louis, Mo.). Gentamicin, X-Gal, and Bluo-Gal were purchased from GIBCO/BRL. X-gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid) was purchased from Clontech (Palo Alto, Calif.). Transformation and storage (TSS) solution, containing polyethylene glycol and dimethyl sulfoxide, was purchased from Epicentre Technologies (Madison, Wis.).

Bacterial transformation. Plasmids were transformed into frozen competent *E. coli* DH10B cells (18) by the procedure recommended by the manufacturer. Colonies were purified by restreaking on fresh selection plates prior to analysis of drug resistance phenotype and isolation of plasmid DNAs. Plasmids were also transformed into competent *E. coli* DH10B cells prepared by suspending early-log-phase cells in TSS solution (8).

DNA preparation and plasmid manipulation. Large amounts of DNA were prepared from 250-ml cultures grown in 2×YT medium supplemented with appropriate antibiotics. Cultures were harvested and lysed by an alkaline lysis method, and the plasmid DNA was purified over QIAGEN (Studio City, Calif.) Tip-500 resin columns as described by the manufacturer. Small amounts of DNA from high-copy-number plasmids were prepared from 2-ml cultures by a modified rapid boiling method (21, 50) or by an alkaline lysis

TABLE 2. Plasmids

Designation	Marker(s)	Size (bp)	Description	Source or reference
F'lacIQ	Kan^r	>90,000	F' *proAB*$^+$ *lacI*^{q}ZΔM15 *zzf*::Tn*5* (Kan^r) isolated from strain DH5αF'IQ	GIBCO/BRL (24)
pBCSKP	Cam^r, *lacZα*	3,400	pBC SK(+) phagemid cloning vector	Stratagene
pBS2SKP	Amp^r, *lacZα*	2,961	pBlueScriptII SK(+) phagemid cloning vector	Stratagene (1)
pRAJ275	Amp^r	4,516	pUC19 *Sal*I-*Eco*RI + 1,863-bp *Sal*I-*Eco*RI fragment encoding β-glucuronidase	Clontech (23)
pSL301	Amp^r, *lacZα*	3,284	pBluescript KS(+) derivative with SL2 super polylinker	Invitrogen (6)
pUC-4K	Amp^r Kan^r	3,914	pUC4-Kan (Tn*903*)	Pharmacia (51, 54)
pMON3327	Amp^r	2,923	pUC8 *Bam*HI + 237-bp *Bam*HI-*Bgl*II fragment containing SV40 poly(A) signal	Paul Hippenmeyer (19a)
pMON7104	Gen^r	5,218	pEMBL19P *Hin*cII + 1,258-bp *Alu*I fragment encoding the gene (*aacC1*) for gentamicin acetyltransferase-3-I	This study
pMON7117	Amp^r	~11,200	pUC8-*att*Tn*7*::Tn*7*L-P_{iuc}A-*lacZ lacY lacA'*-Tn*7*R	3
pMON7124	Tet^r	~13,200	pBR322-Tn*7 tnsABCDE* genes-Tn*7*R	3
pMON7134	Amp^r	4,483	pEMBL9-*att*Tn*7* (523-bp *Hin*cII fragment into *Hin*cII site)	This study
pMON14007	Amp^r	11,517	pVL1393 *Bam*HI + 1,867-bp *Bam*HI fragment encoding hLTA$_4$H	17
pMON14102	Amp^r Kan^r	4,201	pBS2SKP *Pst*I + 1,240-bp *Pst*I fragment of pUC-4K	This study
pMON14118	Amp^r	9,515	pVL1393 *Eco*RV-*Sma*I to remove polyhedrin promoter	This study
pMON14181	Kan^r	7,965	6,707-bp *Bam*HI-*Eco*RI fragment of F'lacIQ + 1,258-bp *Eco*RI-*Bam*HI Kan^r fragment of pMON14102	This study
pMON14189	Amp^r Gen^r	4,783	pMON7117 *Pst*I-*Xba*I + pMON7104 *Xba*I-*Pst*I fragment encoding gentamycin acetyltransferase-3-I	This study
pMON14192	Cam^r, *lacZα*	3,463	pBCSKP *Sal*I-*Eco*RI + 90-bp *Sal*I-*Eco*RI PCR fragment of pMON7134 containing mini-*att*Tn*7*	This study
pMON14209	Amp^r	5,293	pSL301 *Stu*I-*Not*I + pMON14007 *Eco*RV-*Not*I	This study
pMON14214	Amp^r Gen^r	4,984	pMON14189 *Bam*HI-*Xba*I + 244-bp *Bam*HI-*Xba*I SV40 poly(A) fragment of pMON3327	This study
pMON14221	Amp^r	11,510	pMON14007 *Nco*I-*Eco*RI + *Nco*I-*Eco*RI fragment of pRAJ275 encoding β-glucuronidase	This study
pMON14231	Kan^r	8,538	pMON14181 *Eco*RI-*Sal*I + *Bbs*I-cleaved *lacZα*-mini-*att*Tn*7* PCR fragment of pMON14192	This study
pMON14239	Amp^r Gen^r	4,526	pMON14214 *Nco*I-*Not*I/mung bean nuclease	This study
pMON14255	Amp^r Gen^r	4,554	pMON14239 *Bam*HI + I-*Sce*I polylinker	This study
pMON14271	Amp^r Kan^r, *lacZα*	18,053	pMON14118 *Bgl*II + pMON14231 *Bam*HI partial (A orientation)	This study
pMON14272	Amp^r Kan^r, *lacZα*	18,053	pMON14118 *Bgl*II + pMON14231 *Bam*HI partial (B orientation)	This study
pMON14314	Amp^r Gen^r	6,719	pMON14255 *Xba*I + pMON14209 *Spe*I-*Nhe*I	This study
pMON14327	Amp^r Gen^r	6,715	pMON14314 *Nco*I-*Eco*RI + pRAJ275 *Nco*I-*Eco*RI fragment encoding β-glucuronidase	This study
pMON22300	Amp^r Gen^r	6,093	pMON14327 *Nco*I-*Sac*I + pMON9392 *Nco*I-*Sac*I fragment encoding hNMT-P127L	This study

method and purification over Magic Mini Prep resin (Promega, Madison, Wis.) as described by the manufacturer. All other standard genetic and cloning procedures were performed as described elsewhere (46). Simulated cloning and manipulation of plasmid maps were facilitated through use of the POLLUX plasmid data base and display program (11).

Insect cell culture and propagation of baculoviruses. Sf9 cells (50), a clonal isolate of the IPLB-SF21-AE (Sf21) cell line (52) derived from the ovarian tissue of the fall armyworm *Spodoptera frugiperda*, were used for the routine propagation of wild-type and recombinant baculoviruses. The E2 variant (49) of AcNPV was used throughout these procedures. IPL-41 medium (GIBCO/BRL) supplemented with 2.6 g of tryptose phosphate broth (GIBCO/BRL) per liter and 10% fetal bovine serum (J.R.H. Biosciences, Lenexa, Kan.) was used for the routine propagation of Sf9 and Sf21 cells. Transfections and plaque assays were performed as described by Summers and Smith (50). DNA dot-blot hybridizations and all other routine cell culture methods have been described by O'Reilly et al. (43). Radiolabeling of infected cells with [^{35}S]methionine was performed as described by Luckow and Summers (34).

Construction of baculovirus transfer vectors. Plasmid pMON14007 (17) is a derivative of the baculovirus transfer vector pVL1393, containing the gene for human leukotriene A_4 hydrolase (hLTA$_4$H) under the control of the AcNPV polyhedrin promoter. Plasmid pMON14221 was constructed by replacing an *Nco*I-*Eco*RI fragment of pMON14007 containing the LTA$_4$H gene with an *Nco*I-*Eco*RI fragment of pRAJ275 containing the β-glucuronidase gene. pRAJ275 is a derivative of pRAJ255 (23) containing a consensus *E. coli* translational initiator in place of deleted 5' β-glucuronidase sequences. Viruses constructed from pMON14221 are used as controls for comparing levels of expression of β-glucuronidase with composite bacmids. Recombinant viruses expressing β-glucuronidase were easily identified as blue plaques on agarose plates containing the chromogenic indicator X-gluc (36).

Construction of an infectious baculovirus shuttle vector. A schematic outline for the generation of recombinant baculovirus shuttle vectors is shown in Fig. 1, and a flow chart describing the construction of mini-Tn*7* target plasmids is shown in Fig. 2. Plasmid pMON14102 was constructed by cloning a 1,240-bp *Pst*I fragment of pUC4-K (51, 54) into the *Pst*I site of pBluescriptII SK(+) (1). F' plasmid DNA prepared from strain DH5α/(F' *lac-pro*)::Tn*5* was digested with *Bam*HI and *Eco*RI and ligated to an agarose gel-purified

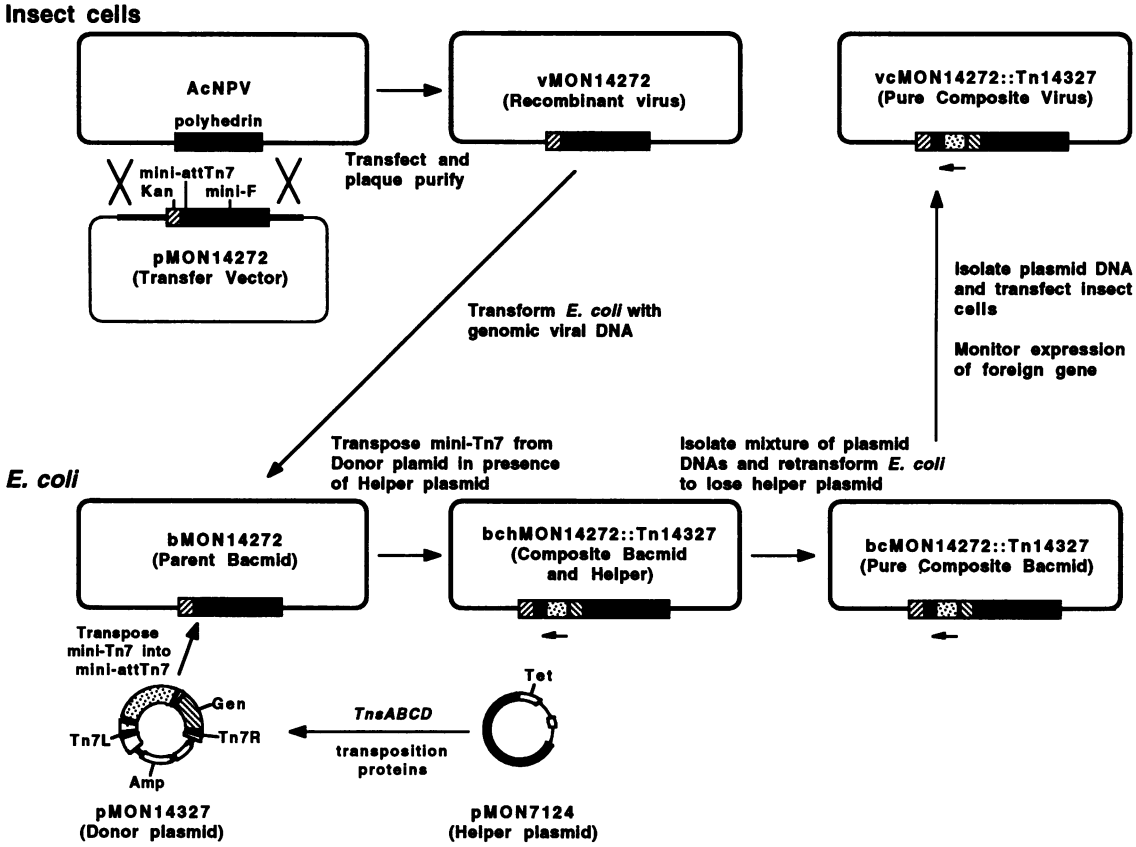

FIG. 1. Schematic outline for the generation of recombinant baculovirus shuttle vectors (bacmids) and site-specific transposon-mediated insertion of foreign genes into the baculovirus genome propagated in *E. coli*. Recombinant viruses containing a mini-F replicon, a kanamycin resistance marker, and a mini-*att*Tn*7* inserted into the *lacZ*α region of a pUC-based plasmid were constructed by homologous recombination into the polyhedrin locus of AcNPV in insect cells. Viral DNA was purified from insect cells and introduced into *E. coli*, where it propagates as a large low-copy-number plasmid. A mini-Tn*7* element from a donor plasmid inserts into the mini-*att*Tn*7* site on the shuttle vector when transposition functions are provided in *trans* by a helper plasmid. Pure composite bacmid DNAs introduced into insect cells are infectious and express the foreign gene under the control of the baculovirus promoter in the mini-Tn*7* element. See text for additional details.

*Bam*HI-*Eco*RI fragment from pMON14102 that confers resistance to kanamycin (Tn*903*). After transformation into *E. coli* DH10B, kanamycin-resistant colonies were selected and shown to contain plasmids of the desired structure. The plasmid of one such transformant was designated pMON14181.

Plasmid pMON7134 was constructed by inserting a 523-bp *Hin*cII fragment of pEAL1 (32) containing the attachment site for Tn*7* (*att*Tn*7*) into the *Hin*cII site of pEMBL9 (12). A 112-bp mini-*att*Tn*7* sequence was amplified by PCR from plasmid pMON7134 with two primers, AttRP-PR1 (5′-agatctgcaggaattcacataacaggaagaaaaatgc-3′) and AttSP-PR1 (5′-ggatccgtcgacagccgcgtaacctggcaaa-3′), designed to amplify a short DNA sequence containing a functional *att*Tn*7* with *Eco*RI (G′AATT,C) and *Sal*I (G′TCGA,C) sites (underlined above) at either end. PCRs were carried out with a DNA thermal cycler and GeneAmp PCR reagent kit (Perkin Elmer Cetus, Norwalk, Conn.). Thirty cycles consisting of three steps (94°C, 1 min; 50°C, 2 min; 72°C, 3 min) were used to amplify the mini-*att*Tn*7*. The amplified segment contains an 87-bp *att*Tn*7* (numbered −23 to +61 as described by Craig [10]). The 112-bp amplified fragment was digested with *Eco*RI and *Sal*I and cloned into the *Eco*RI and *Sal*I sites within the *lacZ*α region of the cloning vector pBCSKP to generate pMON14192. The *Eco*RI-*Sal*I mini-*att*Tn*7* does not disrupt the reading frame of the *lacZ*α region of pBCSKP and has the *E. coli glmS* transcriptional terminator inserted in the opposite orientation from transcription directed by the *lac* promoter, so colonies of *E. coli* DH10B harboring pMON14192 are blue on agar plates containing X-Gal or Bluo-Gal and IPTG.

Plasmid pMON14192 was linearized with *Sca*I and used as a template for PCR in the presence of two new primers, *lacZ*α-PR1 (5′-tgatcattaattaagtcttcgaaccaatacgcaaaccgcctctc cccgcgcg-3′) and *lacZ*α-PR2 (5′-cgatcgactcgagcgtcttcgaagc gcgtaaccaccacacccgccgcgc-3′), as described above except that the reaction buffer contained 5% (vol/vol) dimethyl sulfoxide to permit less-stringent annealing. Thirty cycles consisting of three steps (94°C, 1 min; 55°C, 2 min; 72°C, 3 min) were used to amplify the mini-*att*Tn*7*. The PCR primers were designed to amplify the entire *lacZ*α region of pMON14192 and any pUC-based cloning vectors. Each primer contained a *Bbs*I site (GAAGACNN′NNNN, or ,NNNN′NNGTCTTC) near the 5′ end. Primer *lacZ*α-PR1 contains an *Eco*RI-compatible ('AATT,) site, and primer *lacZ*α-PR2 contains a *Sal*I-compatible ('TCGA,) site as part of the cleavage site (double underline above) flanking the *Bbs*I recognition site (single underline above). A *Drd*I site and a *Pac*I site (not underlined) are also present adjacent to the *Bbs*I sites in *lacZ*α-PR1 and *lacZ*α-PR2, respectively.

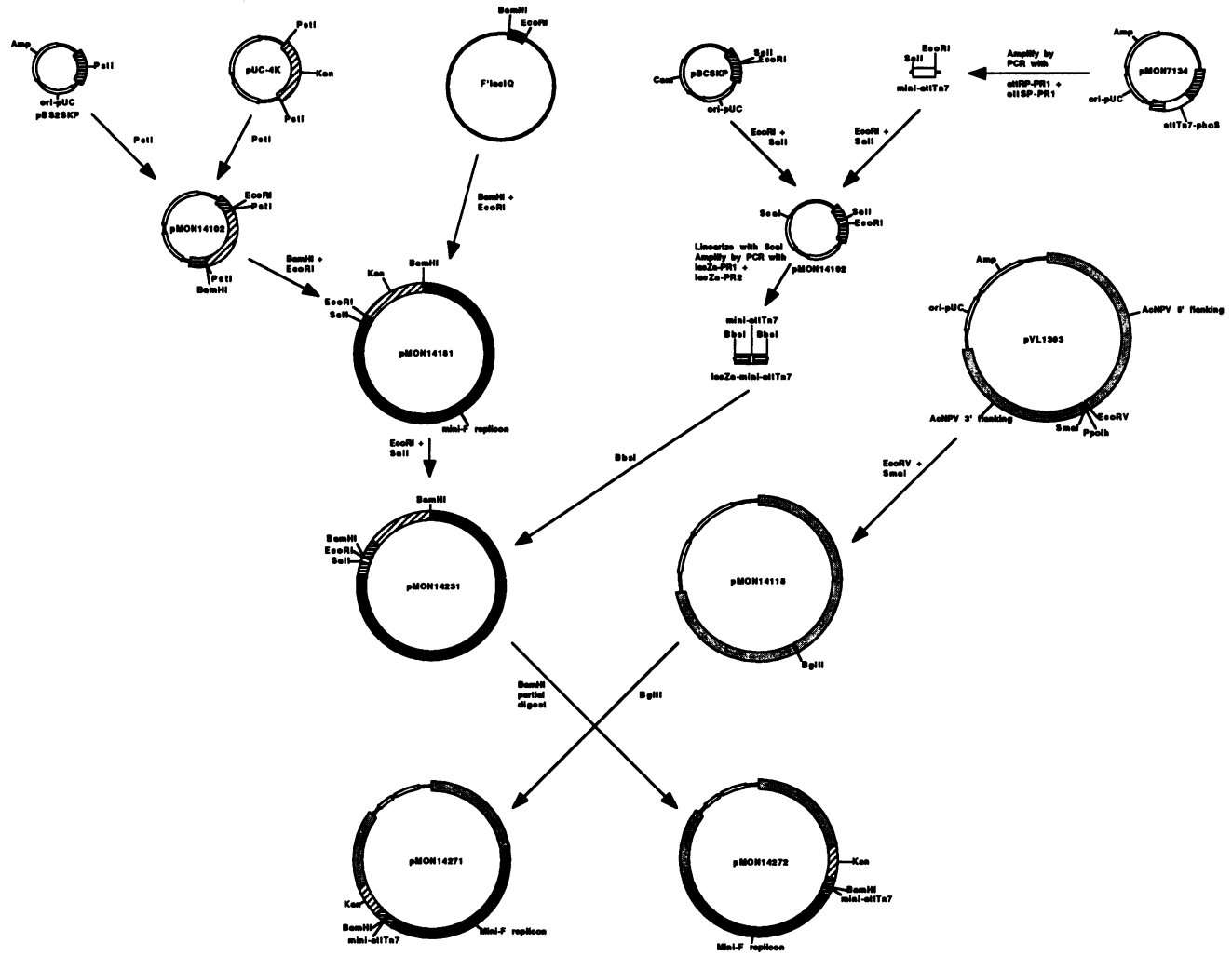

FIG. 2. Flow chart for construction of the bacmid transfer vectors pMON14271 and pMON14272. See Materials and Methods for additional details. The light gray sections represent baculovirus sequences flanking the polyhedrin promoter (P_{polh}) in the 7,327-bp AcNPV *Eco*RI fragment I. The dark gray region represents the mini-F replicon derived as a *Bam*HI-*Sal*I fragment from the F' plasmid isolated from the *E. coli* strain DH5αF'IQ. The horizontal-striped section with a white center represents the *lacZ*α region derived from pBCSKP, containing an in-frame insertion of the attachment site for Tn*7* (mini-*att*Tn*7*). The leftward diagonally striped section represents a segment conferring resistance to kanamycin.

The amplified 728-bp double-stranded DNA fragment could therefore be cleaved with *Bbs*I to generate *Eco*RI- and *Sal*I-compatible sticky ends, even though there were internal *Eco*RI and *Sal*I sites flanking the mini-*att*Tn*7* region towards the center of the fragment. The 708-bp *Bbs*I-cleaved PCR fragment was ligated to pMON14181 (mini-F-Kan) that was cleaved with *Eco*RI and *Sal*I and transformed into *E. coli* DH10B. Several kanamycin-resistant Lac^+ transformants were obtained, and all had the expected DNA structure. One clone, designated pMON14231 (mini-F-Kan-*lacZ*α-mini-*att*Tn*7*) was chosen for subsequent work. Its structure was verified by digestion with *Bam*HI, *Eco*RI, *Eco*RV, *Kpn*I, *Bgl*II, *Hin*dIII, and *Hin*dIII plus *Bgl*II (data not shown).

Plasmid pMON14118 was constructed by digesting pVL1393 (33, 43) with *Eco*RV and *Sma*I and recircularizing in the presence of T4 DNA ligase to remove the AcNPV polyhedrin promoter. Plasmid pMON14231 has two *Bam*HI sites, one within the *lacZ*α-mini-*att*Tn*7* region and the other at the junction between the mini-F and Kan^r genetic elements, so it was digested with a low concentration of *Bam*HI to generate full-length linear molecules and ligated to pMON14118 cleaved with *Bgl*II to generate pMON14271 and pMON14272. Plasmids pMON14271 and pMON14272 differ only in the orientation of the mini-F-Kan-*lacZ*α-mini-*att*Tn*7* cassette inserted into the pMON14118 transfer vector. Their structures were verified by digestion with *Bam*HI, *Eco*RI, and *Xho*I (data not shown). Detailed maps and the predicted sequences of these plasmids are available from the authors. Upon transformation into *E. coli* DH10B, both plasmids confer resistance to ampicillin and kanamycin and a Lac^+ phenotype on plates containing X-Gal or Bluo-Gal and IPTG.

Both transfer vectors, pMON14271 and pMON14272, were introduced into insect cells along with wild-type genomic AcNPV DNA by a calcium phosphate-mediated transfection protocol (50). Putative recombinant viruses were identified by their occlusion-minus phenotype under a stereo dissecting microscope and confirmed by DNA dot-

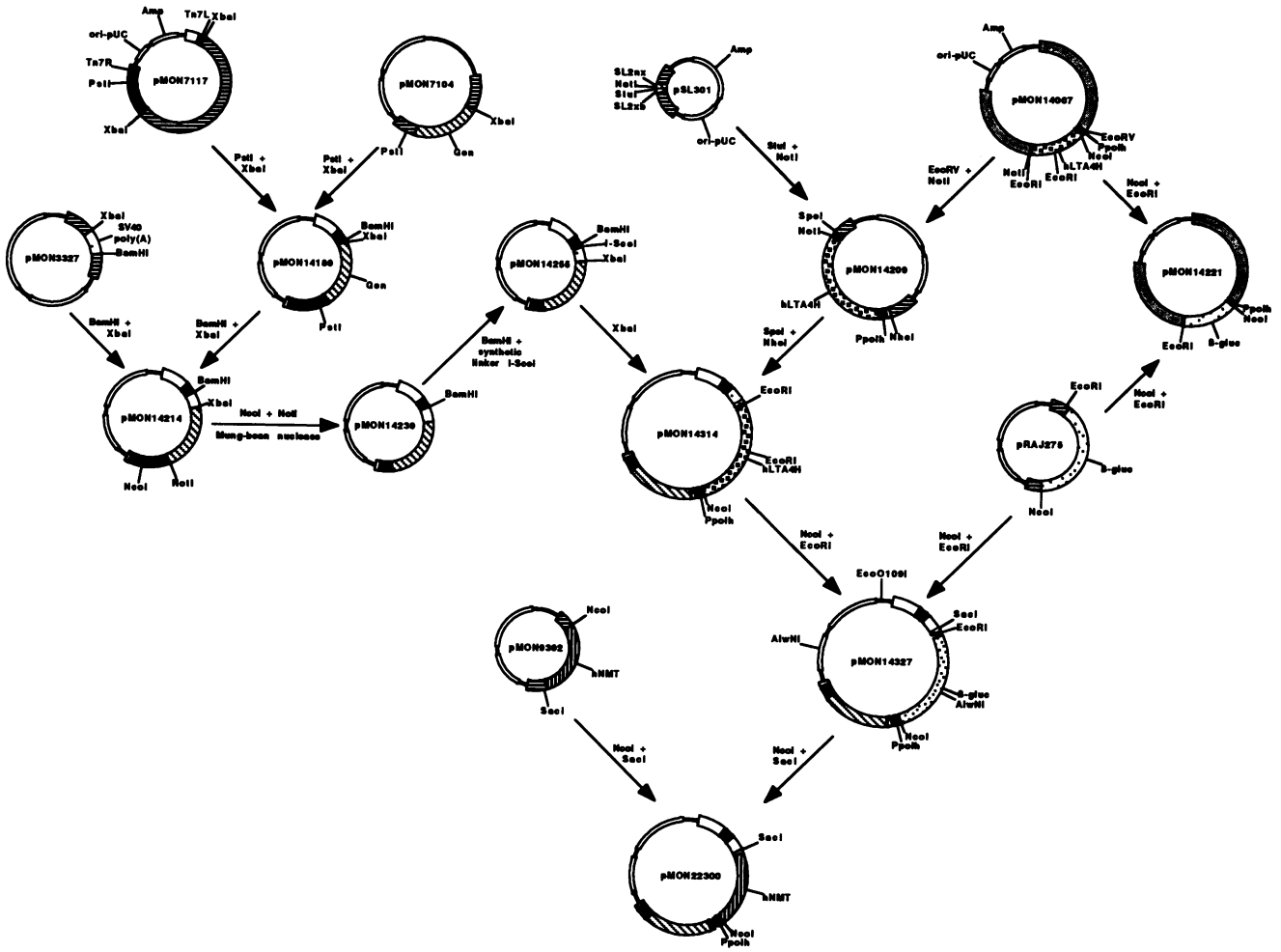

FIG. 3. Flow chart for construction of the mini-Tn7 donor plasmids. See Materials and Methods for additional details. The left and right ends of Tn7 and the polyhedrin promoter are indicated by solid areas. The heavy and light stippled areas represent the β-glucuronidase gene and the SV40 poly(A) termination signals, respectively. The leftward diagonally striped section represents a segment conferring resistance to gentamicin. Wide hatched regions (SL2nx and SL2xb) represent synthetic polylinker regions derived from the superpolylinker plasmid pSL301. Open regions represent sections derived from the *E. coli phoS* and *glmS* genes flanking the target site *att*Tn7 and sections containing the pUC origin of replication and the ampicillin resistance gene.

blot hybridization with ^{32}P-labeled pMON14181 DNA prepared by random priming (43) as a probe to cell lysates (50) blotted 48 h postinfection onto nitrocellulose filter paper (34). Three viruses were selected for each construct and purified of wild-type parental virus by sequential plaque assays (50), and passage 1 stocks of each purified virus (vMON14271 and vMON14272) were prepared. The prefix v is used to designate the source of viral stocks or viral DNA, in this case prepared from infected insect cells.

Genomic viral DNA was prepared from the infected cells used to generate the passage 1 stock of virus by the protocol described by Summers and Smith (50). Viral DNA constitutes approximately 25% of the total nucleic acid content of an infected-cell nucleus very late in infection (>48 h postinfection). Briefly, cells were lysed with lysis buffer (30 mM Tris-HCl [pH 8.0], 10 mM magnesium acetate, 1% Nonidet P-40), and the nuclei were pelleted by centrifugation at 2,000 rpm for 3 min. The nuclei were washed once in cold phosphate-buffered saline (PBS) and lysed with 4.5 ml of extraction buffer (100 mM Tris-HCl [pH 8.0], 100 mM EDTA, 200 mM KCl). Approximately 200 μg of proteinase K was added and incubated at 50°C for 1 h before 0.5 ml of 10% sarcosyl was added and then incubated at 50°C overnight. The DNA was purified by extracting once with buffer-saturated phenol and once with phenol-chloroform-isoamyl alcohol (25:24:1) before precipitation with ethanol.

Viral DNA was transformed into *E. coli* DH10B by using frozen competent cells obtained from GIBCO/BRL. Colonies on plates transformed with the viral DNA vMON14271 or vMON14272 were kanamycin resistant and had a Lac$^+$ (blue) phenotype in the presence of Bluo-Gal or X-Gal and IPTG, indicating complementation between the *lacZα* peptide expressed by the plasmid and the *lacZ*ΔM15 acceptor polypeptide expressed from the chromosome of *E. coli* DH10B. The transformants were designated bMON14271 and bMON14272 to indicate their origin as *E. coli*-derived baculovirus shuttle vectors (bacmids).

Construction of a mini-Tn7 donor plasmid. A flow chart describing the construction of mini-Tn7 donor plasmids is shown in Fig. 3. Plasmid pMON7104 is a derivative of

pEMBL19P containing a 1,258-bp *Alu*I fragment encoding the gene (*aacC1*) for gentamicin acetyltransferase-3-I (56). The gentamicin resistance gene of pMON7104 was released by *Xba*I and *Pst*I digestion, and the purified fragment was ligated to *Pst*I- and *Xba*I-digested pMON7117 (3), producing pMON14189. The simian virus 40 (SV40) poly(A) transcription termination signal of pMON3327 (19a) was released as a 244-bp fragment by *Bam*HI plus *Xba*I digestion and ligated to *Bam*HI- and *Xba*I-digested pMON14189, resulting in plasmid pMON14214. pMON14214 was digested with *Nco*I and *Not*I, and the sticky ends of the restriction fragment were removed by treatment with mung bean nuclease (Promega) under the conditions described by the manufacturer. This fragment was recircularized by ligation, producing pMON14239. pMON14239 was digested with *Bam*HI and ligated to the synthetic double-stranded polylinker shown below (Boehringer Mannheim, Indianapolis, Ind.), resulting in plasmid pMON14255. *omega* nuclease I-*Sce*I recognizes the 18-bp sequence TAGGG'ATAA'CAGGGTAAT and generates a 4-bp 3′ hydroxyl overhang (9):

*Bam*HI I-*Sce*I *Bgl*II

Linker I-*Sce*I: 5′-gatccgctaggg ataa'cagggtaatata -3′
Megalinker: 3′- gcgatccc'tatt gtcccattatatctag -5′

Plasmid pMON14007 (17) was digested with *Eco*RV and *Not*I, and the fragment containing the AcNPV polyhedrin promoter and the $hLTA_4H$ cDNA (16, 40) was ligated to *Stu*I- and *Not*I-digested pSL301 (6), producing plasmid pMON14209. pMON14209 was digested with *Spe*I and *Nhe*I, and the fragment containing the polyhedrin promoter and $hLTA_4H$ gene was ligated to *Xba*I-digested pMON14255, resulting in plasmid pMON14314. Plasmid pMON14327 was constructed by replacing the $hLTA_4H$ gene of pMON14314 with an *Nco*I-*Eco*RI fragment of pRAJ275 (23), which contains the coding sequences for the β-glucuronidase gene. Plasmid pMON22300 is a derivative of the donor plasmid pMON14327, which has the cDNA for human myristoyl coenzyme A:protein *N*-myristoyl transferase (hNMT) (13) under the control of the polyhedrin promoter. The hNMT cDNA in this plasmid encodes a Pro to Leu mutation at amino acid position 127 (P127L). Detailed maps and the predicted sequences of all the donor plasmids and their intermediates are available from the authors.

Transposition of mini-Tn*7* elements from donor plasmid to target bacmid. Transposition experiments were carried out by transforming a donor plasmid (pMON14314, pMON14327, or pMON22300) into *E. coli* DH10B harboring the transposition helper plasmid pMON7124 (3) and the bacmid bMON14271 or bMON14272. Competent cells were prepared by diluting an overnight culture 1:50 in 2×YT medium containing kanamycin (50 μg/ml) and tetracycline (10 μg/ml) and growing it to an A_{600} reading of 0.3 to 0.5 (30 to 50 Klett units). One milliliter of culture was pelleted by centrifugation for 30 s at 14,000 rpm and resuspended in 100 μl of 1× TSS solution. Between 0.1 and 1.0 μg of donor plasmid DNA was added to the competent cells, held on ice for 30 to 60 min, and outgrown at 37°C for 3 h in 1.0 ml of S.O.C. medium in the absence of antibiotic selection. Then, 100 μl of the outgrown cells were plated on LB plates containing kanamycin (50 μg/ml), tetracycline (10 μg/ml), gentamicin (7 μg/ml), X-Gal or Bluo-Gal (100 μg/ml), and IPTG (40 μg/ml). White kanamycin-, gentamicin-, and tetracycline-resistant but ampicillin-sensitive colonies harboring the helper plasmid and the bacmid with the mini-Tn*7* element inserted into the mini-*att*Tn*7* region of the *lacZ*α region were purified, and plasmid DNA was prepared over QIAGEN resin columns. This mixture of plasmid DNAs was used to retransform *E. coli* DH10B, selecting for kanamycin and gentamicin resistance, and colonies were scored to confirm the absence of the tetracycline resistance marker present on the helper plasmid.

Restriction analysis and Southern blotting of shuttle vector DNAs. Plasmid and viral DNAs digested with the appropriate restriction enzymes were separated on 1% agarose gels and bidirectionally blotted to nitrocellulose filters overnight by established protocols (46). Radiolabeled probes were prepared with a random DNA priming kit (GIBCO/BRL). Hybridizations were carried out as described previously (34).

RESULTS

Construction of a baculovirus shuttle vector capable of propagation in *E. coli*. The baculovirus shuttle vectors (bacmids) that were constructed contain a cassette that includes several distinct genetic elements (a mini-*att*Tn*7* inserted in frame within the *lacZ*α region derived from a pUC-based plasmid, a selective kanamycin resistance gene, and the *rep* and *par* functions derived from the F factor) inserted into the polyhedrin locus of AcNPV (Fig. 1 and 2). Baculovirus transfer vectors pMON14271 and pMON14272 containing these elements in either orientation were constructed and transfected into cultured Sf9 insect cells with wild-type genomic AcNPV DNA. Recombinant viruses, identified by their occlusion-minus phenotype and confirmed by DNA dot-blot hybridization, were purified by sequential plaque assays.

Crude genomic viral DNA isolated from the nuclei of cells infected with the purified viruses vMON14271 and vMON14272 was transformed into *E. coli* DH10B. Transformants (designated bMON14271 and bMON14272) were distinguishable as light blue colonies on agar plates containing kanamycin, X-Gal or Bluo-Gal, and IPTG. The amino-terminal portion of β-galactosidase encoded by the *lacZ*α region on the bacmid is capable of intra-allelic (α) complementation with the defective form of β-galactosidase (*lacZ*ΔM15) encoded by the host when both are expressed in the presence of the inducer IPTG (54). *E. coli* DH10B harboring cloning vectors such as pUC19, which are also capable of α complementation, form dark blue colonies on plates containing the same concentration of X-Gal or Bluo-Gal and IPTG. The difference in color intensity is probably due to the low copy number of the bacmid driven by the mini-F replicon compared with the high copy number conferred by the ColE1-based replicon present in pUC-like cloning vectors. Small amounts of pure bacmid DNA could be isolated from *E. coli* after alkaline lysis and purification over resin columns. Yields of pure bacmid DNAs were typically 50 to 100 μg per 250 ml of *E. coli* grown overnight in 2×YT medium, which are 50- to 250-fold lower than that observed for small high-copy-number plasmids isolated under the same conditions. These results indicated that the insect cell-derived baculovirus DNA could be propagated in *E. coli* by using the mini-F replicon, which ensures stable replication of plasmid DNAs at a low copy number. No transformants were observed when wild-type viral DNA or recombinant virus DNA lacking the mini-F region was introduced into *E. coli*.

Bacmids are infectious in insect cells. Bacmid DNA was introduced into insect cells by the calcium phosphate transfection protocol (50). At 3 to 5 days posttransfection, the

cells appeared swollen and detached easily from the plastic bottom of the flask, as is seen for cells infected with viral DNA isolated originally from insect cells. Mock-infected cells attached tightly to the monolayer. Plaques produced by budded virus generated from transfections with *E. coli*-derived bacmid DNA were all occlusion minus (data not shown).

Construction of mini-Tn*7* donor plasmids. To facilitate the construction and delivery by transposition of mini-Tn*7* elements from a donor plasmid to the *att*Tn*7* sequence present in a target plasmid, the replicon containing the element should be of small size and high copy number and contain a drug resistance marker and a polylinker with unique restriction sites between the left (Tn*7*L) and right (Tn*7*R) arms of Tn*7*. To accomplish this, pMON14255 was constructed through a series of steps (outlined in Materials and Methods) by replacing the P_{iuc}-*lacZ* cassette within pMON7117 with the gentamicin resistance marker of pMON7104, an SV40 poly(A) signal from pMON3327, and a synthetic polylinker containing a site for the *omega* nuclease I-*Sce*I. Plasmid pMON14314 was constructed by inserting the cDNA for $hLTA_4H$ under the control of the AcNPV polyhedrin promoter from pMON14007 into a polylinker region in pMON14255. The β-glucuronidase gene from pRAJ275 was substituted for the $hLTA_4H$ cDNA in pMON14314 to generate pMON14327. The β-glucuronidase gene in pMON14327 was replaced with a gene encoding hNMT-P127L to generate pMON22300. The resulting donor plasmids, pMON14314, pMON14327, and pMON22300, therefore have mini-Tn*7* elements on a pUC-based plasmid containing a gentamicin resistance marker, the polyhedrin promoter driving expression of a foreign gene, a polylinker, an SV40 poly(A) transcriptional termination signal, and an I-*Sce*I site between the left and right arms of Tn*7*. The gentamicin resistance marker is used to select for transposition events to the target plasmid, and the I-*Sce*I site is used to facilitate the mapping of mini-Tn*7* elements inserted into the genome of the target bacmids. There are no I-*Sce*I sites in the genome of wild-type AcNPV.

Transposition of mini-Tn*7* elements to target bacmids. Transposition experiments were carried out by transforming a donor plasmid (pMON14314, pMON14327, or pMON22300) conferring ampicillin and gentamicin resistance into competent *E. coli* DH10B cells harboring the tetracycline-resistant helper plasmid pMON7124 and a kanamycin-resistant, *lacZ*α$^+$ bacmid (bMON1427 or bMON14272) and plating the cells on LB agar plates containing kanamycin, tetracycline, gentamicin, X-Gal or Bluo-Gal, and IPTG. White (Lac$^-$) kanamycin-, gentamicin-, and tetracycline-resistant but ampicillin-sensitive colonies harboring the helper plasmid and the bacmid with the mini-Tn*7* element inserted into the mini-*att*Tn*7* region of the *lacZ*α region, which arose at a frequency of between 5 and 25% of total colonies, were identified and purified by restreaking on fresh plates. Blue (Lac$^+$) kanamycin-, gentamicin-, and tetracycline-resistant but ampicillin-sensitive colonies, which probably represent insertions of the mini-Tn*7* element into the *att*Tn*7* site in the *E. coli* chromosome between the *glmS* and *phoS* genes, occurred at a nearly equivalent frequency. The remainder of the colonies were blue (Lac$^+$) and showed resistance to all four antibiotics. Although these colonies simultaneously harbored the bacmid shuttle vector and the helper and donor plasmids, this situation appeared to be unstable, as white (Lac$^-$) and blue (Lac$^+$) colonies that were also kanamycin, tetracycline, and gentamicin resistant but ampicillin sensitive appeared upon restreaking.

Plasmid DNAs containing a mixture of the composite bacmid and the helper plasmid were purified and used to retransform *E. coli* DH10B, selecting for kanamycin and gentamicin resistance, and colonies were scored to confirm the absence of the tetracycline resistance marker present on the helper plasmid. No kanamycin- and gentamicin-resistant colonies were obtained when DNA was isolated from a colony originally observed to have a blue (Lac$^+$) kanamycin-, gentamicin-, and tetracycline-resistant but ampicillin-sensitive phenotype, suggesting that the mini-Tn*7* element (carrying the gentamicin resistance gene) in this strain had transposed into the chromosome.

Structure of baculovirus shuttle vector DNAs. DNAs from donor plasmids, the parent bacmids, and the composite bacmids isolated from *E. coli* and from insect cells were examined by digestion with *Bgl*II, *Eco*RI, I-*Sce*I, *Not*I, *Pst*I, *Sse*8387I, and *Xho*I, and the restriction patterns were compared with the pattern generated by cleavage of wild-type AcNPV DNA purified from budded virus. Bacmid DNAs isolated from *E. coli* and digested with *Bgl*II, *Pst*I, or *Xho*I exhibit no gross structural differences from the corresponding viral DNAs isolated originally from insect cells. A schematic depiction of the structures of the parent bacmids bMON14271 and bMON14272, the composite bacmids bcMON14271::Tn14327 and bcMON14272::Tn14327, and the donor plasmid pMON14327 is shown in Fig. 4.

To illustrate the structural analysis of the parent and composite shuttle vectors, selected plasmid and viral DNAs were digested with *Eco*RI or *Eco*RI plus *Sse*8387I, separated on an agarose gel, and blotted onto two nitrocellulose membranes. These were hybridized to radiolabeled probes prepared from the transfer vector pMON14272 and from the donor plasmid pMON14327. The results and interpretation of these experiments are shown in Fig. 5. The ethidium bromide-stained agarose gel (Fig. 5A) displays all of the relevant DNA bands above 400 bp in size. The composite bacmid DNA bcMON14272::Tn14327 (Fig. 5A, lanes 10 and 11) isolated from *E. coli* over QIAGEN resin columns was remarkably free of contaminating chromosomal DNA compared with the crude viral DNAs prepared from vMON14272-infected insect cells, which were contaminated with insect chromosomal DNA (Fig. 5A, lanes 8 and 9). Little or no chromosomal DNA was present in the preparations of pMON14272 (Fig. 5A, lanes 4 and 5) or pMON14327 (Fig. 5A, lanes 12 and 13) plasmid DNA or from wild-type AcNPV DNA prepared from purified budded virus (Fig. 5A, lanes 6 and 7).

Figure 5B and C are autoradiograms of the Southern blot of the same gel displayed in Fig. 5A probed with radiolabeled pMON14272 and pMON14327, respectively. A summary and interpretation of these results is shown in Fig. 5D. The transfer vector pMON14272 has two *Eco*RI sites, one at the junction of the kanamycin resistance marker and the 5′-flanking viral sequences and one located in the polylinker region encoding the *lacZ*α peptide. There are also two *Sse*8387I sites, one in the mini-F replicon and the other in a polylinker region at the junction of the *lacZ*α region and the kanamycin resistance gene. Wild-type AcNPV is cleaved into more than 25 *Eco*RI fragments but does not appear to be cleaved by *Sse*8387I. The 9,327-bp *Eco*RI I fragment that contains the polyhedrin gene hybridizes strongly to the pMON14272 probe (Fig. 5B, lanes 6 and 7) but only very weakly (upon long exposure; not shown) to pMON14327 (Fig. 5C, lanes 6 and 7). This is not surprising, since the only homology between AcNPV and pMON14327 is a short (~120-bp) segment containing the polyhedrin promoter. The

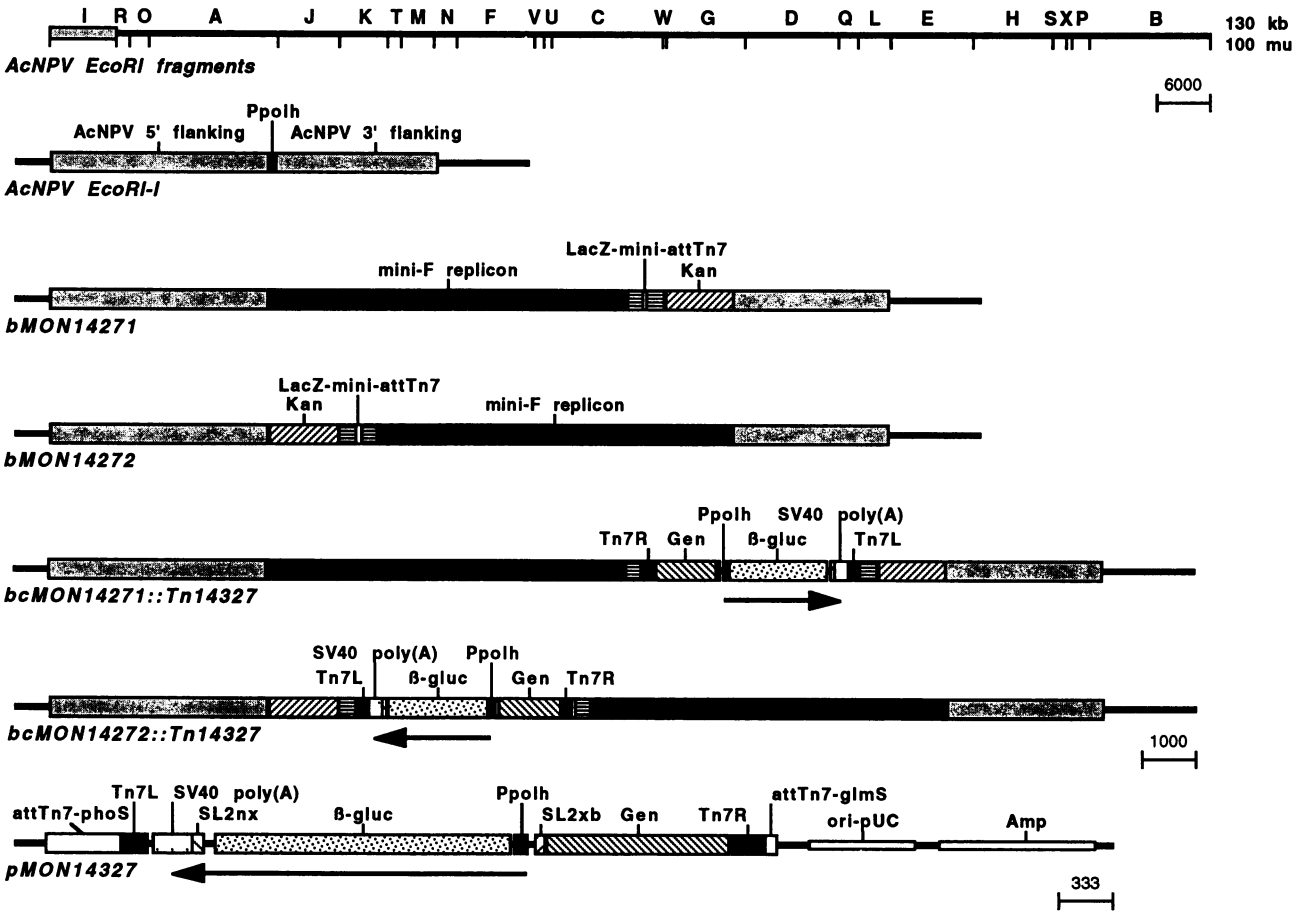

FIG. 4. Structures of baculovirus shuttle vectors (bacmids). The top map shows positions of *Eco*RI sites on a linear map of the AcNPV genome. The light gray section highlights *Eco*RI fragment I, containing the polyhedrin gene and flanking regions. The maps of bMON17271 and bMON14272 are linear representations of the mini-F-*lacZ*α-mini-*att*Tn*7*-Kan cassette inserted into the genome of AcNPV at the polyhedrin locus by homologous recombination. The maps of the bcMON14271::Tn14327 and bcMON14272::Tn14327 are linear representations of a portion of the composite bacmids derived by transposition of the mini-Tn*7* element from the donor plasmid pMON14327. A threefold enlargement of a linear representation of the entire donor plasmid pMON14327 is shown at the bottom. The arrow indicates the direction and expected size of a transcript containing the β-glucuronidase sequences initiated from the polyhedrin promoter. The shading of different genetic elements is the same as described in the legends to Fig. 2 and 3. The maps are drawn to the scale (in bases) indicated by the bars at the right. mu, map units.

parent virus vMON14272, generated by homologous recombination between pMON14272 and wild-type AcNPV, contains the mini-F-Kan-*lacZ*α-mini-*att*Tn*7* cassette present in pMON14272 inserted into the AcNPV *Eco*RI-I fragment, since this fragment disappears and is replaced by three new *Eco*RI fragments of the expected sizes (Fig. 5A and B, lanes 8 and 9; Fig. 5D), two of which are cleaved by *Sse*8387I. Additional digests confirmed that the cassette was inserted into the polyhedrin locus located in the AcNPV restriction fragments *Bgl*II-C, *Pst*I-D, and *Xho*I-D (data not shown). None of the viral bands in vMON14272-digested DNAs hybridize to the donor plasmid pMON14327 (Fig. 5C, lanes 8 and 9). The composite bacmid bcMON14272::Tn14327 has a single new insertion of the expected size and location in the mini-*att*Tn*7*, as judged from the insertion of one or more additional restriction sites (*Eco*RI, I-*Sce*I, or *Not*I) present in the mini-Tn*7* donor cassette (Fig. 5, lanes 10 and 11 in all panels, and data not shown). The donor plasmid pMON14327 contains one *Eco*RI site but no *Sse*8387I sites (Fig. 5, lanes 12 and 13).

Introduction of a composite bacmid into insect cells and expression of the heterologous gene. Composite bacmid DNAs were isolated from *E. coli* and transfected into insect cells. Cytopathic effects became apparent after 3 days in culture. The cells became swollen and were more easily detached from the monolayer than mock-infected cells. Cells transfected with a donor plasmid alone did not appear to be infected.

To assess the ability of the composite viruses to express a heterologous gene in a rapid but qualitative manner, 100 μl of medium from the transfected cells was mixed with 10 μl of X-gluc (20 mg/ml in dimethyl sulfoxide), a chromogenic substrate for β-glucuronidase. A dark blue product was observed only in samples taken from cells infected with the composite viruses vcMON14271::Tn14327, vcMON14272::Tn14327, and vcMON14271::Tn14327/pMON7124 and with the control virus vMON14221 that was constructed by homologous recombination in insect cells. The virus stock vcMON14271::Tn14327/pMON7124 was prepared by transfecting insect cells with a mixture of composite DNA and noninfectious pMON7124 helper plasmid DNA. No β-glucuronidase activity was detectable from uninfected cells or

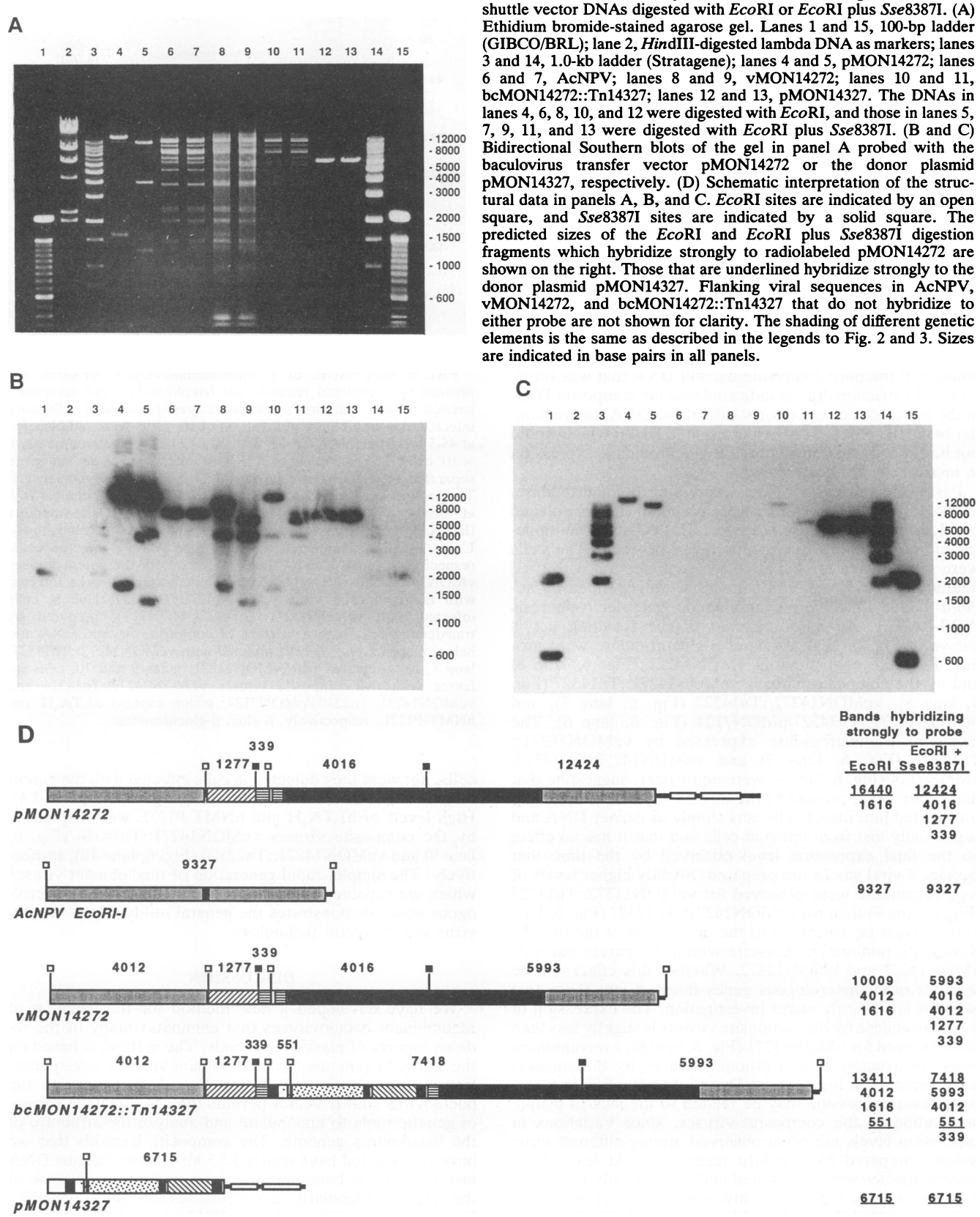

FIG. 5. Restriction analysis and Southern blotting of baculovirus shuttle vector DNAs digested with *Eco*RI or *Eco*RI plus *Sse*8387I. (A) Ethidium bromide-stained agarose gel. Lanes 1 and 15, 100-bp ladder (GIBCO/BRL); lane 2, *Hin*dIII-digested lambda DNA as markers; lanes 3 and 14, 1.0-kb ladder (Stratagene); lanes 4 and 5, pMON14272; lanes 6 and 7, AcNPV; lanes 8 and 9, vMON14272; lanes 10 and 11, bcMON14272::Tn14327; lanes 12 and 13, pMON14327. The DNAs in lanes 4, 6, 8, 10, and 12 were digested with *Eco*RI, and those in lanes 5, 7, 9, 11, and 13 were digested with *Eco*RI plus *Sse*8387I. (B and C) Bidirectional Southern blots of the gel in panel A probed with the baculovirus transfer vector pMON14272 or the donor plasmid pMON14327, respectively. (D) Schematic interpretation of the structural data in panels A, B, and C. *Eco*RI sites are indicated by an open square, and *Sse*8387I sites are indicated by a solid square. The predicted sizes of the *Eco*RI and *Eco*RI plus *Sse*8387I digestion fragments which hybridize strongly to radiolabeled pMON14272 are shown on the right. Those that are underlined hybridize strongly to the donor plasmid pMON14327. Flanking viral sequences in AcNPV, vMON14272, and bcMON14272::Tn14327 that do not hybridize to either probe are not shown for clarity. The shading of different genetic elements is the same as described in the legends to Fig. 2 and 3. Sizes are indicated in base pairs in all panels.

cells infected with wild-type AcNPV or viruses expressing $hLTA_4H$ or hNMT-P127L (data not shown). All of the plaques produced by budded virus generated from transfections with composite bacmids containing the β-glucuronidase gene formed blue plaques when the agarose overlay contained X-gluc (data not shown). Also, we have not been able to detect any difference in the relative infectivity of viral DNA isolated from insect cells or from *E. coli* (data not shown).

When equivalent amounts of pure composite bacmid DNA (bcMON14271::Tn14327) and a mixture of helper plasmid and composite bacmid DNA that contained the β-glucuronidase gene (bcMON14271::TN14327/pMON7124) were transfected into insect cells, expression of β-glucuronidase (assessed by reaction of the infected-cell supernatants with X-gluc) differed at 3 days posttransfection but not at 5 days posttransfection (data not shown). These results suggest that the difference in expression (lower for the transfected mixture of helper plasmid and composite bacmid) at the early time point may be the result of a lower molar ratio of infectious composite bacmid DNA in the mixture than the amount of the pure composite bacmid DNA that was transfected. Restriction digests indicated that the composite DNA in the mixture accounted for <10% of the DNA, the remainder being the pMON7124 helper plasmid DNA, which would not be expected to contribute to β-glucuronidase expression in insect cells (data not shown).

Passage 2 stocks of viruses expressing β-glucuronidase, $hLTA_4H$, and hNMT-P127L were prepared (but not plaque purified), titered, and used to infect Sf21 cells at a multiplicity of infection of 10 virus particles per cell. The cells were radiolabeled for 4 h at 44.5 h postinfection with [^{35}S] methionine and lysed, and samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). An autoradiogram of the resulting gel is shown in Fig. 6. High levels of β-glucuronidase were produced by the control virus vMON14221 (Fig. 6, lane 8) and by the composite viruses vcMON14271::Tn14327 (Fig. 6, lane 5), vcMON14272::Tn14327 (Fig. 6, lane 7), and vcMON14271::Tn14327/pMON7124 (Fig. 6, lane 6). The levels of β-glucuronidase expressed by vcMON14271::Tn14327 (Fig. 6, lane 5) and vcMON14271::Tn14327/pMON7124 (Fig. 6, lane 6) were equivalent, suggesting that the helper DNA present in the mixture of DNAs originally transfected into insect cells acts simply as carrier DNA and is gradually lost from infected cells and that it has no effect on the final expression level observed by the time that passage 2 viral stocks are prepared. Slightly higher levels of β-glucuronidase were observed for vcMON14272::Tn14327 (Fig. 6, lane 7) than for vcMON14271::Tn14327 (Fig. 6, lane 5) that might be attributed to the orientation of the mini-F-Kan-*lacZ*α-mini-*att*Tn*7* cassette within the parent bacmids bMON14271 and bMON14272. Whether this effect will be seen for other heterologous genes inserted into these two bacmids is currently under investigation. The expression of β-glucuronidase by the composite viruses is slightly less than that observed for vMON14221 (Fig. 6, lane 8), a recombinant virus constructed in a traditional manner by homologous recombination in insect cells. This slight reduction in β-glucuronidase expression may be related to the lack of plaque purification of the composite viruses, since variations in expression levels are often observed among different virus isolates prepared by standard techniques. At least three smaller species were also noted and are probably related to β-glucuronidase, since they are not present in wild-type AcNPV-infected (Fig. 6, lane 2) or uninfected (Fig. 6, lane 1) cells, nor were they detected in cells infected with the parent viruses vMON14271 or vMON14272 (Fig. 6, lanes 3 and 4). High levels of $hLTA_4H$ and hNMT-P127L were expressed by the composite viruses vcMON14271::Tn14314 (Fig. 6, lane 9) and vcMON14271::Tn22300 (Fig. 6, lane 10), respectively. The simple, rapid generation of recombinant viruses which are capable of abundant expression of these heterologous genes demonstrates the general utility of the baculovirus shuttle vector technology.

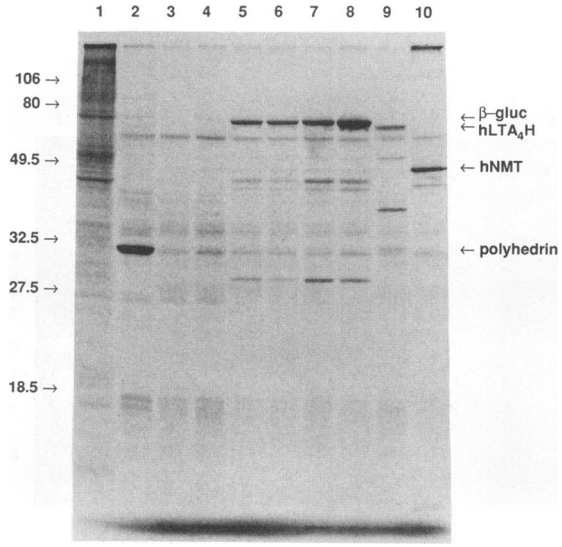

FIG. 6. SDS-PAGE of [^{35}S]methionine-labeled proteins expressed by traditional recombinant baculoviruses and composite bacmid vectors. All viral stocks were titered, and Sf21 cells were infected at a multiplicity of infection of 10. Cells were radiolabeled at 44.5 h postinfection for 4 h with 10 μCi of [^{35}S]methionine per 6 × 10^5 cells. The equivalent of 3.75 × 10^4 infected cells per lane were separated by electrophoresis on a 12% SDS–polyacrylamide gel. The gel was fixed, dried, and exposed to Kodak X-AR film for 76 h at room temperature. The positions of prestained size markers (Bio-Rad) and expressed proteins are indicated (in kilodaltons). Lanes 1 and 2, uninfected and wild-type AcNPV-infected cells, respectively; lanes 3 and 4, cells infected with the parent viruses vMON14271 and vMON14272, respectively; lane 5, cells infected with the composite virus vcMON14271::Tn14327; lane 6, cells infected with vcMON14271::Tn14327/pMON7124, prepared by transfecting cells with a mixture of composite bacmid DNA and helper plasmid; lane 7, cells infected with vcMON14272::Tn14327; lane 8, cells infected with vMON14221; lanes 9 and 10, cells infected with the composite viruses vcMON14272::Tn14314 and vcMON14271::Tn22300/pMON7124, which express $hLTA_4H$ and hNMT-P127L, respectively. β-gluc, β-glucuronidase.

DISCUSSION

We have developed a new method for the isolation of recombinant baculoviruses that eliminates many of the tedious aspects of classical methods. The method is based on the ability to generate the recombinant virus by site-specific transposition in *E. coli*. Using *E. coli* to propagate the baculovirus shuttle vector permits the use of a wide variety of genetic tools to manipulate and analyze the structure of the baculovirus genome. The composite bacmids that we have constructed have nearly 12.5 kb of heterologous DNA inserted into the baculovirus genome and represent some of the largest molecularly cloned viruses currently available. Recombinant (composite) virus DNA isolated from selected

colonies is not mixed with parental, nonrecombinant virus, eliminating the need for multiple rounds of plaque purification. As a result, the time required to identify and purify a recombinant virus is reduced from 4 to 6 weeks (typical for conventional methods, or slightly less if linearized parental virus DNA is used) to only 7 to 10 days. In the most rapid mode, this can be accomplished as follows: day 1, transform donor plasmid into competent cells harboring the shuttle vector and helper plasmid; day 2, identify colonies containing composite shuttle vectors, purify by restreaking on selection plates, and inoculate a purified colony into an overnight culture; day 3, prepare composite bacmid DNAs and transfect them into insect cells; and day 7 and beyond, harvest the stocks of pure budded virus for subsequent expression studies. Although we do not believe that it is routinely necessary at this stage, plaque purification may be desirable for virus stocks that will be used on a continuous basis, particularly as inoculum for protein production in large-scale bioreactors. One of the greatest advantages of this method is that it permits the rapid and simultaneous isolation of multiple recombinant viruses and is particularly suited for the expression of protein variants for structure-function studies.

Using site-specific transposition to insert foreign genes into a baculovirus shuttle vector that is propagated in *E. coli* has a number of advantages over generation of recombinant baculoviruses in insect cells by homologous recombination. The mini-Tn*7* donor plasmids that we describe are small compared with traditional baculovirus transfer vectors and are easily manipulated to add or remove restriction sites. The modular approach used in the construction of donor plasmids also permits rapid substitution or insertion of additional genetic elements (e.g., promoters, foreign genes, drug resistance markers) for customization of expression cassettes designed for different purposes, including use in different host organisms. The efficiency of transposition of the mini-Tn*7* element from the donor plasmid into the attachment site on the bacmid is higher than in recombinants generated by homologous recombination. Insertions into the mini-*att*Tn*7* located in frame with a segment of DNA on the bacmid encoding the α peptide of *lacZ* prevent complementation between the α peptide and the acceptor polypeptide produced from a *lacZ* deletion located on the *E. coli* chromosome. Therefore, transposition events into the bacmid can be easily distinguished from transposition into the chromosome or the absence of transposition by screening for white colonies in a background of blue colonies on agar plates containing chromogenic substrates for β-galactosidase, such as X-Gal or Bluo-Gal. Pure composite bacmid DNA or a mixture of a composite bacmid DNA and a helper plasmid can be transfected into insect cells to generate viruses which will express the foreign gene. Finally, the expression levels of foreign genes under the control of the polyhedrin promoter and inserted as a DNA cassette into the baculovirus genome by transposition are similar to or only slightly less than those observed for recombinant viruses generated by homologous recombination in insect cells and purified by traditional methods. Modifications to the transcriptional cassette in the donor plasmid or alteration of the position of the target site in the shuttle vector may provide enhanced expression levels for foreign genes within the mini-Tn*7* element compared with the initial results reported in this study.

This method of generating recombinant baculoviruses does not suffer from some of the disadvantages of the yeast shuttle vector system (44) or from those inherent in the in vitro site-specific recombination method mediated by bacteriophage P1 enzymes (45). Although the yield of DNA is not high, it is relatively easy to isolate pure bacmid DNA from *E. coli* over resin columns for use in transfections and to analyze its structure by restriction analysis or by DNA amplification by PCR techniques, compared with the more tedious methods involving sucrose gradients to purify shuttle vector DNAs from yeast cells. Our method may also be more suited for the generation of cDNA libraries in baculovirus vectors for use in expression cloning because of the higher transformation efficiency possible with *E. coli* ($>10^9$ transformants per μg of DNA) than with yeast (up to 5×10^5 transformants per μg of DNA) (4). Although the total number of recombinant viruses that can be generated by the P1 Cre-*lox* system may be high (up to 5×10^7 recombinant plaques per μg of ploxZ transfer vector DNA), no more than 50% of the viral progeny contain an inserted plasmid. Careful standardization of the in vitro reaction conditions (particularly the amounts of Cre enzyme, transfer vector DNA, and viral DNA and the incubation time) is also required to maximize single insertion events and prevent multiple tandem plasmid insertions as the result of sequential Cre-mediated recombination events, which may adversely affect the total number of desired progeny. This is not a problem for the shuttle vector system described here, as transposition immunity, conferred by the ends of Tn*7*, inhibits secondary insertions into the target plasmid (10) and will thereby prevent multiple insertions of the mini-Tn*7* elements from the donor plasmid into the target bacmid.

We have not carried out systematic studies to examine the structural integrity and segregation properties of the baculovirus shuttle vectors after many generations of growth in *E. coli*. Recent studies, however, have demonstrated that 300-kb fragments of human DNA cloned into mini-F-based plasmids propagated in *E. coli* are stably maintained for over 100 generations (25, 48). Structural instability of the bacmids propagated in *E. coli* does not appear to be a significant problem during the relatively few generations needed to perform the transposition experiments and to outgrow the cultures used for the isolation of composite bacmid DNAs. We have not examined the structure of the genomic viral DNA isolated from insect cells infected with composite bacmids.

Several modifications to the system described here may further improve the speed of identifying composite shuttle vectors and enhance the levels of heterologous gene expression. These modifications include altering the selection scheme through the use of different selectable or scorable markers and the use of different promoters to drive expression of the heterologous gene located within the mini-Tn*7* cassette on the donor plasmid. Modifications to the bacterial host strain, the donor plasmid, or the helper plasmid may also increase the efficiency of the system by eliminating the helper functions after transposition has occurred.

This approach not only greatly facilitates the use of baculovirus vectors for the expression of cloned foreign genes but also permits the development of new strategies for rapid protein engineering of eukaryotic proteins and expression cloning of previously uncharacterized genes from cDNA libraries. Similar genetic approaches can also be used to aid in the construction of other large plasmid- and mammalian virus-based expression vectors.

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REFERENCES

1. **Alting-Mees, M. A., and J. M. Short.** 1989. pBluescript II: gene mapping vectors. Nucleic Acids Res. **17**:9494.
2. **Barry, G. F.** 1986. Permanent insertion of foreign genes into the chromosomes of soil bacteria. Bio/Technology **4**:446–449.
3. **Barry, G. F.** 1988. A broad host-range shuttle system for gene insertion into the chromosomes of Gram-negative bacteria. Gene **71**:75–84.
4. **Becker, D. M., and L. Guarente.** 1991. High-efficiency transformation of yeast by electroporation. Methods Enzymol. **194**:182–187.
5. **Berg, C. M., D. E. Berg, and E. A. Groisman.** 1989. Transposable elements and the genetic engineering of bacteria, p. 879–925. *In* D. E. Berg and M. M. Howe (ed.), Mobile DNA. American Society for Microbiology, Washington, D.C.
6. **Brosius, J.** 1989. Superpolylinkers in cloning and expression vectors. DNA **8**:759–777.
7. **Capone, J.** 1989. Screening recombinant baculovirus plaques *in situ* with antibody probes. Gene Anal. Tech. **6**:62–66.
8. **Chung, C. T., S. L. Niemela, and R. H. Miller.** 1989. One-step preparation of competent *Escherichia coli*: transformation and storage of bacterial cells in the same solution. Proc. Natl. Acad. Sci. USA **86**:2172–2175.
9. **Colleaux, L., L. d'Auriol, F. Galibert, and B. Dujon.** 1988. Recognition and cleavage site of the intron-encoded *omega* transposase. Proc. Natl. Acad. Sci. USA **85**:6022–6026.
10. **Craig, N. L.** 1989. Transposon Tn*7*, p. 211–225. *In* D. E. Berg and M. M. Howe (ed.), Mobile DNA. American Society for Microbiology, Washington, D.C.
11. **Dayringer, H. E., and S. A. Sammons.** 1991. POLLUX: a program for simulated cloning, mutagenesis and database searching of DNA constructs. CABIOS **7**:161–167.
12. **Dente, L., G. Cesareni, and R. Cortese.** 1983. pEMBL: a new family of single-stranded plasmids. Nucleic Acids Res. **11**:1645.
13. **Duronio, R. J., S. I. Reed, and J. I. Gordon.** 1992. Mutations of human myristoyl-CoA:protein N-myristoyltransferase cause temperature-sensitive myristic acid auxotrophy in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **89**:4129–4133.
14. **Farmer, J. L., R. G. Hampton, and E. Boots.** 1989. Flow cytometric assays for monitoring production of recombinant HIV-1 gp160 in insect cells infected with a baculovirus expression vector. J. Virol. Methods **26**:279–290.
15. **Fung, M. C., K. Y. M. Chiu, T. Weber, T. W. Chang, and N. T. Chang.** 1988. Detection and purification of a recombinant human B lymphotropic virus hhv-6 in the baculovirus expression system by limiting dilution and DNA dot-blot hybridization. J. Virol. Methods **19**:33–42.
16. **Funk, C. D., O. Rådmark, J. Y. Fu, T. Matsumoto, H. Jörnvall, T. Shimizu, and B. Samuelsson.** 1987. Molecular cloning and amino acid sequence of leukotriene A_4 hydrolase. Proc. Natl. Acad. Sci. USA **84**:6677–6681.
17. **Gierse, J. K., V. A. Luckow, L. J. Askonas, K. L. Duffin, S. Aykent, G. S. Bild, C. P. Rodi, P. M. Sullivan, M. K. Bourner, N. M. Kimack, and G. G. Krivi.** High-level expression and purification of human leukotriene A_4 hydrolase from insect cells infected with a baculovirus vector. Protein Expression Purification, in press.
18. **Grant, S. G. N., J. Jessee, F. R. Bloom, and D. Hanahan.** 1990. Differential plasmid rescue from transgenic mouse DNAs into *Escherichia coli* methylation-restriction mutants. Proc. Natl. Acad. Sci. USA **87**:4645–4669.
19. **Hartig, P. C., and M. C. Cardon.** 1992. Rapid efficient production of baculovirus expression vectors. J. Virol. Methods **38**: 61–70.

19a. **Hippenmeyer, P.** Unpublished data.

20. **Holloway, B., and K. B. Low.** 1987. F-prime and R-prime factors, p. 1145–1153. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
21. **Holmes, D. S., and M. Quigley.** 1981. A rapid method for the preparation of bacterial plasmids. Anal. Biochem. **14**:193–197.
22. **Hosoda, F., S. Nishimura, H. Uchida, and M. Ohki.** 1990. An F factor based cloning system for large DNA fragments. Nucleic Acids Res. **18**:3863–3869.
23. **Jefferson, R. A., S. M. Burgess, and D. Hirsh.** 1986. β-Glucuronidase from *Escherichia coli* as a gene fusion marker. Proc. Natl. Acad. Sci. USA **86**:8447–8451.
24. **Jessee, J., and K. Blodgett.** 1988. MAX Efficiency *E. coli* DH5αF′IQ^{TM}: a new M13 and phagemid host. Focus **10**:69.
25. **Kim, U.-J., H. Shizuya, P. J. de Jong, B. Birren, and M. I. Simon.** 1992. Stable propagation of cosmid sized human DNA inserts in an F factor based vector. Nucleic Acids Res. **20**:1083–1085.
26. **King, L. A., and R. D. Possee.** 1992. The baculovirus expression system: a laboratory guide, p. 1–229. Chapman & Hall, London.
27. **Kitts, P. A.** Production of recombinant baculoviruses using linearized viral DNA. *In* C. Richardson (ed.), Baculovirus expression protocols, methods in molecular biology, in press. Humana Press, Clifton, N.J.
28. **Kitts, P. A., and R. D. Possee.** 1993. A method for producing recombinant baculovirus vectors at high frequency. BioTechniques **14**:810–817.
29. **Kitts, P. A., M. D. Ayres, and R. D. Possee.** 1990. Linearization of baculovirus DNA enhances the recovery of recombinant virus expression vectors. Nucleic Acids Res. **18**:5667–5672.
30. **Kline, B.** 1985. A review of mini-F plasmid maintenance. Plasmid **14**:1–16.
31. **Leonardo, E. D., and J. M. Sedivy.** 1990. A new vector for cloning large eukaryotic DNA segments in *Escherichia coli*. Bio/Technology **8**:841–844.
32. **Lichtenstein, C., and S. Brenner.** 1982. Unique insertion site of Tn*7* in the *E. coli* chromosome. Nature (London) **297**:601–603.
33. **Luckow, V. A.** 1991. Cloning and expression of heterologous genes in insect cells with baculovirus vectors, p. 97–152. *In* A. Prokop, R. K. Bajpai, and C. Ho (ed.), Recombinant DNA technology and applications. McGraw-Hill, New York.
34. **Luckow, V. A., and M. D. Summers.** 1988. Signals important for high-level expression of foreign genes in *Autographa californica* nuclear polyhedrosis virus expression vectors. Virology **167**:56–71.
35. **Luckow, V. A., and M. D. Summers.** 1988. Trends in the development of baculovirus expression vectors. Bio/Technology **6**:47–55.
36. **Luckow, V. A., and M. D. Summers.** 1989. High level expression of nonfused foreign genes with *Autographa californica* nuclear polyhedrosis virus expression vector. Virology **170**:31–39.
37. **Maeda, S.** 1989. Expression of foreign genes in insects using baculovirus vectors. Annu. Rev. Entomol. **34**:351–372.
38. **Malitschek, B., and M. Schartl.** 1991. Rapid identification of recombinant baculoviruses using PCR. BioTechniques **11**:177–178.
39. **Miller, L. K.** 1988. Baculoviruses as gene expression vectors. Annu. Rev. Microbiol. **42**:177–199.
40. **Minami, M., S. Ohno, H. Kawasaki, O. Rådmark, B. Samuelsson, H. Jörnvall, T. Shimizu, Y. Seyama, and K. Suzuki.** 1987. Molecular cloning of a cDNA coding for human leukotriene A_4 hydrolase. Complete primary structure of an enzyme involved in eicosanoid synthesis. J. Biol. Chem. **262**:13873–13876.
41. **Murhammer, D. W.** 1991. The use of insect cell cultures for recombinant protein synthesis: engineering aspects. Appl. Biochem. Biotechnol. **31**:283–310.
42. **Oka, A., H. Sugisaki, and M. Takanami.** 1981. Nucleotide sequence of the kanamycin resistance transposon Tn*903*. J. Mol. Biol. **147**:217–226.
43. **O'Reilly, D. R., L. K. Miller, and V. A. Luckow.** 1992. Baculovirus expression vectors: a laboratory manual, p. 1–347. W. H. Freeman and Company, New York.
44. **Patel, G., K. Nasmyth, and N. Jones.** 1992. A new method for the isolation of recombinant baculovirus. Nucleic Acids Res. **20**:97–104.
45. **Peakman, T. C., R. A. Harris, and D. R. Gewert.** 1992. Highly

efficient generation of recombinant baculoviruses by enzymatically mediated site-specific *in vitro* recombination. Nucleic Acids Res. **20**:495–500.

46. **Sambrook, J., E. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
47. **Sewall, A., and N. Srivastava.** 1991. Linear viral DNA improves baculovirus transfections. The Digest **4**:1.
48. **Shizuya, H., B. Birren, U.-J. Kim, V. Mancino, T. Slepak, Y. Tachiiri, and M. Simon.** 1992. Cloning and stable maintenance of 300-kilobase-pair fragments of human DNA in *Escherichia coli* using an F-factor-based vector. Proc. Natl. Acad. Sci. USA **89**:8794–8797.
49. **Smith, G. E., and M. D. Summers.** 1979. Restriction maps of five *Autographa californica* nuclear polyhedrosis virus (*M*NPV) variants, *Trichoplusia ni* MNPV and *Galleria mellonella* MNPV DNAs with endonucleases *Sma*I, *Kpn*I, *Bam*HI, *Sac*I, *Xho*I, and *Eco*RI. J. Virol. **30**:828–838.
50. **Summers, M. D., and G. E. Smith.** 1987. A manual of methods for baculovirus vectors and insect cell culture procedures. Texas Agric. Exp. Station Bull. **1555**:1–57.
51. **Taylor, L., and R. E. Rose.** 1988. A correction in the nucleotide sequence of the Tn*903* kanamycin resistance determinant in pUC4K. Nucleic Acids Res. **16**:358.
52. **Vaughn, J. L., R. H. Goodwin, G. J. Tompkins, and P. McCawley.** 1977. The establishment of two cell lines from the insect *Spodoptera frugiperda* (Lepidoptera: Noctuidae). In Vitro **13**: 213–217.
53. **Vialard, J., M. Lalumière, T. Vernet, D. Briedis, G. Alkhatib, D. Henning, D. Levin, and C. Richardson.** 1990. Synthesis of the membrane fusion and hemagglutinin proteins of measles virus, using a novel baculovirus vector containing the β-galactosidase gene. J. Virol. **64**:37–50.
54. **Vieira, J., and J. Messing.** 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19**:259–268.
55. **Webb, A., M. Bradley, S. Phelan, J. Wu, and L. Gehrke.** 1991. Use of the polymerase chain reaction for screening and evaluation of recombinant baculovirus clones. BioTechniques **11**: 512–519.
56. **Wohlleben, W., W. Arnold, L. Bissonnette, A. Pelletier, A. Tanguay, P. H. Roy, G. C. Gamboa, G. F. Barry, E. Aubert, J. Davies, and S. A. Kagan.** 1989. On the evolution of Tn*21*-like multiresistance transposons: sequence analysis of the gene (*aacC1*) for gentamicin acetyltransferase-3-I (AAC(3)-I), another member of the Tn*21*-based expression cassette. Mol. Gen. Genet. **217**:202–208.
57. **Zuidema, D., A. Schouten, M. Usmany, A. J. Maule, G. J. Belsham, J. Roosien, E. C. Klinge-Roode, J. W. M. van Lent, and J. M. Vlak.** 1990. Expression of cauliflower mosaic virus gene I in insect cells using a novel polyhedrin-based baculovirus expression vector. J. Gen. Virol. **71**:2201–2209.