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Assay for Movement of Lepidopteran Transposon IFP2 in Insect Cells Using a Baculovirus Genome as a Target DNA

MALCOLM J. FRASER,[1] LYNNE CARY,[2] KITIMA BOONVISUDHI, and HWEI-GENE HEIDI WANG[3]

Department of Biological Sciences, The University of Notre Dame, Notre Dame, Indiana 46556

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Mutagenesis of baculoviruses by host mobile elements occurs spontaneously and frequently during propagation of the viruses in Lepidopteran cell cultures. Most of the transposons identified as insertions in baculovirus genomes are relatively small Class II elements that exhibit a remarkable specificity for TTAA target sites. We have developed a transposition assay to analyze the movement of these TTAA-specific Lepidopteran transposons using the baculovirus genome as a target and a lacZ gene under control of the polyhedrin gene promoter as a selective marker for the transposon. This assay provides the first demonstration that a Lepidopteran transposon is capable of transposing while carrying a marker gene in insect cells. The data generated from this assay provide strong evidence that IFP2 encodes a protein that facilitates its own movement. This element may be used in a manner analogous to the P-element to mobilize genes in at least some Lepidopteran insect cells. Transposon tagging within the baculovirus genome identified several known genes and two previously undescribed open reading frames as nonessential to *in vitro* replication of the virus.

INTRODUCTION

Baculoviruses have dynamic genomes that are capable of rearrangements and recombination during propagation in their hosts. Recombination events, both homologous and illegitimate, that insert foreign DNA into the baculovirus genome are easily demonstrated during the generation of recombinants for foreign gene expression or upon cointroduction of plasmids containing nonhomologous DNAs into cultured insect cells (Smith *et al.*, 1983a,b; Pennock *et al.*, 1984; Xiong *et al.*, 1991). Nonhomologous recombination with sequences originating from the host genome occurs under normal propagation conditions as well (Miller and Miller, 1982; Fraser *et al.*, 1983; Cary *et al.*, 1989; Kumar and Miller, 1987; Beames and Summers, 1987).

Aside from the occasional phenotypic alterations that these recombination events impart to the virus, the virus might derive some evolutionary advantages from such genetic scavenging of host sequences (Blissard and Rohrmann, 1990). One example of an apparent host-derived gene that confers an advantage to the virus may be the viral-encoded ecdysteroid UDP-glucosyltransferase gene (O'Reilly and Miller, 1989; O'Reilly *et al.*, 1992) which prolongs the susceptible state of the host thus increasing the virus yield.

Transposon mutagenesis of baculoviruses is a well-defined subset of the several kinds of recombination events possible during propagation of these viruses in insect cells, and it is the most frequently documented recombination event observed following serial propagation of the virus in insect cell cultures (Fraser *et al.*, 1983, 1985; Cary *et al.*, 1989; Kumar and Miller, 1987; Wang and Fraser, 1993). Inserted host sequences resembling transposons have also been identified as a result of sequence variations between virus strains (Schetter *et al.*, 1990). The insertion of transposons into baculovirus genomes can occur during larval propagation of the virus as well (Jehle *et al.*, 1995). The potential involvement of transposon mutagenesis in the evolution of baculoviruses, or possibly even their hosts, is only beginning to be appreciated (Miller and Miller, 1982; Fraser, 1986; Blissard and Rohrmann, 1990; Kidwell, 1993; McDonald, 1993).

We have been studying the Lepidopteran transposon IFP2, which is repeatedly isolated as an insertion within the baculovirus genome following maintenance of the virus in the *Trichoplusia ni* TN-368 cell line (Fraser *et al.*, 1983; Cary *et al.*, 1989). The IFP2 element is 2.7 kb in length and is bounded by 13-bp inverted terminal repeats, with additional internal 19-bp inverted repeats located asymmetrically with respect to the ends. The element is dispersed in the genome of the TN-368 cell line and has been identified in one of two other independently established cell lines derived from *T. ni* (Cary *et al.*, 1989). Five independent mutant baculoviruses having IFP2 element insertions within the 25K gene have been characterized (Fraser *et al.*, 1983; Cary *et al.*, 1989), each of which duplicates a tetranucleotide target site, TTAA (Cary *et al.*, 1989).

[1] To whom reprint requests should be addressed.

[2] Present address: American Biogenetic Sciences, Inc., Notre Dame, IN 46556.

[3] Present address: Bristol-Meyers Squibb, Pharmaceutical Research Institute, 5 Research Parkway, P.O. Box 5100, Wallingford, CT 06492-7660.

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The IFP2 transposon sequence contains a consensus RNA polymerase II promoter region and a polyadenylation signal (Cary *et al.,* 1989) flanking a single major overlapping open reading frame (Elick *et al.,* submitted for publication), and encodes a single transcript of approximately 2.1 kb in length (Elick *et al.,* submitted for publication). Primer extension analysis with polyadenylated mRNAs from TN-368 cells harboring the IFP2 element positions the 5′ end of the transcripts near the identified consensus promoter region (Cary *et al.,* 1989). The IFP2 sequence is unique. As a consequence we are unable to determine whether or not transposition-associated functions are encoded by the element from simple sequence comparisons with the GenBank database.

The specificity for inserting at TTAA target sites is a property this element shares with several other Lepidopteran transposon-like insertions identified in baculovirus genomes (Fraser *et al.,* 1985; Wang *et al.,* 1989; Wang and Fraser, 1993; Schetter *et al.,* 1990; Beames and Summers, 1990; Carstens, 1987). All of these elements terminate with two (Carstens, 1987; Schetter *et al.,* 1990; Beames and Summers, 1990) or three C residues (Fraser *et al.,* 1985; Cary *et al.,* 1989; Wang and Fraser, 1993) at the 5′ ends of their inverted repeats, while the sequences of the inverted repeats and the internal domains differ substantially.

The common features of target site specificity and terminal C residues likely reflect a similar mechanism of transposition operating among these different transposable sequences. The TTAA target site specificity is evidenced among some characterized representatives of these elements upon insertion within the uninfected cell genome as well (Beames and Summers, 1990; Wang and Fraser, 1993). In the case of the TTAA-specific TFP3 element, PCR analyses demonstrated that movement to a particular site in the cellular genome resulted in the duplication of a TTAA target site, establishing that the transposition mechanism for these elements is not viral encoded (Wang and Fraser, 1993).

This report presents the first experimental assay of transposition for TTAA-specific Lepidopteran elements from plasmid clones into the baculovirus genome. Using this assay we have confirmed that IFP2 elements always insert with specificity for TTAA target sites during transposition, regardless of their ultimate position in the virus genome. The data provide strong evidence for the involvement of IFP2 in transactivating the movement of genetically tagged copies of itself from plasmids into the baculovirus genome and demonstrate for the first time the potential utility of these TTAA-specific transposons for genetic engineering in insects. The assay provides an experimental basis for examining the molecular aspects of IFP2 mobilization. This assay has demonstrated the potential utility of transposon tagging to assist in the identification and confirmation of nonessential baculovirus genes.

MATERIALS AND METHODS

Cell lines and virus

The IFP2-deficient insect cell line IPLB-SF21 AE (SF21AE; Vaughn *et al.,* 1977; Cary *et al.,* 1989) was maintained in TNM-FH medium supplemented with 8% fetal bovine serum and antibiotics as previously described (Fraser *et al.,* 1983; Cary *et al.,* 1989). The *Autographa californica* NPV strain E2 (AcMNPV; Smith and Summers, 1979) was used as a target genome for these analyses.

Virus DNA was purified from occlusion bodies (OBs) isolated from infected *T. ni* larvae by a modification of a previously described method (Corsaro and Fraser, 1988). Briefly, OBs were purified from infected *T. ni* larvae through sucrose gradients, washed and pelleted, and resuspended in 2× volumes of guanidinium isothiocyanate buffer (Corsaro and Fraser, 1988). The released DNA was fractionated in a step gradient composed of a 3-ml layer of 5.7 *M* CsCl in TE buffer, 2.5 ml of the OB lysate containing 1 g of CsCl and 300 μl of ethidium bromide, and a 3-ml layer of the guanidinium isothiocyanate buffer and was centrifuged at 100,000 *g* in an SW40 rotor for 24 hr. The single band of nucleic acid that formed just below the interface of the CsCl and the lysate layers was harvested and overlaid onto 3 ml of 50% (w/v) CsCl in TE buffer and centrifuged at 100,000 *g* for 12 hr. Open circular and supercoiled forms were harvested separately and dialyzed as described (Corsaro and Fraser, 1988) prior to use in transfections.

Cloning and preparation of lacZ-tagged IFP2 constructs

A series of lacZ-tagged IFP2 elements were prepared from the pGmFP2 clone (Fraser *et al.,* 1983; Cary *et al.,* 1989; Fig. 1). The polyhedrin/lacZ fusion gene (polh/lacZ) was constructed in the plasmid pD-2/β-gal obtained from Dr. Jim McLinden (American Biogenetic Sciences, Inc.). The lacZ ORF of pMC1871 (Pharmacia) was excised as a *Bam*HI cartridge, blunt ended with Klenow, and inserted into a *Sma*I site that was part of a multiple cloning site linker that replaced the polyhedrin sequence from −8 to the *Kpn*I site at +630 nt. A unique *Nru*I site was engineered at nucleotide −146 relative to the start of translation in the polyhedrin sequence. The entire promoter region and polh/lacZ fusion gene was excised using the engineered *Nru*I site and the natural *Dra*I site (+907) and was cloned into the *Hpa*I site of the pGmFP2 clone (Cary *et al.,* 1989; Fig. 1). Two separate constructs were prepared for each orientation of the polh/lacZ cartridge relative to the direction of IFP2 transcription. These were designated pLCND 1.1 and 4.1 for the negative orientation and pLCND 1.2 and 4.2 for the positive orientation.

Transfection of insect cells with plasmid and viral DNAs

In our initial trials of the transposition assay we isolated each recombinant from an individual cotransfection

of SF21AE cells. Each cotransfection was carried out in 60 × 15-mm plastic petri plates seeded with 3.5×10^6 cells using the standard $CaPO_4$ technique previously described (Graham and Van der Eb, 1973; Summers and Smith, 1987; Corsaro and Fraser, 1988). For each cotransfection we used 1 μg of open circular viral DNA and 10 μg each of supercoiled pLCND 1.1 and the helper p3E1.2 plasmid DNAs (Fig. 1). The control transfection employed 1 μg of viral DNA and 20 μg of pLCND 1.1.

The supernatants containing both recombinant and wild-type virus were harvested after 7 days. Plaque assays were performed as previously described (Fraser, 1982) with the addition of 50 μl/100 ml of stock X-gal solution (50 mg/ml in dimethylformamide; Promega) to the overlay medium (Pennock *et al.*, 1984). Individual plaques were picked using a 200-μl micropipettor, and agarose plugs were expelled into 1 ml of medium. Three serial plaque assays utilizing 10^{-1} or 10^{-2} dilutions of picked plaque preparations were used to purify each recombinant virus.

We later modified the recombinant generation and selection procedure by first cotransfecting 3×10^6 cells with viral and plasmid DNAs in 60 × 15-mm petri plates and, following the 8-hr exposure to the precipitate, the cells were partitioned equally among wells of a 24-well plate. Addition of 5 μl of X-gal (see above) to the cell culture medium at 5 days posttransfection allowed determination of those cultures which harbored recombinant viruses. Plaque assays were then performed as above to purify recombinants from each culture well.

Detection, purification, and amplification of recombinant viruses

Plaque-purified lacZ-positive viruses were initially amplified in 24-well plate cultures for preparation of extracellular virus DNA by adding 100 μl of a 1-ml plaque pick solution to 2.5×10^5 cells per well. Subsequent amplifications were performed in 6-well plate cultures by seeding each well with 3.5×10^6 cells in a total volume of 3 ml of complete TNM-FH medium and inoculating 100 μl of a 1-ml plaque-picked virus preparation. Following a 5-day incubation the infected cell culture supernatants were clarified by centrifugation at 1000 rpm for 5 min in a table-top centrifuge, and extracellular virus was pelleted from infectious supernatants by centrifugation at 15,000 *g* in an Eppendorf microfuge for 15 min. Viral DNA was extracted from the pelleted virus using standard phenol and chloroform–isoamyl alcohol extraction procedures (Sambrook *et al.*, 1989). The extracted viral DNA was ethanol precipitated and resuspended in 100 μl of distilled water.

pBR322 libraries and selection for lacZ

A *Hin*dIII-cleaved, alkaline phosphatase-treated pBR322 plasmid (New England Biolabs) was used for cloning libraries of *Hin*dIII fragments of the recombinant virus genomes into DH5α *Escherichia coli* cells (Gibco/BRL). The polh/lacZ marker gene is active enough in bacterial cells to allow detection of the lacZ-positive plasmid-transformed colonies on ampicillin plates overlaid with 40 μl of 2% X-gal. Plasmid DNAs were isolated using the rapid boiling procedure (Holmes and Quigly, 1981) and analyzed by digesting with *Eco*RV and separating fragments on 1.5% agarose gels. Positive clones were amplified and purified in CsCl gradients for double-stranded dideoxy sequence analysis as previously described (Sanger *et al.*, 1977; Wang and Fraser, 1993; Ma *et al.*, 1993).

Polymerase chain reaction

We employed an inverse polymerase chain reaction (Ochman *et al.*, 1988; Wang and Fraser, 1993) for the specific amplification of IFP2 terminal fragments using the left and right terminal-specific primer pairs indicated in Fig. 3 and the purified extracellular virus DNA. Two separate inverse PCR strategies were performed to specifically amplify either end of the inserted IFP2 element. For the left end, we chose to digest the viral genome with *Taq*I, ligate, and amplify using the primer combination JF-2 and MF01 (Fig. 3A). For the right end we chose *Sau*3A digests, and amplified the ligated circles with the primers JF-1 and MF04 (Fig. 3B). The conditions for renaturation during the amplification reactions were optimized for each of the primer combinations used. Bands generated with each primer combination were cloned using the TA cloning kit and the plasmid pCRII (Invitrogen) and, if warranted, sequenced as above. Double-stranded sequencing was carried out with either JF-1 or JF-2 (Fig. 3) using protocols and conditions as previously described (Wang and Fraser, 1993; Ma *et al.*, 1993).

RESULTS

Construction of polh/lacZ-tagged IFP2 transposons

We constructed a polh/lacZ-tagged IFP2 transposon for our analyses using the previously cloned and sequenced element of pGmFP2 (Fraser *et al.*, 1983; Cary *et al.*, 1989). This element has 89 bp of flanking viral DNA from the 25K gene on the left end and 459 bp of 25K sequence on the right end. These flanking viral sequences resulted in a significant number of background homologous recombination events in our assays.

We utilized a polh/lacZ fusion gene that had been engineered to contain a unique *Nru*I site at the 5′ end which allowed excision of the *Nru*I to *Dra*I fragment and insertion of this cassette into the unique *Hpa*I site of the IFP2 element of pGmFP2 (Fig. 1). In preparing these constructs we observed that plasmids with the inserted polh/lacZ gene generated blue colonies in the presence of X-gal. This facilitated selection of the appropriate clones. Two constructs were selected for each orientation of the polh/lacZ gene in the IFP2 element. The constructs were

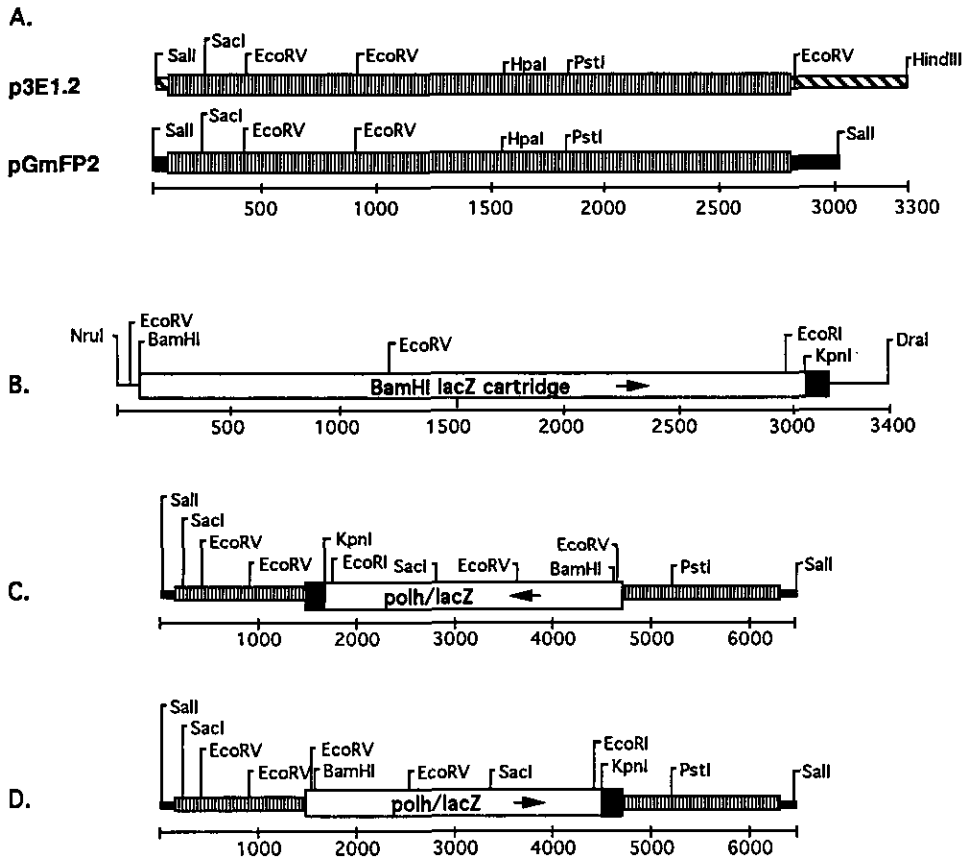

FIG. 1. Plasmid constructs prepared for this study. (A) The p3E1.2 IFP2 element (vertically striped box) was previously cloned into the plasmid pUC18 as a *SalI/HindIII* fragment comprising the amino-terminal region of the 25K gene from the AcMNPV FP mutant 3E1 (Cary *et al.,* 1989). The pGmFP2 IFP2 element (vertically striped box) was previously cloned into pUC8 as a *SalI* fragment that originated from the carboxy-terminal region of the 25K gene of GmMNPV FP mutant GmFP2 (Fraser *et al.,* 1983). Both plasmids contain flanking viral sequences from the FP locus of their respective viruses. The physical maps of both IFP2 elements are identical, and the unique *HpaI* site used for cloning is in the middle of the coding domain of the transposon. (B) The polh/lacZ gene fusion was prepared by inserting a blunt-ended *Bam*HI cartridge from pMC1871 into a *Sma*I site constructed as part of a multiple cloning site linker replacing −8 to +630 of the polyhedrin sequence. This lacZ gene fusion was altered by engineering a unique *NruI* site at −146 relative to the translational start codon, and the construct was designated as pD-2/Beta-gal. The polh/lacZ cartridge used for tagging the IFP2 element was excised as a blunt-ended *NruI/DraI* fragment for insertion at the unique *HpaI* blunt-end cloning site within the IFP2 coding domain. (C) Physical map of the pLCND 1.1 and 4.1 constructs. These constructs were prepared with the polh/lacZ transcriptional alignment in the opposite direction with respect to the IFP2 ORF. (D) Physical map of the pLCND 1.2 and 4.2 constructs. The polh/lacZ gene was oriented within pGmFP2 so that transcription proceeded in the same direction as the IFP2 ORF.

designated pLCND 1.1 and 4.1 if the lacZ and IFP2 open reading frames were in the opposite direction and pLCND 1.2 and 4.2 if they were in the same orientation (Fig. 1).

Cotransfection and selection of lacZ-positive recombinant virus

Our first experiments utilized the pLCND 1.1 construct in individual cotransfections of SF21AE cell cultures with AcMNPV genomic DNA. In the experimental cotransfections we added an equivalent amount of both pLCND 1.1 and the helper plasmid p3E1.2. The control transfections employed twice the amount of pLCND 1.1 to negate the possibility of a stabilizing carrier effect for the added p3E1.2 plasmid in the experimental cotransfections. A single recombinant was purified from each cotransfection. Twenty-five recombinant blue viruses (B1–B25) were purified and analyzed from these initial assays.

An alternative protocol was devised in an attempt to speed the identification and characterization of additional transposition events. Similar cotransfections with viral and plasmid DNAs were performed using each of the pLCND constructs and the p3E1.2 helper plasmid, and then the cells were partitioned among individual wells in 24-well plates. After 3 days we added X-gal to the culture media to score wells for lacZ-positive recombinants. A 2-fold concentration of the pLCND 1.1 plasmid alone was used for the control cotransfections.

During the plaque purifications of the blue virus we noted many were unstable recombinants that could not be purified to homogeneity. This was true in both the control cotransfections and in the cotransfections with the helper plasmid p3E1.2. In all cases the unstable recombinants could not be fully characterized and were eliminated from further analysis.

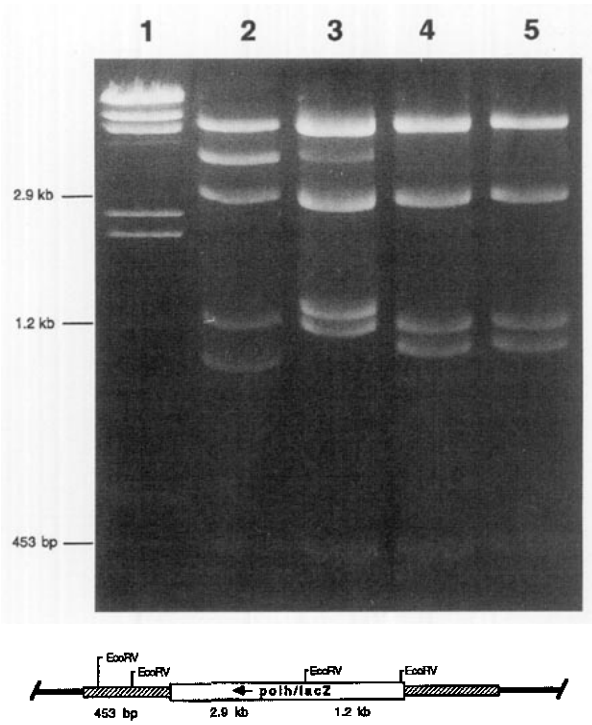

FIG. 2. Restriction endonuclease analysis of lacZ-positive pBR322 clones obtained from recombinant virus libraries. *Eco*RV restriction digests of pBR322 *Hin*dIII fragment clones from recombinant viruses generated in the transposition assay. Lane 1, λ *Hin*dIII digest standards. Lanes 2–5, plasmid clones from recombinant viruses B1 (lane 2), B12 (lane 3), and B17 (lanes 4 and 5) that exhibit the characteristic banding pattern diagnostic for insertion of the entire IFP2 and the polh/lacZ marker. The 453-bp fragment representing the left end of the IFP2/polh/lacZ construct and the 1.2- and 2.9-kb fragments that are diagnostic for the polh/lacZ gene (see map below gel) are clearly present in all these clones. Subsequent sequencing analysis confirmed that while the polh/lacZ-specific *Eco*RV fragments were present in nearly every clone that yielded blue colonies, the 453-bp IFP2-specific *Eco*RV fragment was diagnostic for insertion of the entire transposon.

Transposition events screened from plasmid libraries of purified recombinant viruses

Stable recombinant viruses were amplified by culture in SF21 cells for subsequent analysis by cloning and sequencing. *Hin*dIII or *Sal*I libraries of purified recombinant viral DNAs were randomly cloned into pBR322 and transformed into bacteria. These enzymes were chosen for generating plasmid libraries because neither enzyme site was present within either the IFP2 element or the polh/lacZ gene fusion.

Transformed *E. coli* were plated in the presence of X-gal to allow detection of the polh/lacZ fusion gene. The plasmids from lacZ-positive colonies were further screened by digestion with *Eco*RV (Fig. 2) which generated diagnostic fragments that identified the presence of the lacZ gene (1.2 kb) and the left end of the IFP2 transposon (450 bp). This screening eliminated those recombinants that did not result from insertion of the entire IFP2 element and therefore could not be transposition events. However, this analysis did not distinguish homologous recombination events at the 25K gene.

Dideoxy sequencing was performed on the *Eco*RV-positive clones using the end-specific primers JF-1 and JF-2 (Fig. 3). These primers initiated chain polymerization from within the ends of the IFP2 element toward their junctions with the viral DNA, allowing discrimination of transposition events as opposed to homologous recombination or illegitimate recombination events. Several recombinants, B1, B17, B19, and CHb3, were analyzed successfully using this plasmid library screening protocol (Fig. 4).

Transposition events screened by PCR amplification of IFP2 insertion sites within the viral genome

The pBR322 libraries did not permit a complete analysis of all of the blue virus recombinants isolated, especially those from the control transfections with twice the amount of pLCND 1.1 construct alone. We were also concerned that some transposition events might have been missed among those purified recombinants from which no blue plasmids were recovered in the *Hin*dIII or *Sal*I pBR322 library constructions.

An inverse PCR strategy was developed to allow full characterization of all purified lacZ-positive recombinant viruses (Fig. 3). Amplified fragments of either *Taq*I or *Sau*3A inverse PCR products were cloned and sequenced using the JF-1 or JF-2 primers in combination with other end-specific primers (Fig. 3). The results of much of the PCR analysis (Table 1) reflected a conservation of the *Taq*I end in nearly all of the lacZ-positive recombinants, while the *Sau*3A end was significantly absent from recombinants that resulted from illegitimate recombination events. These results reflected those of the pBR322 library cloning in that the 450-bp *Eco*RV fragment containing the leftmost region of IFP2 was diagnostic for an intact IFP2 element and a transposition event. The amplified end-specific fragments were cloned using the pCRII vector (see Materials and Methods), and dideoxy sequencing analysis was performed using the primers JF-1 or JF-2.

Twenty-six stable control recombinants were plaque purified from three separate cotransfections of pLCND 1.1 alone. We were able to fully characterize 20 of these control recombinants. Hybridization and sequencing analyses demonstrated that 9 of these recombinants resulted from apparent homologous recombination events that positioned the lacZ-tagged IFP2 element back at the GmFP2 TTAA target site within the FP locus. The other 11 control recombinants apparently resulted from illegitimate recombination events since they did not yield both *Taq*I and *Sau*3A ends in the inverse PCR analysis (Table 1).

In contrast, a total of 37 plaque-purified recombinants were obtained from four separate cotransfections in the experimentals. We were able to confirm 4 transposition events from a total of 11 recombinants finally sequenced. To test the statistical significance of these data we performed a χ^2 analysis by taking the pooled data from the

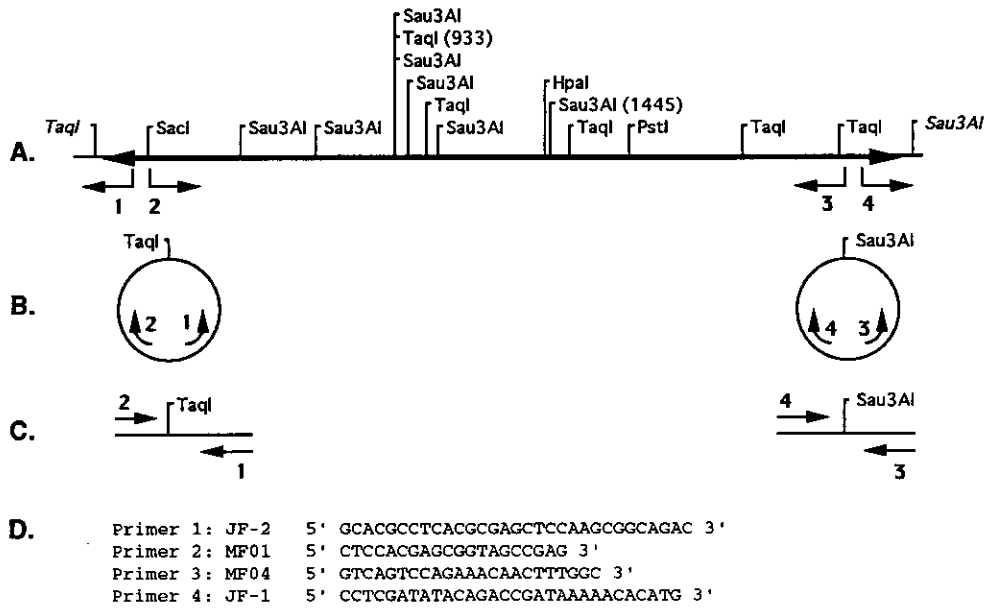

FIG. 3. PCR strategy for identifying IFP2 transposition events. (A) Map of IFP2 (thick line bounded by arrows representing terminal inverted repeats) showing the position of *Sau*3AI (1445) and *Taq*I (933) sites used for preparation of end-specific circles that encompassed the joining sequences between the inserted IFP2 element and the viral genome. The italicized *Sau*3AI and *Taq*I sites represent sites in the flanking viral sequences. Primer pairs (1 and 2 or 3 and 4) were designed to be specific for each terminus of the element. The sequences of each primer are presented in D. (B) *Taq*I or *Sau*3AI circles were generated following separate digestions of the viral genome with each enzyme and ligation with T4 ligase. Primer pairs annealed to the terminal regions of IFP2 in these end-specific circles. (C) PCR amplification generated linear fragments that included IFP2 termini and flanking viral sequences. Subsequent cloning and sequencing (see Materials and Methods) allowed identification of transposition, illegitimate recombination, or homologous recombination events. (D) Primer sequences used for amplification and sequencing of IFP2 terminal domains.

four experimental transfections (4 transpositions per 37 purified recombinants) and comparing it with the similar pooled data from the control transfections (0 transpositions per 26 purified recombinants). The experimentals were significantly different from the controls ($\chi^2 = 9.5$, 1 *df*, $P < 0.005$) confirming that the number of transposition events that occurred in the presence of the helper plasmid was significantly different from the control.

Virus Mutant	Sequence Surrounding the Insertion Site
GmFP2	ACAGAGCGTCGCGAGTTTTT TTAA GTAACAGCTTTTGCTCCGCT
GmFP4	GAGTAAAGCGCAAATCTTTT TTAA ATAATAGTTTCTAATTTTTT
GmFP6	ACTGTTCAAATTGATCCATA TTAA CGATATCAACCCGATGCGTA
B17	CTTTATCGGCAATTTTTTGC TTAA GATCGGCCACCGTCTCTGCC
B1	CATTACAATGGCCGTTATTT TTAA CAACAAGCAACTGCTCGCAG
CIa6d1	TTGTACAACACAAATACTCG TTAA TTGTCACAACCGACAAGCAC
CJc2a1	ACAGAGCGTCGCGAGTTTTT TTAA AAAAGATTTTGCAGTTTACT
CGc5a1a	TAAAAGTAAAGCGGCTTGTT TTAA TAAAATGAAAATAAAACGTT
CHb3	GTAATTTACAGTATAGTATT TTAA TTAATATACAAATGATTTGA

FIG. 4. Alignment of the sequences surrounding the identified IFP2 transposition sites in the baculovirus. All of the transposition events observed were identified by the duplication of a TTAA target sequence that occurred once in the uninterrupted viral sequence. Alignment of the sequences flanking the insertion site does not reveal any consensus sequences that might indicate a signal for targeting TTAA sites.

Our combined experiments generated a total of 216 blue recombinant cultures using combinations of the pLCND constructs with p3E1.2 from which we plaque purified a total of 80 recombinants and fully characterized 32 by cloning and DNA sequencing. Six of these 32 recombinants were unambiguous transposition events as determined by sequencing analysis (Table 2). These events were defined by the presence of a duplicated TTAA target site at the point of precise insertion of the IFP2 element.

We aligned the sequences flanking the known insertion sites for the IFP2 element in the baculovirus genome in an attempt to define a consensus sequence configuration that might target insertions to these particular TTAA sites (Fig. 4). We have sequenced a total of seven unique TTAA target sites from 11 independent IFP2 insertion events identified in this assay or a previous analysis (Cary *et al.*, 1989). Comparison of the flanking viral sequences does not reveal any consensus configuration aside from the TTAA target site itself.

Orientation of the polh/lacZ gene has no apparent effect on transposition of IFP2

We performed our experiments utilizing two plasmids that contained the polh/lacZ fusion gene in the same transcriptional orientation relative to the IFP2 element (plus

TABLE 1

PCR Analysis of Recombinants Isolated from Cotransfections with End-Specific Primer Pairs

Plasmid pair	Start	Blue	*Taq*$^+$	*Sau*3A$^+$	*Taq*$^+$ *Sau*3A$^+$	Seq	Transpo
C + G	24	13	10	6	6	3	1
C + H	24	10	10	3	3	3	1
C + I	24	8	8	2	2	2	1
C + J	24	6	5	4	4	3	1
C + G (2)	120	43	29	30	29	NA	NA
2 × G1	24	5	3	4	3	3	0
2 × G2	24	7	4	4	4	3	0
2 × G3	30	14	12	2	2	2	0

Note. The helper plasmid p3E1.2 (C) was cotransfected in SF21AE cells along with AcMNPV viral DNA and one of the polh/lacZ-tagged IFP2 constructs pLCND 1.1 (G), pLCND 1.2 (H), pLCND 4.1 (I), or pLCND 4.2 (J). The cotransfected cells were separated into wells of a 24-well plate (see Materials and Methods) and blue virus generated from each well was plaque purified and characterized. Two sets of cotransfections were done using the C + G plasmid combination. Control transfections were performed simultaneously (2 × G1, 2 × G2) by adding twice the amount of pLCND 1.1 with the viral DNA. Additional controls were analyzed from a separate cotransfection experiment (2 × G3) in which recombinants were isolated from individual cotransfection cultures. *Taq*I or *Sau*3AI circles were prepared separately from each recombinant and used as templates for inverse PCR (Materials and Methods) with the primer pairs indicated in Fig. 3. The number of recombinants yielding a PCR product in the *Taq*I (*Taq*$^+$), *Sau*3AI (*Sau*3A$^+$) are indicated. The *Taq*I$^+$ *Sau*3AI$^+$ column indicates the total number of recombinants that were positive for both reactions. The number of recombinants yielding both *Taq*$^+$ and *Sau*3A$^+$ fragments that we were able to successfully analyze by sequencing (Seq) and the number of transposition events identified from those sequenced (Transpo) are indicated as well. A χ^2 analysis was performed to compare the pooled data from the experimental (C + G, C + H, C + I, and C + J) with the pooled controls (2 × G1, 2 × G2, and 2 × G3). For this analysis we compared the number of transposition events (Transpo) as a proportion of the total number of blue viruses purified and analyzed (Blue).

orientation) and two that were reversed relative to this reading frame (negative orientation). We were uncertain if the generation of complementary transcripts from the IFP2 and polyhedrin promoters might have adversely affected detection of the lacZ phenotype or might have reduced or eliminated transposition of the tagged element. Transfections performed with either orientation yielded transposition events (Table 1). Therefore we concluded that the variable orientation of the inserted marker gene had no appreciable effect on the transposition of the tagged IFP2 element.

Transposition events in the viral genome interrupt open reading frames

We performed hybridizations with the IFP2-containing viral DNA fragments to identify the region of the virus genome into which the elements had inserted (not shown). In addition, we sent the sequences of the insertion sites to Drs. John Kuzio and Bob Possee, Oxford University, for independent computerized comparison of the insertion positions with their genomic sequence of the AcMNPV strain C6. Subsequent publication of the sequence (Ayres *et al.*, 1994) allowed more direct analysis of the insert positions.

Several of the IFP2 insertions were positioned within the coding domains of previously identified genes (Table 2). Some of these genes (e.g., ORF 603, p26) had already been characterized as nonessential through direct mutagenesis (Gearing and Possee, 1990; Rodems and Friesen, 1993). The recombinant mutants B17 and Cla4 (nontranspositional insertion event) provided a direct demon-

TABLE 2

Sequences Associated with IFP2 Insertion Events within the Baculovirus Genome

Recombinant designation	m.u. Gene	TTAA site	Reference for genetic locus	GenBank Accession No.
B1, B19	3.2 ORF 603	2015–2018	Gearing and Possee, 1990	D00700
B17	21.6 vUBI	308–311	Guarino, 1990	M30305
CGc5	45.4 ORF 870	60896–60899	Ayres *et al.*, 1994	L22858
CHb3	88.6 p26	1310–1313	Liu *et al.*, 1986	X04611
Cla6	33.2 ORF 369	44499–44502	Ayres *et al.*, 1994	L22858
Cla4	14.7 ORF 2070	at 8076	Braunagel *et al.*, 1992	M96361

Note. Recombinants B1, B17, and B19 were purified from progeny of cotransfected pLCND 1.1 and p3E1.2 along with AcMNPV-E2 viral DNA in individual 60 × 15-mm plate cultures of SF21AE cells. Recombinants CGc5, CHb3, Cla4, and Cla6 were recovered from cotransfections of the p3E1.2 (C) helper plasmid and AcMNPV-E2 pLCND 1.1 (G), pLCND 1.2 (H), or pLCND 4.1 (I) using the 24-well plate culture strategy (see Materials and Methods). The exact positions of the insertion sites within the identified gene sequences are indicated (TTAA site). The reference and GenBank Accession No. for each ORF sequence are provided. The Cla4 insertion event appears to have resulted from an illegitimate recombination event that inserted the IFP2/lacZ construct into the 2070 ORF with retention of a single TTAA target site at one terminus of the IFP2 element.

stration that the v-ubi gene (Guarino, 1990) and ORF 2070 (Braunagel *et al.,* 1992), respectively, were nonessential for *in vitro* replication of the virus as well.

The insertions in mutants CGc5 and Cla6 occurred within two previously uncharacterized ORFs. The IFP2 element of CGc5 inserted at a TTAA target site at 60896–60899 in the AcMPNV sequence (Ayres *et al.,* 1994) and within a 870-bp ORF that extended from a MET at 60110 and ends with a TAA stop at 60980. The amino acid sequence from this 870-bp ORF predicted a peptide of 34.4 kDa. The Cla6 insertion was positioned at a TTAA target site from 44499 to 44501 bp and within a 369-bp ORF extending from the MET at 44710 to a TAA stop at 44341. This ORF could encode a peptide with a predicted size of 14.9 kDa. Whether or not these ORFs represent actual genes was not determined.

With the single exception of the ORF 2070 insertion event, all of the other insertions we characterized in this study occurred at and duplicated TTAA target sites present within these genes (Table 2), indicating transposition of IFP2 at these sites. In the v-ubi sequence, the single TTAA within the *Atl*II site (Guarino, 1990) served as the target for the insertion event.

The two separate insertion events of mutants B1 and B19 at the same TTAA site in ORF 603 may have been a coincidence. Alternatively, ORF 603 may have provided a particularly active site for insertions of the IFP2 element into the virus genome. These mutants could not have resulted from a single amplified mutation since they were generated in the first series of cotransfections in which a single recombinant was recovered from each independently transfected 60 × 15-mm culture plate.

Phenotypic characterization of transposon-mutagenized recombinant viruses

The genes that were disrupted by IFP2 insertions were obviously nonessential for virus replication *in vitro.* We were also interested to know if these insertion events had any apparent effects on functions that contributed to assembly of occluded virions during virus replication. Phenotypic characterizations of plaque size (which is reflective of budded extracellular virus produced; Fraser and Hink, 1982a,b), presence of OBs, number of OBs per cell, and estimated relative number of infectious extracellular virus produced per cell did not indicate any significant alterations of phenotype associated with IFP2 insertions in any of these genes. Electron microscopy of recombinant-infected cells revealed no gross alterations in virus maturation and assembly of the occluded form of the virus. In contrast, the single FP mutant control infection exhibited the expected reduction in *de novo* envelope morphogenesis and occlusion of virions (Fraser and Hink, 1982a).

DISCUSSION

Several studies have demonstrated that baculoviruses are suitable targets for the insertion of transposons resident in the genomes of their infected host cells (Miller and Miller, 1982; Fraser *et al.,* 1983, 1985; Beames and Summers, 1987; Carstens, 1987; Schetter *et al.,* 1990). There has been some speculation that the baculovirus might provide a vehicle for the escape of self-propagating sequences, perhaps allowing transduction of genes between the various productive and nonproductive hosts of the virus (Miller and Miller, 1982; Fraser, 1986). Some of these transductions might lead to evolutionarily significant mutations in the alternate host (Fraser, 1986; Kidwell, 1993). Defining the mechanism and extent of genetic interactions between baculoviruses and their hosts is of considerable practical significance considering the ongoing research toward developing these viruses as genetically engineered biopesticides. The acquisition of host-transposable elements by baculoviruses provides a unique opportunity to experimentally examine the potential for evolutionarily significant transduction of genes by DNA animal viruses.

The IFP2 element is representative of a diverse family of Lepidopteran elements that recognize and insert at TTAA target sites within the Lepidopteran genome (Beames and Summers, 1989; Wang and Fraser, 1993) and within the genomes of infecting baculoviruses (Fraser *et al.,* 1985; Carstens, 1987; Beames and Summers, 1989; Cary *et al.,* 1989; Schetter *et al.,* 1990). Previously we established that these TTAA-specific elements move with the same characteristics in the absence of an infecting virus (Wang and Fraser, 1993). This was an important point in considering the virus as a target for analysis of transposition events since we had to be sure that movement in infected cells reflected natural movement of the transposon.

In the case of IFP2, the dispersed repetitive nature of this element within the TN-368 genome is ample evidence of its mobility in the absence of virus infection, while the occurrence of spontaneous FP mutations during serial propagation of the virus in these same cells demonstrates mobility in the presence of virus infection (Fraser *et al.,* 1983). In addition, previous analyses demonstrated that the IFP2 element is highly conserved in the TN-368 cell genome (Fraser *et al.,* 1983), which in turn suggests that the encoded product(s) of the IFP2 element is (are) likely important for maintenance of the element.

We have developed an assay that provides a close approximation of the transposon mutagenesis phenomenon as it actually occurs in baculovirus-infected Lepidopteran cells. Using this assay we have defined several important features of IFP2 mobilization in these cells.

Transpositional movement of lacZ-tagged IFP2 elements from the plasmid into the virus genome was only detected in cotransfections with the added p3E1.2 helper plasmid. We were unable to detect transposition in the absence of the intact IFP2 element of p3E1.2 even though the amount of lacZ-tagged IFP2 was 2-fold greater in the control reactions and all of the recombinant viruses that

we purified from these control reactions were fully analyzed. If a transacting factor supplied by the helper plasmid is not required for transposition, then the probability of detecting transposition in the controls should have been as good as, if not better than, the probability of detecting transposition in the experimental cotransfections.

Only 2 of 3 of the stable recombinant blue viruses recovered from the cotransfections with the p3E1.2 helper were analyzed fully, and as a result several transposition events likely escaped detection. Based upon our rate of detecting transposition events in the fully characterized recombinants (approximately 1 per 10 plaque-purified recombinants) we estimate at least 3 or 4 additional events would be among the remaining recombinants that were plaque purified but not fully analyzed (Table 1).

From the results of our transposition assay we may infer that the movement of IFP2 is at least enhanced, if not directly controlled by the IFP2 encoded product(s). Transposition is only demonstrated in SF21AE cells when the p3E1.2 helper transposon is added. The statistical probability of obtaining a transposition event in our assay by chance alone is less than 1/200. The fact that we have obtained four transposition events from only 37 purified recombinants presents a strong argument for the involvement of the IFP2 ORF in mobilization of the element.

Other TTAA-specific insertion elements have been isolated from AcMNPV FP mutants derived by passage in SF21AE cells (Beames and Summers, 1989). This means that the machinery for movement of at least some of these TTAA-specific elements is also present in these cells. However, the complete lack of transposition for tagged IFP2 elements in transfections of SF21AE cells lacking helper p3E1.2 argues that some portion of the transposition mechanism for these elements is specific, perhaps involving recognition of the terminal repeat sequences. Future analyses are planned to determine relative importance of these terminal sequences in transposition and excision of these elements. Alternatively, the relative level of background mobilization of IFP2 by TTAA-specific elements present in SF21AE cells may simply be undetectable in our assay.

The IFP2 element shares some characteristics of movement with other eukaryotic transposable elements that have proven useful for genetic engineering of insect and plant species, such as the P-element of *Drosophila melanogaster* (Rubin and Spradling, 1982) and the Ac/Ds element of *Maize* (Baker *et al.*, 1986). The assay has demonstrated that the IFP2 element is capable of carrying active genes from plasmids into new genomes and allowing them to be expressed within the context of these new genomes. The results also demonstrate that factors facilitating movement of the transposon vector can be provided *in trans* by an intact element.

The IFP2 element also has some features that contrast with the P-element. The IFP2 element does not seem to be restricted in its movement to species closely related to the one from which it was isolated. Transposition of the tagged element from the plasmid into the virus genome was effected in a cell line established from *Spodoptera frugiperda*, a species distinct from that which harbored the original IFP2 element and which does not contain IFP2-homologous sequences in its genome. In addition, the IFP2 element does not appear to be germline restricted in its movement, since it moved within cultured somatic cells. These properties make the IFP2 element an interesting candidate for potential use in the genetic transformation of some insects.

All previously characterized IFP2 insertions into the baculovirus genome were localized within the FP locus. Similarly, most previously characterized transposon insertion events for TTAA-specific elements were localized to the FP locus as well. These observations implied that the FP locus might be a prefered location for transposition events in the baculovirus genome. Our results establish that other points in the virus genome are at least equally susceptible to mutagenic attack by the IFP2 element in particular and TTAA-specific elements in general. Our limited analyses have established that the other positions detected in our assay are nonessential to the survival of the virus in cell cultures, although we have not examined their importance in establishing or maintaining infections in larvae.

We believe that the current assay could be modified to allow generalized screening for nonessential baculovirus genes. The illegitimate recombination events that seem to predominate in our assay could be useful in this vein as well (e.g., the Cla4 mutant with an insertion in ORF 2070), since insertion by whatever mechanism will inactivate genes. While most of the transposon insertions in our assay occurred within the coding domains of known genes, two insertions (CGc5 and Cla6, Table 2) occurred within the coding domains of previously uncharacterized ORFs and established the nonessential nature of those regions. However, no further characterization of these ORFs was performed to establish if they are transcriptionally active, functional genes.

For gene tagging experiments, plasmid constructs that eliminate homologous recombination events and allow for more direct cloning and identification of transposon insertions would improve the recovery of transposition and illegitimate recombination events since nearly half of the putative recombinants detected in the control assay were homologous recombinations at the FP locus. The availability of the complete AcMNPV sequence (Ayres *et al.*, 1994) allows rapid identification and analysis of recombination sites using inverse PCR strategies.

Our results and previous analyses (Fraser *et al.*, 1985; Beames and Summers, 1990; Xiong *et al.*, 1991) have demonstrated a propensity for random recombination of DNA within the baculovirus infected cell. Illegitimate recombination seems to be a highly active mechanism in

baculovirus-infected cells and may be related to DNA repair during replication. The frequency of such events suggests that random recombination has probably contributed significantly to the building of the baculovirus genome. The ability of the virus to undergo recombination events with portions of the host cell genome may provide some evolutionary advantages such as adaptation to enhance replication in a new host.

The propensity for illegitimate and transpositional recombination events between baculoviruses and the genomes of their hosts could have some important consequences in the application of recombinant baculoviruses for insect control. The fact that these mutations do not necessarily lead to defective viruses and the inability to control these events in the production process are factors to consider in evaluating the genetic homogeneity of a candidate biopesticide prior to release. Understanding the underlying mechanisms of these events could lead to eventual control of at least some of these recombination events and could contribute to an increased stability for genetically engineered derivatives of these viruses.

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