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Strong and Regulated Expression of *Escherichia coli* β-Galactosidase in Insect Cells with a Baculovirus Vector†

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The N-terminal region of the gene encoding polyhedrin, the major occlusion protein of the insect baculovirus *Autographa californica* nuclear polyhedrosis virus (AcNPV), has been fused to DNA encoding *Escherichia coli* β-galactosidase. The fused gene was inserted into the AcNPV DNA genome by cotransfection of insect cells with recombinant plasmid DNA and wild-type AcNPV genomic DNA. Recombinant viruses were selected as blue plaques in the presence of a β-galactosidase indicator, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside. Studies of one such virus, L1GP-gal3, indicated that the synthesis of β-galactosidase is temporally controlled beginning late (20 h) in infection after the release of infectious virus particles from the cell. By 48 h postinfection, a remarkably high level of expression is achieved. On the basis of these results, AcNPV should be a useful vector for the stable propagation and expression of passenger genes in a lepidopteran cell background. A generalized transplacement vector that facilitates the construction and selection of recombinant viruses carrying passenger genes under their own promoter control has also been developed.

The 128-kilobase (kb) circular, supercoiled, double-stranded DNA genome of the insect baculovirus *Autographa californica* nuclear polyhedrosis virus (AcNPV) replicates in the nucleus of the host cell, where it is also assembled into rod-shaped nucleocapsids. By 10 to 12 h postinfection (p.i.), infectious extracellular virus (EV) is produced as the nucleocapsids leave the nucleus and bud through the plasma membrane. By 18 to 24 h p.i., the synthesis of a second form of the virus, occluded virus (OV), begins. Nucleocapsids of OVs acquire a membrane within the nucleus and are then embedded within a crystalline protein matrix composed primarily of a single 33,000-dalton polypeptide known as polyhedrin. OVs only initiate the primary infection of the host organism, whereas EVs are responsible for the secondary infection within the organism and infection in cell cultures. Rapid advances have been made in understanding the molecular biology, biochemistry, and genetics of this interesting virus (for reviews, see references 4, 6, 13, and L. K. Miller, Methods Virol., in press).

Many features of AcNPV make this virus a promising candidate as a vector for the propagation and expression of passenger genes (13). First, the rod-shaped capsid apparently extends to accommodate additional DNA sequences with a vector capacity estimated to exceed 100 kb. Second, EV is the infectious form in cell cultures, and the genes for occlusion are nonessential. Third, the gene encoding polyhedrin is an ideal site for passenger gene insertion, since it is abundantly expressed. At between 18 and 72 h p.i., the polyhedrin mRNA transcript constitutes 20% or more of the total polyadenylic acid-containing mRNA (1, 19), and by the time of cell death, the polyhedrin protein constitutes 20% or more of the total protein of the infected cell. Finally, the synthesis of the 1.2-kb polyhedrin mRNA is temporally controlled (15), and infectious EVs are produced before polyhedrin synthesis. This temporal regulation may be particularly advantageous in expressing cytotoxic gene products. The location of the polyhedrin gene on the AcNPV physical map and the direction of the transcription are known (1, 29), and AcNPV mutants with deletions in the polyhedrin gene have been constructed (28).

In this report, we demonstrate that AcNPV can be successfully employed as a recombinant DNA vector by stably propagating a 9.2-kb plasmid inserted into the polyhedrin gene. We also demonstrate that the polyhedrin promoter can drive the high-level expression of a passenger gene, *Escherichia coli* β-galactosidase, when fused in frame with polyhedrin. Finally, we describe a plasmid which facilitates the insertion of foreign genes into AcNPV and the selection of the recombinant viruses as blue plaques under semisolid overlay medium containing the β-galactosidase indicator 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal).

MATERIALS AND METHODS

Virus and cells. The wild-type (wt) virus used in the recombinant DNA constructions was the L-1 variant of AcNPV that was previously described (9, 14). A continuous cell line of *Spodoptera frugiperda* (a lepidopteran noctuid), IPLB-SF-21, was used for virus propagation, transfections, and plaque assays. This cell line and a line derived from the lepidopteran noctuid *Trichoplusia ni*, TN-368, were propagated in TC-100 medium (Microbiological Associates, Bethesda, Md.) supplemented with 0.26% tryptose broth, 10% fetal bovine serum, 2 mM L-glutamine, and an antibiotic-antimycotic preparation (GIBCO Laboratories, Grand Island, N.Y.). A cell line of *Drosophila melanogaster*, DL-1 (26), was propagated in Schneider's revised medium (25) supplemented with 15% fetal bovine serum and 5 mg of bacteriological peptone per ml. Mouse L-cells (L-929) were propagated in RPMI 1640 medium (GIBCO; powdered, with glutamine added) supplemented with 15% fetal bovine serum and antibiotics.

Plaque assays. Plaque assay procedures involving neutral red staining of the lepidopteran cell monolayers have been

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previously described (9). For plaque assays involving blue color production, a similar procedure was used, except 120 μg of X-gal per ml was included in the 0.75% agarose overlay medium. Instead of neutral red staining, the plaques were visualized by their blue color, which could be enhanced by warming the plates at 37°C for 4 to 6 h and then allowing the color to develop at room temperature overnight. Alternatively, for quick visualization, the tissue culture dishes were frozen in liquid N_2 for 5 to 10 s and thawed at room temperature, and the color was then allowed to develop for 2 or 3 h. Infectious viruses were picked from the plates and plaqued directly or grown into a virus stock.

DNA preparations. Plasmid DNAs were prepared by a rapid cleared lysate procedure (5). AcNPV DNA was prepared from EV which was partially purified from infected culture medium by centrifugation through a 20% sucrose cushion at 90,000 × *g* for 90 min at 5°C. The pellet was suspended in 200 μl of 10 mM Tris-hydrochloride (pH 7.6)–10 mM EDTA–0.25% sodium dodecyl sulfate (SDS). Proteinase K was added to a final concentration of 0.5 mg/ml, and the mixture was incubated for 30 min at 37°C and then phenol extracted twice before the DNA was ethanol precipitated.

Transfections. The calcium phosphate precipitation method of Potter and Miller (18) was used without the glycerol boost step. After the 20-min incubation with the DNA precipitate, the *S. frugiperda* cells were overlaid with liquid medium for 2 to 3 h at room temperature. This overlay was then replaced with either fresh liquid medium or agarose-containing medium for plaque development at 27°C. For transplacement experiments, a molecular ratio of 100 plasmid DNAs per viral DNA was routinely used. The DNAs to be used in the transfection procedure were dissolved in water rather than a Tris-EDTA solution.

Nucleotide sequencing of the N-terminal region of the polyhedrin gene. The 1.2-kb *Sal*I-*Hin*dIII fragment of *Eco*RI-I spanning the *Bam*HI site at the *Bam*HI-B/F junction was subcloned with plasmid pUC8 as the vector (10, 31). DNA from the plasmid was digested with restriction enzymes, and the desired fragments were resolved by agarose gel electrophoresis and purified (33). When restriction enzyme cleavage sites could not be directly cloned into an M13 polylinker, the site was enzymatically blunted with the Klenow fragment of DNA polymerase I for ligation to the *Sma*I site with mp8 and mp9 vectors. M13 clones containing a polyhedrin sequence were obtained, and templates were prepared and sequenced by the methods of Sanger et al. (23) with the gel system of Sanger and Coulsen (22).

***E. coli* plasmid constructions.** The 25.2-kb plasmid pGPB6874 was constructed by digesting plasmids pEXS942B6 (15) and pMC874 (3) with *Bam*HI, followed by ligation at high DNA concentrations to favor bimolecular fusions. After transformation of *E. coli* HB101 with the ligation products, the plasmid fusions were selected by their resistance to ampicillin (supplied by pEXS942B6) and kanamycin (from pMC874). Blue colonies on plates containing ampicillin, kanamycin, and X-gal were selected. The colonies contained the fusion plasmid pGPB6874 (see Fig. 2) with β-galactosidase fused to the polyhedrin N-terminus, as demonstrated by restriction endonuclease digestion with *Sal*I, *Eco*RI, and *Hin*dIII enzymes. These enzyme analyses were capable of distinguishing the two possible orientations of the fused plasmids.

The pGPB6874/*Sal* plasmid was constructed by partially digesting pGPB6874 with *Sal*I and ligating the two at a low DNA concentration. After transformation of *E. coli* HB101, blue colonies were selected on kanamycin plates and then tested for their sensitivity to ampicillin. The plasmids of Amp^s Kan^r blue colonies were characterized by restriction endonuclease analysis. The pGPB6874/*Sal* plasmid (see Fig. 7) was found to contain the region of pGPB6874 extending from the *Sal*I site in *Eco*RI-I(l) through pMC874 to the *Sal*I site of *Eco*RI-I(s) (see Fig. 2).

β-Galactosidase assays. Levels of β-galactosidase were determined by the *o*-nitrophenyl-β-D-galactopyranoside (ONPG) assay (11). Cells of the eucaryotes tested were inoculated with virus at a multiplicity of infection of 20 PFU per cell. The virus was allowed to adsorb for 1 h at room temperature with gentle rocking. The inoculum was removed and replaced with appropriate medium, and incubation was carried out at 27°C for insect cells or 33°C for mammalian cells for the time indicated (see Fig. 4). The cells were washed with a phosphate-buffered saline solution (9), scraped from the plate, and pelleted at 200 × *g* for 5 min. The supernatant was discarded, and the cell pellet was frozen in liquid N_2 and stored at −70°C until assayed. The cells were suspended in distilled water and then diluted appropriately (from 1:1 to 1:1,000) in Z buffer so that a faint yellow color developed between 15 min and 1 h after the ONPG addition. The cells were disrupted with chloroform–0.1% SDS (11). Specific activity was defined according to Miller as nanomoles of ONPG cleaved per minute per milligram of protein (11). Protein was determined by Lowry assays; the standard curve was based on bovine serum albumin (24).

Pulse-labeling and electrophoretic separation of proteins. The procedure of Miller et al. (16) was used for the analysis of virus-induced proteins in infected *S. frugiperda* cells except that 10^6 cells per 35-mm plate were used, the cells were washed with medium after being pelleted at low speed, and a constant volume of cell extract was loaded per lane. Immune precipitations with polyhedrin antiserum were performed by a slight modification of a previously described procedure (7). Forty microliters of ^{35}S-labeled protein from 48 h p.i. was incubated with 40 μl of polyhedrin antiserum for 1 h at room temperature, and then 160 μl of a 2% suspension of *Staphylococcus aureus* (Sigma Chemical Co., St. Louis, Mo.) in precipitation buffer (0.15 M NaCl, 5 mM EDTA, 50 mM Tris-hydrochloride, 0.2% NaN_3, 0.05% Nonidet P-40, 0.1% bovine serum albumin) was added. The mixture was incubated at room temperature for 20 min, and the resulting precipitate was collected by centrifugation at 15,000 × *g* for 1 min. The supernatant was discarded, and the pellet was washed with 0.5 ml of precipitation buffer. The pellet was then suspended in solubilization buffer (1% SDS, 0.5% β-mercaptoethanol, 0.5 M urea) with vortexing and heated for 5 min at 100°C. The debris was pelleted at 15,000 × *g* for 5 min, and the supernatant was analyzed by SDS-polyacrylamide gel electrophoresis.

RESULTS

Determination of the nucleotide sequences encoding the N-terminus of polyhedrin. Plasmid pEXS942B6 contains the 0.0 to 8.7% region of AcNPV with one *Bam*HI site near or within the polyhedrin gene (15). To determine whether the single *Bam*HI site is within the N-terminal region of the polyhedrin gene and to determine the reading frame at the *Bam*HI site, the *Sal*I-*Hin*dIII fragment spanning the *Bam*HI site was subcloned, and the nucleotide sequence from the *Hin*dIII site to the N-terminal AUG was determined (Fig. 1A). The nucleotide sequence and the deduced amino acid sequence of the polyhedrin N-terminus are presented in Fig.

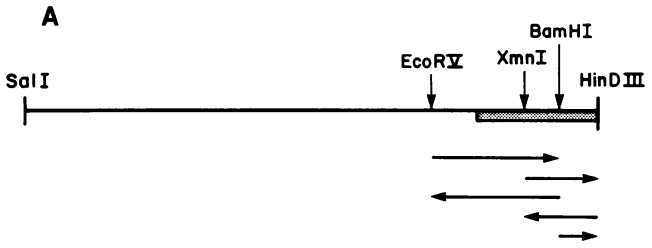

FIG. 1. (A) Strategy for sequencing the N-terminal coding region of the polyhedrin gene. The location of critical restriction sites on the 1.26-kb *Sal*I-*Hin*dIII fragment are indicated by vertical arrows, and the direction and regions sequenced are indicated by horizontal arrows. (B) DNA sequence and deduced amino acid sequence of the N-terminal coding region of the polyhedrin gene. The *Bam*HI site, used in fusing *E. coli* β-galactosidase to the polyhedrin gene, is underlined.

1B. Although the amino acid sequence of the AcNPV polyhedrin protein has not been determined, this deduced amino acid sequence matches the highly conserved consensus sequence previously determined for a variety of other polyhedrins by protein sequencing (8, 20, 21). The strict correspondence of the nucleotide sequence with the polyhedrin amino acid consensus sequence demonstrates the lack of introns within the N-terminal polyhedrin coding region. The absence of introns in the C-terminal region of the polyhedrin genes by S1 nuclease digestion (29) has been previously demonstrated; thus, polyhedrin may be a rare example of an abundantly expressed gene lacking introns within the coding sequence. The *Bam*HI site lies within the sequence GAG GAT CCT encoding Glu, Asp, and Pro and corresponds to the 57th to 59th amino acids from the NH_2 terminus.

Construction of a polyhedrin/β-galactosidase fusion gene in *E. coli*. The pMC874 plasmid constructed by Casadaban et al. (3) possesses a single *Bam*HI site at the eighth codon of the *E. coli* β-galactosidase gene in the same reading frame as the *Bam*HI site of polyhedrin (GAT CCG: Asp, Pro). In addition to the β-galactosidase gene, the 9.2-kb pMC874 plasmid contains the *lacY* gene, part of the *lacA* gene, an inactive portion of the ampicillin resistance gene, a ColE1 replication origin, and a gene for kanamycin resistance (3; Fig. 2, shaded region of). A fusion of *Bam*HI-digested pMC874 and pEXS942B6 DNAs (see above) by in vitro ligation resulted in the formation of the 25.2-kb plasmid pGPB6874 (Fig. 2). *E. coli* colonies with a functionally defective chromosomal *lac* operon but containing pGPB6874 are blue on X-gal plates and red on MacConkey plates.

Transfer of the polyhedrin/β-galactosidase fusion gene to AcNPV. To transfer the polyhedrin/pMC874 fusion sequences into the AcNPV DNA genome, an allelic replacement, or "transplacement" technique, that was originally developed for the marker rescue of AcNPV temperature-sensitive mutants was employed (12, 17). Monolayers of *S. frugiperda* cells were cotransfected with intact wt AcNPV and a 100-fold molar excess of pGPB6874 DNA by a calcium phosphate coprecipitation technique (18). Viruses expressing the β-galactosidase gene were detected as blue-colored plaques in the presence of X-gal.

Comparison of neutral red-stained monolayers and X-gal-stained monolayers indicated that ca. 0.1% of the plaques expressed β-galactosidase after cotransfection of AcNPV and pGPB6874. Several blue plaques were picked and directly plaqued on fresh *S. frugiperda* cell monolayers. Blue plaques free of occlusion bodies were picked, developed into virus stocks, and characterized by restriction endonuclease analysis.

Restriction endonuclease analysis of blue-plaque AcNPV. The *Pst*I and *Bam*HI patterns of one of these blue-plaque viruses, L1GP-gal3, are presented in Fig. 3. As was expected

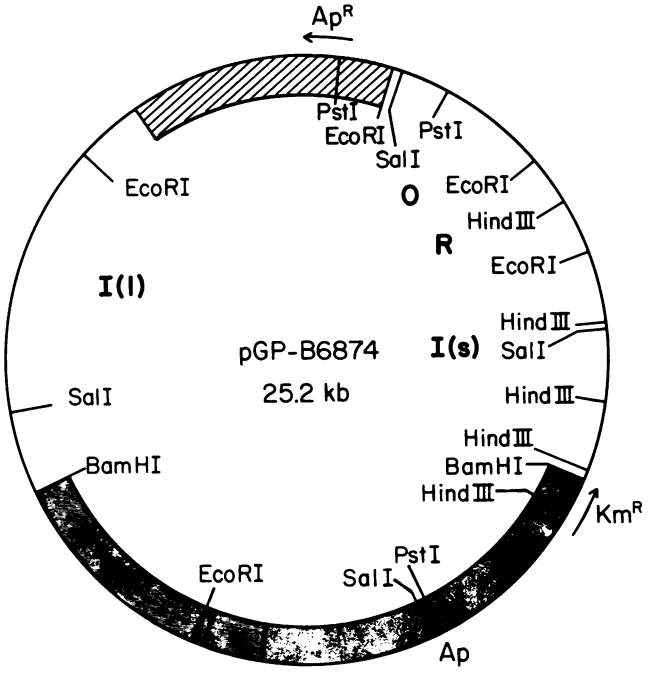

FIG. 2. Restriction map of *E. coli* plasmid pGPB6874 containing the polyhedrin/β-galactosidase fusion gene. The pGPB6874 plasmid was constructed by fusing *Bam*HI-digested pMC874 (shaded region) and pEXS942B6 DNAs. The pEXS942B6 plasmid contains the *Eco*RI-I, R, and O fragments in the 0.0 to 8.7% region of AcNPV (single line) and the large *Eco*RI-*Sal*I fragment of pBR322 (hatched region) containing a replication origin and the ampicillin resistance gene (Ap^r). The I(l) region of AcNPV is the 0.0- to 4.21-kb region of *Eco*RI-I, and I(s) is the *Eco*RI-I region from 4.21 to 7.30 kb. The pMC874 plasmid (shaded region) contains *E. coli* β-galactosidase (β-gal), the *lacY* gene (*Y*), a portion of the *lacA* gene, an inactive portion of the ampicillin resistance gene (Ap^r), a replication origin (*rep*), and a kanamycin resistance gene (Km^r). In pGPB6874, the 8th amino acid codon of *E. coli* β-galactosidase is fused in frame to the 58th amino acid codon of the AcNPV polyhedrin gene.

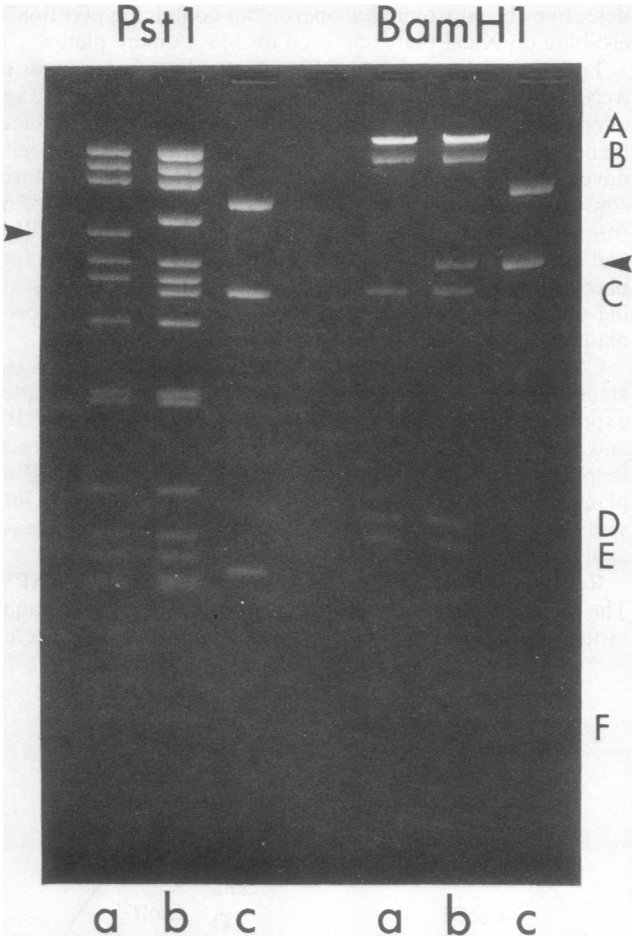

FIG. 3. Restriction endonuclease analysis of the blue AcNPV L1GP-gal3. wt AcNPV DNA (a), AcNPV L1GP-gal3 (b), and pGPB6874 DNA (c) were digested with either *Pst*I or *Bam*HI restriction endonuclease. The resulting fragments were separated by agarose gel electrophoresis and visualized by ethidium bromide staining under UV light. The arrowhead on the left points to the *Pst*I-D fragment missing in L1GP-gal3. Capital letters on the right refer to the *Bam*HI fragments. The arrowhead on the right marks the position of the new 9.2-kb insert in L1GP-gal3 equivalent to *Bam*HI-linearized pMC874.

for an insertion in the polyhedrin gene, *Pst*I-D was missing in L1GP-gal3, and two new fragments were observed; one new fragment was found between *Pst*I-C and *Pst*I-D, and the other was found below *Pst*I-F. The latter fragment comigrated with a *Pst*I fragment of pGPB6874 extending from the Ap^s region of pMC874 to the *Eco*RI-O region of AcNPV (Fig. 2). The new *Pst*I fragment between *Pst*I-C and *Pst*I-D corresponded to the *Pst*I-O/N junction in *Eco*RI-B through *Eco*RI-I(l) of AcNPV and the *E. coli* β-galactosidase, *lacY*, and *lacA* regions. The *Bam*HI pattern confirmed that the entire pMC874 was properly inserted into AcNPV and that the *Bam*HI sites were intact. No wt *Bam*HI fragments were missing in L1GP-gal3, but a new 9.2-kb fragment comigrating with pMC874 was found. *Bam*HI-F was also observed in the L1GP-gal3 virus. Since the *Bam*HI site at the AcNPV *Bam*HI-F/C junction was site-specifically mutagenized in the construction of pEXS942B6, the presence of *Bam*HI-F indicated that homologous recombination between AcNPV and pGPB6874 occurred near the pMC874 and the *Eco*RI-I(s) junction (Fig. 2). It is possible that the alteration of the *Bam*HI site originally introduced into pEXS942B6 was lethal to the virus. *Sal*I and *Eco*RI analyses of L1GP-gal3 also confirmed the insertion of intact pMC874 into AcNPV, as did Southern blots of the *Pst*I and *Bam*HI gel of Fig. 3 when probed with pMC874 (data not shown).

Regulation of expression of β-galactosidase activity. To determine whether the expression of the β-galactosidase gene was under the same temporal regulation as was the polyhedrin gene expression, monolayers of *S. frugiperda* were infected with wt or L1GP-gal3 virus at a multiplicity of infection of 20. At various times p.i. the infected cells were assayed for β-galactosidase activity and total protein content. β-galactosidase activity first appeared at between 18 and 24 h and rose dramatically through 48 h p.i. (Fig. 4). The observed specific activity for 48 h p.i. of 8,000 nmol of ONPG cleaved per min per mg of protein represented an approximately 900-fold increase in β-galactosidase activity. This observed specific activity is very high for a crude cell lysate. At 72 h p.i. the specific activity began to drop; this may have been due to the decrease in the rate of protein synthesis with the approach of cell death and some instability of β-galactosidase within the cell.

Regulation of the polyhedrin/β-galactosidase gene expression was also analyzed by pulse-labeling infected cells with [^{35}S]methionine at various times p.i. The ^{35}S-labeled proteins were analyzed by SDS-polyacrylamide gel electrophoresis. Coomassie blue staining of total infected-cell proteins isolated at various times p.i. (Fig. 5A) showed that early in infection (at 6 and 12 h) there was no significant difference between the wt and the L1GP-gal3 profiles. From 24 to 72 h p.i., the 33,000-dalton polyhedrin protein was synthesized in wt-infected cells but not in L1GP-gal3-infected cells. In contrast, L1GP-gal3 profiles showed a new protein of ca. 120,000 daltons as well as several smaller proteins in the 95,000- to 100,000-dalton range from 20 through 72 h p.i. that were not found in wt-infected cells. The largest protein

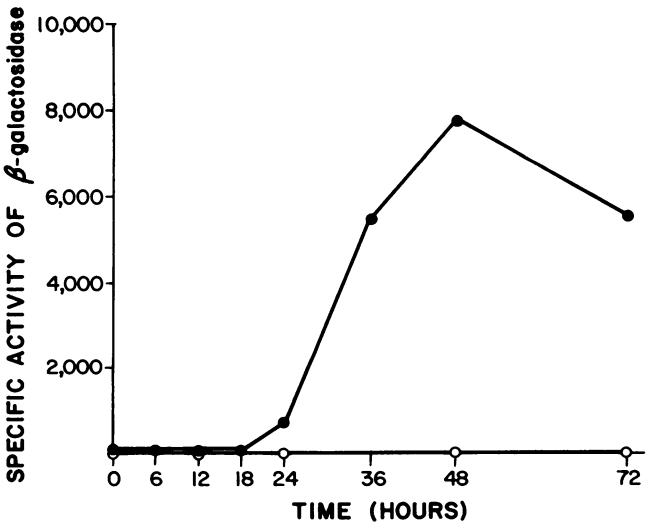

FIG. 4. Temporal regulation of polyhedrin/β-galactosidase fusion gene expression after infection with AcNPV-L1GP-gal3. *S. frugiperda* cells were infected with wt AcNPV (○) or with AcNPV-L1GP-gal3 (●) at a multiplicity of infection of 20. The specific activity of β-galactosidase in the infected cells was determined at various times p.i. Specific activity is reported as nanomoles of ONPG cleaved per minute per milligram of protein.

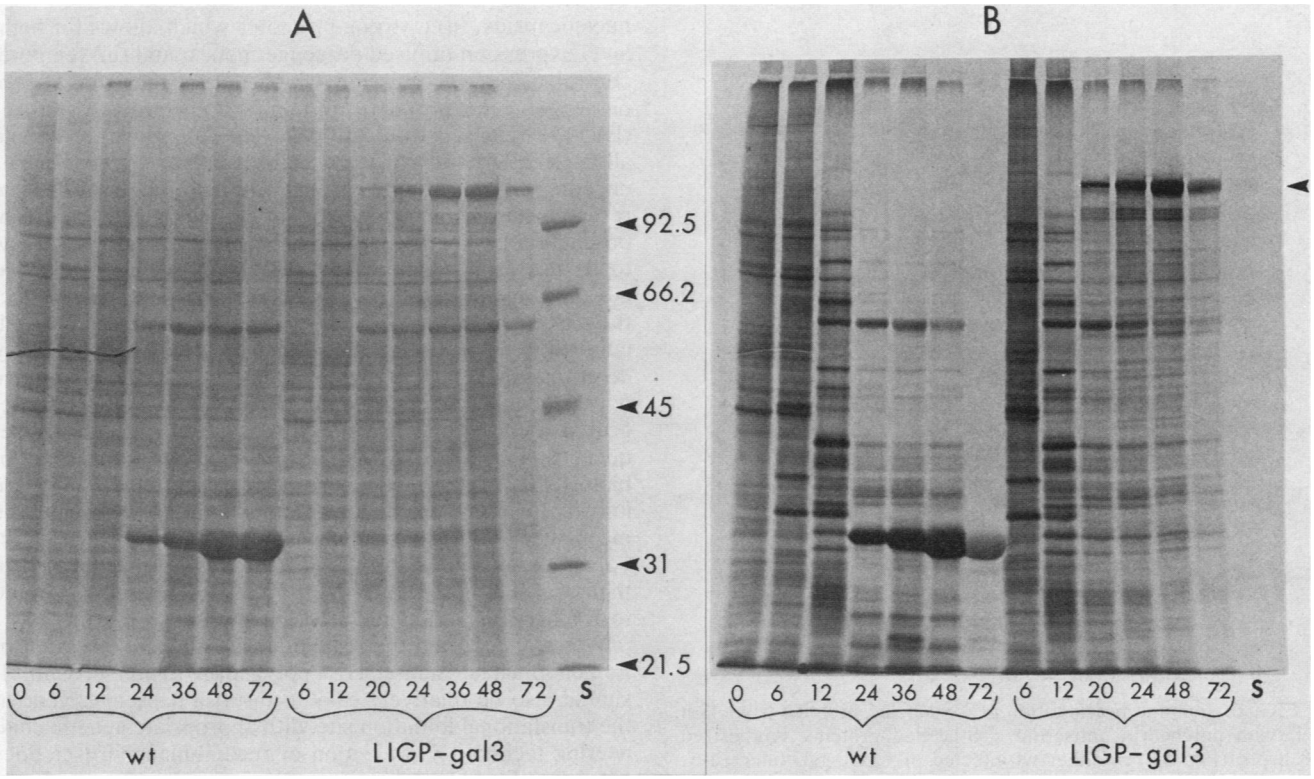

FIG. 5. SDS-polyacrylamide gel electrophoresis profiles of proteins from *S. frugiperda* cells infected with wt AcNPV or the recombinant virus, L1GP-gal3, expressing *E. coli* β-galactosidase. Proteins were pulse-labeled with [^{35}S]methionine and isolated from infected cells at various times (0, 6, 12, 20, 24, 36, or 72 h) after infection. Proteins synthesized in wt-infected cells are compared with proteins from L1GP-gal3-infected cells. (A) Coomassie blue-stained gel; (B) autoradiogram of the same gel. The size of the protein standards in kilodaltons is shown in the middle. An arrowhead on the right indicates the positions of the 120,000-dalton polyhedrin/β-galactosidase fusion protein.

(120,000 daltons) was the appropriate size for a polyhedrin/β-galactosidase fusion polypeptide. The nature of the smaller polypeptides is not known, but it is interesting that they were of discrete size classes. The intensity of the Coomassie blue stain in the 120,000-dalton region reinforced the specific activity data indicative of exceptionally high levels of active β-galactosidase.

The autoradiogram of the ^{35}S-labeled proteins (Fig. 5B) is similar in general features to the Coomassie blue-stained gel but provides some additional information. Again, there was no detectable difference between the wt- and the L1GP-gal3-infected cells at 6 and 12 h p.i. but by 24 h p.i. numerous differences were observed. In addition to the 120,000-dalton protein, the three 95,000- to 100,000-dalton proteins found in L1GP-gal3- but not in wt-infected cells by 20 h p.i. were more evident. A protein of ca. 58,000 daltons was found in L1GP-gal3-infected cells with ^{35}S pulse-labeling that was not observed by Coomassie blue staining, suggesting that this protein is particularly unstable. A virus-induced protein was observed in the 33,000-dalton region of the L1GP-gal3 profile, but it was not synthesized as abundantly as was polyhedrin in wt infection and apparently represented another 33,000-dalton virus-induced protein that comigrated with polyhedrin (see below). The wt autoradiogram profiles in the 20,000- to 25,000-dalton region at 24 through 48 h p.i. revealed two bands that were not found in the L1GP-gal3 region. A third band was transiently observed in wt-infected cells at 36 h p.i. These polypeptides may be encoded by the polyhedrin gene (see below). Polyhedrin-related polypeptides of less than 33,000 daltons have been noted previously in relationship to (i) polyhedrin deletion mutants (28), (ii) in vitro translation of polyhedrin mRNA (1), and (iii) alkaline dissolution of OV (4).

Immune precipitation with polyhedrin antibody. To further explore the nature of the polypeptides produced at 48 h in wt- and L1GP-gal3-infected cells, the ^{35}S-proteins labeled at 48 h p.i. were immune precipitated with antiserum raised to purified polyhedrin (Fig. 6). The polyhedrin antiserum selectively precipitated the 33,000-dalton polyhedrin protein from wt-infected cells as well as one or two other polypeptides in the 20,000- to 25,000-dalton region, suggesting that these proteins are polyhedrin gene encoded. Polypeptides of this size were not precipitated by polyhedrin antiserum in the L1GP-gal3-infected cells. Instead, the 120,000-dalton protein was the predominant precipitant, indicating that it is indeed a polyhedrin fusion product. The series of polypeptides in the 95,000- and 58,000-dalton range specific to L1GP-gal3-infected cells were not selectively immune precipitated by polyhedrin antiserum.

Expression of polyhedrin/β-galactosidase in other cell lines. The polyhedrin/β-galactosidase fusion protein is also expressed in the fully permissive cells of the lepidopteran noctuid *T. ni* as was demonstrated by vivid blue plaques on *T. ni* monolayers overlaid with X-gal medium (data not shown). Because AcNPV is an enveloped virus and because several reports have suggested that AcNPV can replicate in dipteran and mammalian cell lines, we tested whether the β-galactosidase gene of L1GP-gal3 was expressed after infection of mouse L-cells or *Drosophila* cells (see above). No increase in the specific activity of β-galactosidase could be detected in mouse L-cells assayed at 6 or 48 h p.i. at 33°C (a permissive temperature for L1GP-gal3 replication). Similar-

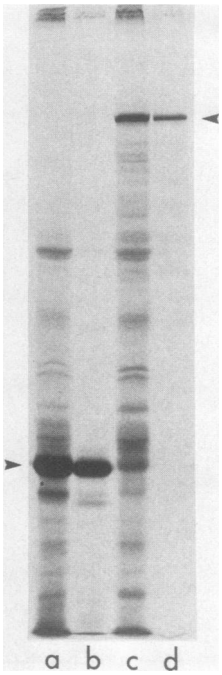

FIG. 6. Immune precipitation of ^{35}S-labeled proteins from 48 h p.i. with polyhedrin antiserum. ^{35}S-labeled proteins synthesized 48 h p.i. (Fig. 5B) in either wt-infected or L1GP-gal3-infected *S. frugiperda* cells were immune precipitated with polyhedrin antibody, and the precipitates were analyzed by SDS-polyacrylamide gel electrophoresis. The autoradiogram of the ^{35}S-labeled protein gel is presented. Lane a, wt-infected cell proteins at 48 h; lane b, wt-infected cell proteins immune precipitated; lane c, L1GP-gal3-infected cell proteins; lane d, L1GP-gal3-infected cell proteins immune precipitated. An arrowhead on the left points to the 33,000-dalton polyhedrin protein, and an arrowhead on the right points to the polyhedrin/β-galactosidase fusion protein.

ly, no increase in β-galactosidase activity could be detected in *Drosophila* cells assayed at 6 h or 48 h p.i. with L1GP-gal3 at 27°C. The background levels of β-galactosidase in both the *Drosophila* and mouse cells were less than 10 nmol of ONPG cleaved per min per mg of protein.

Construction of a generalized transplacement vector. For some types of recombinant DNA research, it would be of interest to insert passenger genes under their own promoter control into the vector. We constructed a plasmid, pGPB6874/*Sal* (see above; Fig. 7), which provided a single *Pst*I site for the insertion of a passenger DNA and allowed the recombinant plasmid to be inserted into AcNPV when blue plaques were used as a selection for recombinant viruses. The pGPB6874/*Sal* plasmid was essentially pMC874 bordered by the N- and C-termini of the polyhedrin gene as in pGPB6874. The region encompassing the *Pst*I site was not essential for β-galactosidase expression or replication in *E. coli*. *E. coli* colonies containing pGPB6874/*Sal* were blue on X-gal plates and red on MacConkey plates. Cotransfection of *S. frugiperda* cells with pGPB6874/*Sal* and wt AcNPV DNA gave rise to blue plaques at a frequency 1/5 to 1/10 that observed with pGPB6874.

DISCUSSION

AcNPV has many features which make it particularly attractive as a recombinant DNA vector: (i) a capacity to accommodate large passenger DNA inserts in its expandable nucleocapsids, (ii) a strong promoter which allows for high-level expression of fused passenger genes, and (iii) temporal control of gene expression which delays the synthesis of passenger gene products until infectious progeny viruses (EV) are released from the cell. The latter feature should allow AcNPV to propagate stably and to express genes encoding cytotoxic gene products. In addition, considerable research has been conducted on the mass-scale industrial production of AcNPV and its host cell cultures with a view to its use as a biological pesticide (30). The recent rapid advances made in the molecular biology of AcNPV reflect the ease of working with this virus and its permissive cell cultures. The ability to plaque purify AcNPV and readily determine its genotype gives this virus vector an advantage over production schemes employing cell cultures with integrated or minichromosome-propagated passenger sequences. Finally, baculoviruses have an additional level of biological safety which few other viruses have. They are known to infect productively only invertebrates, and the mechanism of organismal transfer in nature primarily involves the occluded form of the virus. Without polyhedrin then, transmission of AcNPV from insect to insect is limited to EV transfer via hemolymph injection.

We have reported the regulated, high-level expression of *E. coli* β-galactosidase as a polyhedrin fusion protein. It should also be relatively easy to insert a passenger gene at the translational initiation site with appropriate genetic engineering technology. Selection of recombinant viruses does not depend on blue-plaque production, since a manageable proportion of recombinant viruses are found after cotrans-

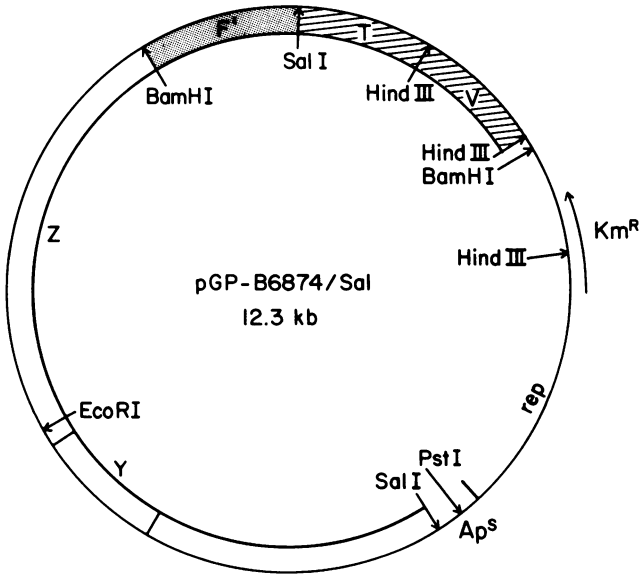

FIG. 7. Restriction map of the transplacement plasmid pGPB6874/*Sal*. The 12.3-kb plasmid pGPB6874/*Sal* contains the entire 9.2-kb plasmid pMC874 composed of the *E. coli* β-galactosidase gene (*Z*), the *lacY* gene (*Y*), a portion of the *A* gene (not marked specifically), a portion of the ampicillin resistance gene (Ap^s), a replication origin (*rep*), and the kanamycin resistance gene (Km^r). In pGPB6874/*Sal*, pMC874 is fused on one side of the *Bam*HI site to the 1.11-kb *Bam*HI-*Sal*I fragment of AcNPV *Hin*dIII-F (the stipled area designated F') and fused on the other side to a 2.04-kb *Bam*HI-*Sal*I fragment of AcNPV *Eco*RI-I containing *Hin*dIII-V and *Hin*dIII-T (hatched region). The single *Pst*I site in the inactive portion of the ampicillin resistance gene (Ap^s) may be used for passenger gene insertion.

fection. Polyhedrin substitution viruses may be screened by the lack of OV in plaques, since OVs are 5 μm in diameter and highly refractive in a light microscope. However, we have found that screening many plaques for the absence of OV is tedious. Furthermore, not all plaques expressing the passenger gene will be completely homogeneous and OV negative. Therefore, an in situ plaque antibody screening procedure or plaque hybridization screening procedure (32) may be desirable for the initial screen for recombinant viruses.

We have developed a transplacement plasmid, pGPB6874/*Sal*, to facilitate the insertion and selection of recombinant viruses carrying passenger genes which are under the control of their own promoter. Passenger genes can be inserted at the unique *Pst*I site next to the *E. coli* replication genes of the plasmid (Fig. 7) and cloned in *E. coli*. The cotransfection of the resulting plasmid with wt AcNPV and subsequent selection for blue viruses should facilitate the selection of recombinant viruses.

The specific activity of β-galactosidase in L1GP-gal3-infected cells is exceptionally high. The highest specific activity reported in yeast cells to our knowledge is one-half that observed in L1GP-gal3-infected cells at 48 h p.i. (27), and transient expression of β-galactosidase under early simian virus 40 control gives much lower specific activities (2). It is possible that the specific activity in our system may be enhanced further by attention to details such as supplying a polyadenylation site for the transcript. We do not know where mRNA chain termination or polyadenylation is occurring. It appears that polyhedrin mRNA is not spliced within the coding region, so it is not surprising that a passenger gene lacking an intron is expressed efficiently in this system.

The multiple polypeptides found in L1GP-gal3-infected cells but not in wt-infected cells from 24 through 48 h p.i. indicate additional products from pMC874-specific sequences. The origins of these polypeptides are not known but may reflect additional or alternate transcriptional, post-transcriptional, or translational phenomena. Since the proteins appear only at very late times p.i. their synthesis is probably under polyhedrin promoter control, and it thus seems likely that some alternate initiation or processing of the *lac* operon transcript or possibly posttranslational cleavage of the 120,000-dalton fusion protein is responsible for generating these discrete polypeptide species. However, none of the smaller L1GP-gal3-specific polypeptides react strongly to polyhedrin antiserum. These smaller polypeptides are individually less abundant than the 120,000-dalton protein, but collectively they constitute a significant proportion of the total cellular protein. It is probable that a passenger gene product of smaller size and greater stability will constitute an even higher proportion of total cellular protein than that observed for β-galactosidase.

The natural host range of AcNPV extends to at least 27 different lepidopteran species. It was of interest to determine whether the polyhedrin/β-galactosidase fusion protein was expressed in mammalian and *Drosophila* cells. No increase in β-galactosidase activity was observed. However, expression of polyhedrin requires the expression and function of other AcNPV gene products (16). Thus, this experiment alone does not test whether AcNPV can enter a nontarget host cell and express a limited number of early genes.

Not only will blue color, provided by β-galactosidase, facilitate passenger gene insertion into AcNPV, but also we anticipate that blue-plaque baculoviruses will be valuable in a variety of molecular biological studies of these unique viruses.

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