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Transient, Nonlethal Expression of Genes in Vertebrate Cells by Recombinant Entomopoxviruses

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The group B entomopoxvirus (EPV) from *Amsacta moorei* (AmEPV) productively infects only insect cells. A series of AmEPV-*lacZ* recombinants was constructed in which the *lacZ* gene was regulated by either late (the AmEPV spheroidin or the cowpox virus A-type inclusion [ATI]) or early (the AmEPV *esp* [early strong promoter; derived from a 42-kDa AmEPV protein] or the *Melolontha melolontha* EPV fusolin, *fus*) virus promoters. When the AmEPV recombinants were used to infect vertebrate cells, β-galactosidase expression occurred (in >30% of the cells) when *lacZ* was regulated by either the *fus* or *esp* early promoters but not when *lacZ* was regulated by the late promoters (spheroidin or ATI). Therefore, AmEPV enters vertebrate cells and undergoes at least a partial uncoating and early, but not late, viral genes are expressed. Neither viral DNA synthesis nor cytopathic effects were observed under any infection conditions. When an AmEPV recombinant virus containing the *Aequorea victoria* green fluorescent protein gene (*gfp*) under the control of the *esp* promoter was used to infect vertebrate cells at a low multiplicity of infection, single fluorescent cells resulted, which continued to divide over a period of several days, ultimately forming fluorescent cell clusters, suggesting that vertebrate cells survive the infection and continue to grow. Therefore, AmEPV may prove to be a highly efficient, nontoxic method of gene delivery into vertebrate cells for transient gene expression.

Current gene transfer vectors, which are derived from animal viruses, such as retroviruses, herpesviruses, adenoviruses, or adeno-associated viruses, are commonly engineered to remove one or more genes involved in viral replication and/or to provide the capacity for insertion and packaging of foreign genes. Each of these known vectors has some unique advantages as well as disadvantages. One primary disadvantage is an inability to readily package large DNA inserts greater than 10 kbp. Recently, baculovirus-mediated gene transfer into human hepatocytes (2, 15) has been demonstrated; this is potentially an attractive system because baculoviruses are insect viruses which do not productively infect vertebrate cells.

Entomopoxviruses (EPVs) are poxviruses of insects (22). They share common morphological and structural features with vaccinia, as well as a similar biochemical strategy, yet are quite distinct (1, 20). The *Amsacta moorei* EPV (AmEPV) is the best-studied EPV and the only one that can be readily grown to high titer in cell culture (10, 14, 30). The spheroidin and thymidine kinase (TK) genes of AmEPV have been identified and sequenced (12, 14). Spheroidin is the most highly produced AmEPV protein and can ultimately comprise >20% of the infected cell protein. The 40-kDa fusolin protein, second in abundance to spheroidin, is found in many EPVs (but is absent from AmEPV) and is associated with paracrystalline, spindle-shaped structures in infected cells. The fusolin (*fus*) genes have been cloned and sequenced from the EPVs of *Choristoneura biennis*, *Melolontha melolontha*, and *Heliothis armigera* (7, 9, 31). The *fus* gene contains a promoter similar to the typical vertebrate poxvirus late promoter. However, instead of having a typical 5′ consensus poxvirus late promoter (TAAATG), *fus* contains TAATG at the site of the ATG transcription initiation codon (1). Expression of the *lacZ* gene driven by the *M. melolontha* EPV (MmEPV) promoter in recombinant-AmEPV-infected LD-652 insect cells was not inhibited by the viral DNA synthesis inhibitor 1-β-D-arabinofuranosylcytosine (AraC) (17a), suggesting that the *fus* promoter functions as an early or an early-late promoter.

The normal host range of AmEPV is limited to lepidopteran insects (11). Indeed, early experiments examining AmEPV infection of mammalian L-929 cells provided no evidence of infection at all, as no viral gene expression was noted and there were no obvious changes in the morphology of the AmEPV-infected cells (17).

A significant feature that EPVs share with the vertebrate poxviruses, such as vaccinia, is a cytoplasmic site of replication, which dictates that all the enzymes necessary for early gene transcription be packaged within the infecting virus particle (19). Early gene expression, therefore, is independent of viral replication and depends only on previous assembly of a competent virus particle and a subsequent partial uncoating of the infecting virus after entry into the infected cell. Therefore, it is possible that early viral genes, or foreign genes under the control of early promoters, can be expressed in AmEPV-infected vertebrate cells even though the infection is abortive. In this paper, we show this to be the case. Expression of a reporter gene (β-galactosidase) was observed in more than 30% of recombinant-AmEPV-infected vertebrate cells when *lacZ* was regulated by either of two early promoters (*fus* or *esp*) but not when *lacZ* was regulated by the late promoters (spheroidin or cowpox virus A-type inclusion [ATI]). No AmEPV viral DNA synthesis was observed in vertebrate cells, nor were any cytopathic effects noted. Furthermore, CV-1 cells infected with recombinant AmEPV containing the green fluorescent protein gene (*gfp*) express GFP and continue to divide, suggesting that AmEPV offers the possibility of a highly efficient, nontoxic method of gene delivery into vertebrate cells for transient gene expression.

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MATERIALS AND METHODS

Cells and virus. AmEPV (14) stocks were prepared in IPLB-LD-652 cells (10), which were maintained at 28°C in a 50:50 mix (TE medium) of TC-100 medium (Gibco, Gaithersburg, Md.) and EX-CELL 401 medium (JRH Biosciences, Lenexa, Kans.) supplemented with 10% fetal bovine serum. A TK-negative cell line designated C11.3 was selected by a process of adaptation of TK^+ LD-652 cells to increasing levels (10-μg/ml steps), at intervals of 5 weeks, of 5-bromo-2′-deoxyuridine (BUdR), up to 100 μg of BUdR/ml over a period of 1 year, and thereafter the cells were maintained in TE medium containing 100 μg of BUdR/ml. Vaccinia virus (VV) WR strain (6) was grown at 37°C in CV-1 cells maintained in Eagle's minimal essential medium with Earle's salts (Gibco) supplemented with 5% fetal bovine serum. Other vertebrate cell lines were grown in the media recommended in the literature or by the American Type Culture Collection.

Construction of transfer vectors. pDU20*lacZ* was used to construct a spheroidin-negative recombinant AmEPV as described previously (13).

(i) pTK-ATI*lacZ*. Oligonucleotide primers RM325 (ACAGGAGCTCGAATTCAAGTTAAATATTTA) and RM326 (CACAGGATCCCTGGCAAAACAACAGAATTG), bp 1 to 20 and 748 to 729, respectively, of GenBank locus AAVTHYKIN, were used to amplify a 748-bp fragment of downstream flanking sequence with plasmid pMEGTK-1 (12). This PCR product contains a 5′ *Sac*I and a 3′ *Bam*HI site. Oligonucleotide primers RM327 (AGAGAAGCTTCAAAATGGATTTACTAAATTC) and RM328 (CACAGTTAACGAATTCATATTCAATTATAT), bp 848 to 868 and 1511 to 1492, respectively, of GenBank locus AVVTHYKIN, were used to amplify a 663-bp fragment of upstream flanking sequence with pMEGTK-1. This fragment contains a 5′ *Hin*dIII and a 3′ *Hin*cII site. These two fragments were separately inserted into the *Sac*I-*Bam*HI and *Hin*dIII-*Hin*cII sites of pBluescript I SK(+) to produce an intermediate plasmid, pDUTK. A cowpox virus ATI promoter-driven *lacZ* gene cassette, which was from plasmid pATI*lacZ* (13), was digested with *Kpn*I, blunted with T4 DNA polymerase, and digested with *Pst*I. This ATI promoter-driven *lacZ* gene cassette was inserted into the *Eco*RV and *Pst*I site of pDUTK to produce pTK-ATI*lacZ*.

(ii) pTK-fus*lacZ*. The MmEPV early *fus* gene promoter was PCR amplified with the following primers and the pHF51 plasmid (9), which contained the whole *fus* gene as a template: RM504 (ACAGGATCCGTACGTATATTAATCATGATT) and RM505 (GACCCATGGTAAAGATCTTTGGTAATAATA). This 275-bp fragment containing 5′ *Bam*HI and 3′ *Nco*I sites was digested by *Bam*HI only and inserted into *Bam*HI and *Sma*I sites of pDUTK to produce the pTK-fus plasmid. The *lacZ* gene digested with *Pst*I and *Sma*I from pMC1871 (Pharmacia Biotech, Inc., Piscataway, N.J.) was inserted into pTK-fus, which was first digested with *Nco*I, blunted with T4 DNA polymerase, and digested with *Pst*I to produce pTK-fus*lacZ*, where the *fus-lacZ* cassette is flanked by AmEPV TK gene sequences.

(iii) pTK-esp*lacZ*. One hundred and sixty-nine additional base pairs of 5′ AmEPV 42-kDa protein gene promoter sequence beyond that already presented (23) was derived by sequencing an appropriate *Ssp*I clone of AmEPV DNA identified from a library prepared by ligating 3- to 4-kb fragments of partially *Ssp*I-digested AmEPV DNA. The DNA fragments were cloned into *Sma*I-cut pUC19, and the library was screened with a 42-kDa gene promoter-specific PCR-produced radiolabeled probe amplified from genomic AmEPV template with primers RM558 (TCAAAAAATATAAATGATTCACC) and RM520 (GACCCATGGCGATTTTATATTGTAATTATA). The design of both primers was based upon the known sequence (23). A 285-bp fragment of the AmEPV early strong promoter (*esp*, derived from the AmEPV 42-kDa protein) (23, 29) was PCR amplified with the following primers and appropriate AmEPV viral DNA as a template: RM519 (ACAAGATCTATAATAATGTAAAATCGCAGT) and RM520. This fragment, containing 5′ *Bgl*II and 3′ *Nco*I sites, was digested with *Nco*I and inserted into *Sna*BI and *Nco*I sites of pTK-fus*lacZ* to produce pTK-esp*lacZ* by replacement of the *fus* promoter.

(iv) pTK-esp*gfp*. A green fluorescent protein gene (*gfp*) was PCR-amplified with the following primers from pTR-UF5 plasmid DNA (Vector Core, University of Florida) (32) as a template: RM547 (AGTCTCATGAGCAAGGGCGAGGAAC) and RM548 (ACCCAAGCTTCCGCGGCCGCTCACTTGTAC). This 285-bp fragment, which contained 5′ *Bsp*HI and 3′ *Hin*dIII sites, was inserted into the *Nco*I (*Nco*I is compatible with *Bsp*HI) and *Hin*dIII sites of pTK-esp*lacZ*; thus, the *lacZ* gene of pTK-esp*lacZ* was replaced by the *gfp* gene to produce pTK-esp*gfp*.

Production and concentration of recombinant viruses. AmEPV recombinants with inserts in the spheroidin gene were obtained as described previously (13).

Selection of AmEPV TK^- recombinants, where reporter gene inserts are targeted for the AmEPV TK gene, was facilitated by the use of C11.3 cells, TK^- derivatives of LD-652 cells. The same conditions of transfection and purification as for preparation of spheroidin-negative AmEPV were used except for the agarose overlay, which contained 100 μg of BUdR/ml, necessary for selection of AmEPV TK^- virus.

Extracellular virus was concentrated by centrifugation in a Beckman SW28 rotor at 20,000 rpm for 2 h. The virus pellet was resuspended in phosphate-buffered saline (PBS). Virus titer was estimated by plaque assay on LD-652 insect cells.

β-Galactosidase activity assay and staining procedures. IPLB-LD-652 cells (2×10^5/well) or CV-1 cells (4×10^5/well) in 12-well plates were infected with recombinant viruses at a multiplicity of infection (MOI) of 10 PFU/cell for 2 h, and after removal of the inoculum, the cells were rinsed with serum-free TE medium or minimal essential medium. One milliliter of fresh medium was added to each well. At appropriate times postinfection, the infected cells were harvested by scraping them into the medium and freezing them at −70°C until assayed. The β-galactosidase activity assays were performed with *o*-nitrophenyl-β-D-galactopyranoside as the substrate as described previously (18). β-Galactosidase activity was estimated by measuring the absorbance at 420 nm and then normalized as optical density at 420 nm per mg of total protein (3).

To visualize β-galactosidase-positive cells, 2 ml of X-Gal (5-bromo-4-chloro-3 indolyl-β-D-galactopyranoside) solution (300 μg/ml in serum-free medium) was added to 65-mm-diameter standard plaque assay dishes 48 h after addition of the agarose overlay. Plaques were evaluated after further incubation for 12 h at 37°C.

Dot blot analysis of viral DNA synthesis. Insect LD-652 or vertebrate CV-1 cells (1.5×10^6) were infected with VV or AmEPV *lacZ* recombinant viruses at an MOI of 5 PFU/cell. Virus was adsorbed for 2 h, the inoculum was removed, the cells were rinsed with PBS, and fresh medium was added. At various times postinfection, the infected cells were harvested by scraping them into the medium, and both released virus and infected cells were collected by centrifugation at 11,700 × *g* for 30 min. DNA was then isolated from samples with the QIAamp tissue kit (Qiagen) as suggested by the manufacturer. The purified DNA was diluted to a final volume of 220 μl in TE. Individual DNA samples (80 μl) were denatured by the addition of 200 μl of 0.125 N NaOH, and 10-fold dilutions were prepared and immobilized on Gene Screen hybridization transfer membranes with a vacuum dot blot apparatus. The membrane was probed with *lacZ* DNA released from plasmid pMC1871 (Pharmacia) by *Bam*HI and radiolabeled with [α-^{32}P]dCTP by random oligonucleotide extension.

RESULTS

Expression of β-galactosidase in recombinant-AmEPV-infected vertebrate cells. In order to more definitively evaluate the ability of EPVs to infect vertebrate cells, we constructed AmEPV recombinants with a *lacZ* reporter gene under the control of either late (the cowpox virus ATI or AmEPV spheroidin) or early (AmEPV *esp* or MmEPV *fus*) promoters. The AmEPV recombinants were then used to infect CV-1 cells, and after 24 h, infected cells were evaluated for β-galactosidase expression by staining with X-Gal. Where *lacZ* was under the control of a late promoter, either the spheroidin (Fig. 1B) or ATI (Fig. 1C) promoter, no β-galactosidase synthesis was observed. However, when the *lacZ* gene was under the control of either of two early EPV promoters, *fus* (Fig. 1D) or *esp* (Fig. 1E), expression of β-galactosidase was readily observed in infected CV-1 or human liver Huh-7 cells (Fig. 1F). Despite the fact that at least 30% of the cells appeared to be positive for the synthesis of β-galactosidase, no cytopathic effects were observed under any conditions. Indeed, in separate experiments, we have analyzed cells infected at MOIs of up to 50 with either *esp* or *fus* promoter-regulated *lacZ* AmEPV recombinant infectious particles per cell. Under these conditions, nearly all the cells become β-galactosidase positive yet >99% of the cells remain viable for up to 48 h after infection, as judged by their exclusion of trypan blue (17b). These results suggest that AmEPV enters the vertebrate cells and undergoes sufficient uncoating to allow early gene expression mediated by the virus core, after which the infection aborts, probably prior to DNA replication, which is required for expression of late genes to occur.

Although within the levels of activity revealed by the staining procedure, recombinant AmEPV *lacZ* infection of vertebrate cells gives no *lacZ* expression when *lacZ* is under the control of a late promoter; if such cells (Fig. 1B and C) are coinfected with VV, β-galactosidase expression from either the recombinant AmEPV ATI *lacZ* or spheroidin *lacZ* gene contained within the recombinant AmEPV is readily observable (Fig. 2B and C, respectively). These results suggest that VV can provide, in *trans*, the viral components necessary to provide at least some activation of late AmEPV gene transcription and that coinfection with AmEPV does not preclude the possibility of late gene expression.

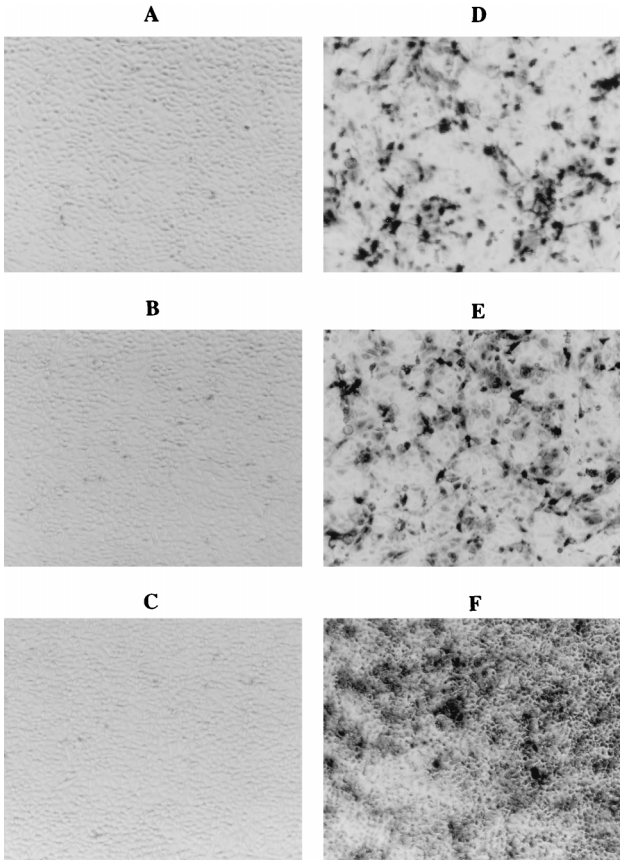

FIG. 1. Expression of *lacZ* in recombinant-AmEPV-infected mammalian cells. CV-1 cells were mock infected (A) or infected with various AmEPV *lacZ* recombinants, where *lacZ* was under the control of the cowpox virus late ATI gene promoter (B), the late AmEPV spheroidin promoter (C), the *M. melolontha* early *fus* promoter (D), or the AmEPV early *esp* promoter (E). Infection of human Huh-7 liver cells with the AmEPV *esp-lacZ* recombinant is also shown (F). The infected cell monolayers were stained with X-Gal 24 h postinfection.

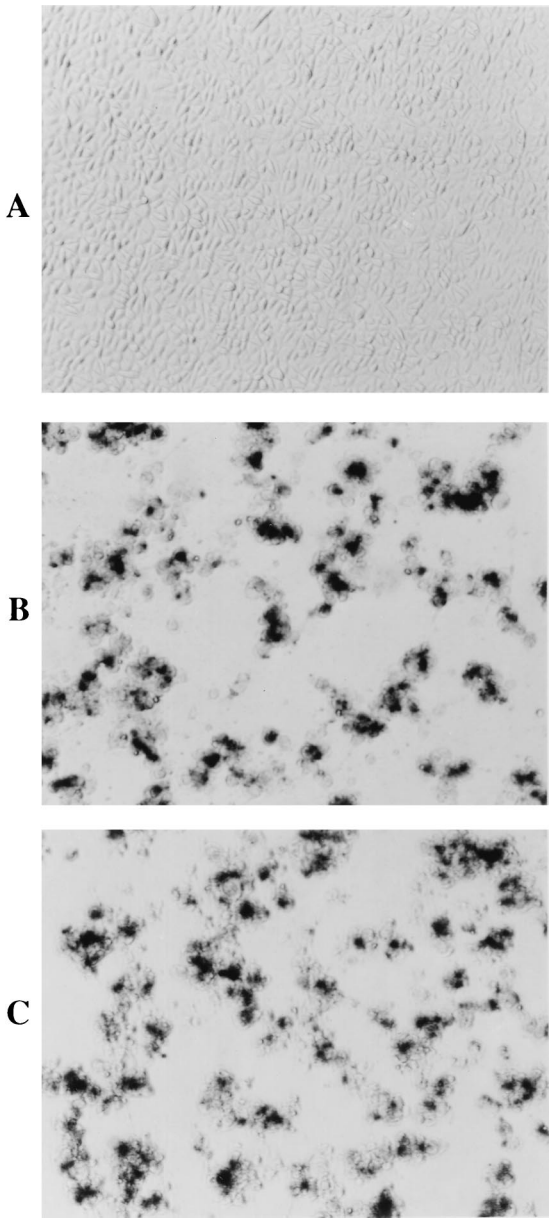

FIG. 2. The effect of vaccinia coinfection on the activation of late AmEPV promoters. CV-1 cells were infected with either AmEPV ATI-*lacZ* or AmEPV spheroidin-*lacZ* recombinants at an MOI of 10 PFU/cell. At 2 h postinfection, the virus was removed and the cells were coinfected with wild-type VV. Twenty-four hours later, the infected cell monolayers were stained with X-Gal. (A) Mock-infected cells; (B) AmEPV ATI-*lacZ* and vaccinia coinfected cells; (C) AmEPV spheroidin-*lacZ* and vaccinia coinfected cells.

Comparison of AmEPV-mediated *lacZ* expression in infected vertebrate and insect cells. Vertebrate CV-1 and insect LD cells were infected with either *fus-lacZ* or *esp-lacZ* AmEPV recombinants, and the synthesis of β-galactosidase was monitored as described in Materials and Methods. As shown in Fig. 3, β-galactosidase activity was detected as early as 2 h after infection for the *esp-lacZ* construct in both vertebrate and insect cells and by 6 h in the case of *fus-lacZ*, after which the levels increased until about 16 h postinfection. The cell lines had little effect on the levels of expression observed for a given promoter, but it does appear that the *esp* promoter is intrinsically somewhat more active than the *fus* promoter.

Susceptibility of different vertebrate cell lines to AmEPV-mediated gene transfer. In order to evaluate the generality of AmEPV's ability to infect vertebrate cells, the *esp-lacZ* AmEPV recombinant was used to infect a variety of vertebrate cell lines and β-galactosidase expression was quantified by measuring levels of β-galactosidase and qualitatively evaluated by determining the percentage of X-Gal staining of infected cells. Mock-infected and AmEPV ATI-*lacZ* recombinant-infected cells were used as controls. While all cell lines except EL-4 were susceptible to infection, it appeared that cell lines derived from liver and kidney were more sensitive than those of lymphoid lineage (Fig. 4). Levels of β-galactosidase-positive cells in excess of 30% as determined by X-Gal staining were readily obtained with 24 h of infection (data not shown). Of the 15 cell lines assayed, no β-galactosidase was detected nor were X-Gal-positive cells observed in either mock-infected or AmEPV ATI-*lacZ* or AmEPV *spheroidin-lacZ* recombinant-infected cells (data not shown).

Persistence of AmEPV particles in vertebrate cells. Although AmEPV infection of vertebrate cells is abortive, the ability of the late-promoter-regulated *lacZ* gene within AmEPV recombinants to be activated by coinfection with VV allows us to ask how long after infection late promoters within AmEPV are subject to activation. CV-1 cells were infected with recombinant AmEPV ATI-*lacZ* at an MOI of 10 PFU/

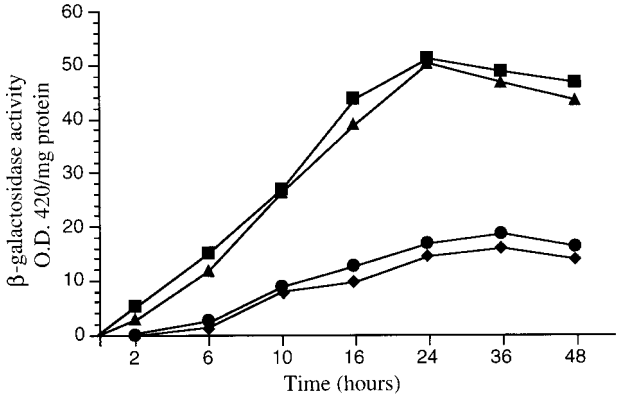

FIG. 3. Comparison of recombinant-AmEPV-mediated β-galactosidase expression in insect and vertebrate cells. CV-1 (37°C) or insect LD-652 cells (28°C) were infected with AmEPV *fus-lacZ* or *esp-lacZ* at an MOI of 10 PFU/cell. After 2 h of incubation, the virus was removed and replaced with fresh medium. Infected cells were harvested at the times indicated, and extracts were prepared and assayed for β-galactosidase activity. Each point represents the average of three independent assays of AmEPV *esp-lacZ*-infected LD (■) or CV-1 (▲) cells and AmEPV *fus-lacZ*-infected LD (●) or CV-1 (◆) cells. O.D. 420, optical density at 420 nm.

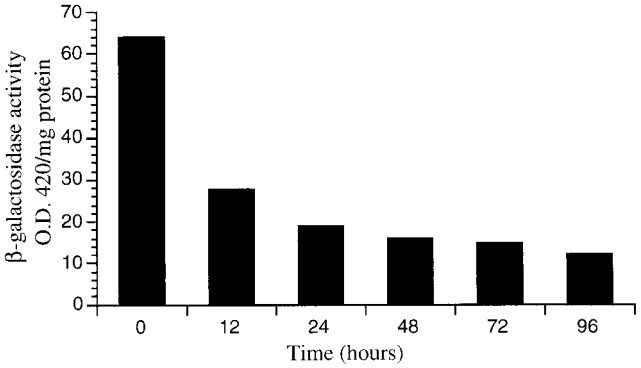

FIG. 5. Persistence of AmEPV in mammalian cells. CV-1 cells were infected with recombinant AmEPV ATI-*lacZ* at an MOI of 10 PFU/cell. At 2 h postinfection, virus was removed and fresh medium was added. After 0, 12, 24, 48, 72, and 96 h of incubation, the medium was removed and the cells were coinfected with wild-type VV. After 24 h of vaccinia infection, cells were harvested and β-galactosidase activity assays were performed as described in Materials and Methods. Each bar is the average of three independent assays. O.D. 420, optical density at 420 nm.

cell. At 2 h postinfection, the virus inoculum was removed and fresh medium was added. Immediately thereafter and 12, 24, 48, 72, and 96 h later, the AmEPV ATI-*lacZ*-infected CV-1 cells were superinfected with VV at an MOI of 10 PFU/cell. At 24 h postinfection with AmEPV or 24 h after the superinfection with VV, infected cells were both stained with X-Gal (data not shown) and assayed for the synthesis of β-galactosidase (Fig. 5). Although interpretation of this experiment may be complicated by superinfection exclusion of the superinfecting VV, particularly at later times (a phenomenon well documented for vertebrate poxvirus infections [5]), a significant induction of β-galactosidase activity was readily detected after VV superinfection even when the superinfection was delayed as long as 4 days after the initial AmEPV infection.

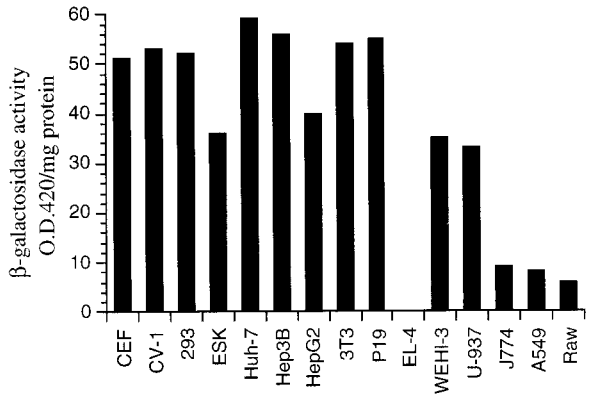

FIG. 4. β-Galactosidase expression of AmEPV *esp-lacZ* in different vertebrate cell lines. Standard conditions for infection were as follows: 4×10^5 cells/well in a 12-well plate, seeded 1 day before infection and infected with AmEPV *esp-lacZ* recombinant virus at an MOI of 10 PFU/cell. Cells were exposed to virus for 2 h, the virus-containing medium was removed, cells were washed with PBS, and fresh medium was added. At 24 h postinfection, β-galactosidase activity assays were performed as described in Materials and Methods. Endogenous background levels of enzyme activity present in uninfected cells were subtracted in all cases. The following cell lines were used: CEF, primary chicken embryo fibroblast cells; CV-1, African green monkey kidney epithelial cells; 293, human kidney cells; ESK, pig kidney cells; Huh-7, human hepatocarcinoma cells; Hep3B, human hepatocarcinoma cells; HepG2, human hepatocarcinoma cells; NIH/3T3, mouse embryo fibroblast; P19, mouse embryonal carcinoma; EL-4, mouse lymphoma; WEHI-3, mouse myelomonocyte; U-937, human histiocytic lymphoma; J774, mouse monocyte macrophage; A549, human lung carcinoma; and Raw, mouse monocyte-macrophage. O.D. 420, optical density at 420 nm.

AmEPV viral DNA is not replicated in vertebrate cells. The data in Fig. 1 clearly show that while AmEPV infection of vertebrate cells results in early-promoter-regulated gene expression, late promoters, whether those derived from vertebrate poxviruses or the extremely active AmEPV late spheroidin promoter, are completely inactive. During the poxvirus replicative cycle, the initiation of viral DNA replication is a prerequisite for late viral gene expression. If AmEPV infection of vertebrate cells were to abort prior to DNA replication, then no late gene expression would occur. Therefore, AmEPV-infected CV-1 cells were examined for viral DNA synthesis (Fig. 6). As expected, examination of AmEPV-infected LD-652 cells showed viral DNA synthesis had begun by 12 h postinfection (Fig. 6A), consistent with our earlier results (30). However, no AmEPV DNA synthesis was observed in infected CV-1 cells (Fig. 6B), as there was no increase in the amount of DNA over that of input virus. As a control, DNA synthesis was readily detected when VV was used to infect CV-1 cells (Fig. 6C).

AmEPV infection of mammalian cells is nonlethal. Normally, poxvirus infections are lethal and are accompanied by extensive cytopathic effects. However, despite the fact that AmEPV infection of vertebrate cells is accompanied by early gene expression, the infected cells failed to exhibit any cytopathic effects, suggesting that such cells might survive the infection. In order to address this question, we constructed a recombinant AmEPV which contained the green fluorescent protein gene (*gfp*) under the control of the *esp* promoter. CV-1 cells were infected at a low MOI, and 18 h later, single infected cells expressing green fluorescent protein were identified by fluorescent microscopy. The pattern of fluorescence emanating from these single cells was then monitored for an additional 3 days. The fate of one such typical cell is shown in Fig. 7. We routinely note that the single fluorescent cells observed 18 h after infection (Fig. 7A) have divided by 24 to 26 h (Fig. 7B and C) and by 50 h have become small fluorescent clusters of cells. The fluorescence intensity per cell steadily diminishes, consistent with the kinetic data for early AmEPV gene expression (Fig. 3), which suggests that active synthesis has ceased by 24 h

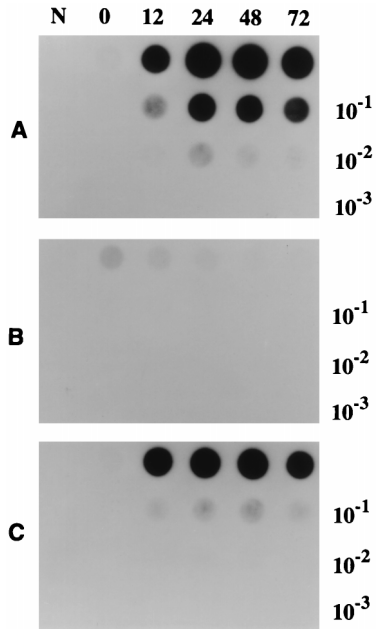

FIG. 6. AmEPV DNA synthesis in insect and vertebrate cells. DNA synthesis was examined in AmEPV spheroidin-*lacZ*-infected insect LD-652 (A) or vertebrate CV-1 (B) cells and vaccinia spheroidin-*lacZ*-infected CV-1 cells (C). Cells were infected at an MOI of 5, the virus was adsorbed for 2 h and removed, and fresh medium was added. Dishes of cells were harvested after 0, 12, 24, 48, and 72 h of subsequent incubation. Lane N, mock infected. DNA synthesis was then measured by dot blot analysis with radiolabeled *lacZ* DNA as a probe as described in Materials and Methods.

postinfection and thereafter the fluorescence per cell diminishes as the GFP is distributed among dividing daughter cells. These results suggest that AmEPV is not toxic to vertebrate cells and that the infected cells survive the infection and continue to divide. Consistent with cell survival are our observations that at multiplicities of up to 50 infectious AmEPV particles per mammalian cell, virtually all the cells continue to exclude trypan blue for at least 48 h (17b).

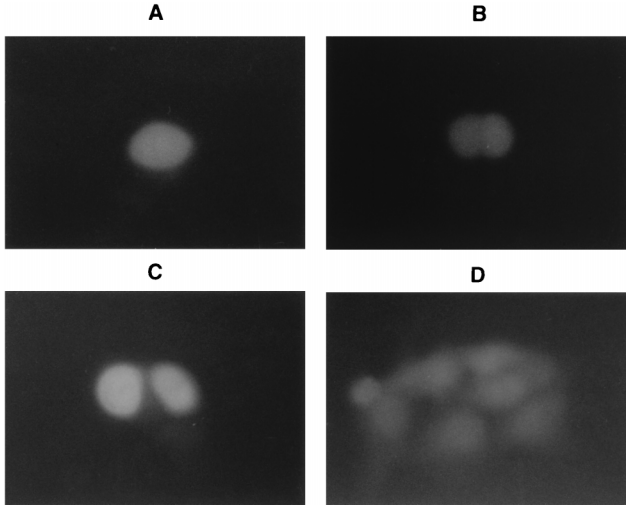

FIG. 7. The survival of mammalian cells following infection by recombinant AmEPV *esp-gfp*. Subconfluent CV-1 cells were infected with AmEPV *esp-gfp* at an MOI of 1 PFU/cell. The individual fluorescent cells were located and followed over a period of 2 to 3 days and periodically photographed with a fluorescence microscope. One fluorescent cell, identified 18 h postinfection (A), had divided into two cells by 24 (B) to 26 (C) h postinfection and by 50 h had become a small cluster of dividing cells (D).

DISCUSSION

It has long been known that AmEPVs do not productively infect vertebrate cells (17). Indeed, in earlier studies, the lack of any cytopathology and the inability of investigators to detect any AmEPV proteins following the addition of virus to mouse L-929 cells raised the possibility that the virus might not even enter the vertebrate cells. The experiments presented here conclusively show that AmEPV does enter vertebrate cells and uncoat sufficiently to express early genes (Fig. 1). Since AmEPV, like other poxviruses, packages the enzymes necessary for early gene transcription from the previously infected cell, the virus need only enter a cell and undergo partial uncoating in order for early genes to be transcribed. Indeed, the transcription of only early genes and the failure to observe any viral DNA replication (Fig. 6) or subsequent late gene expression (Fig. 1) are all consistent with a block early in the viral cycle.

Despite the overall similarities to VV and the fact that AmEPV enters vertebrate cells, partially uncoats, and expresses early genes, the inability of AmEPV to grow in vertebrate cells makes it clear that there are factors required for growth in vertebrate cells, lacking in AmEPV, which preclude a productive infection. A more specific example which reflects this specificity is that the promoter regulating the AmEPV spheroidin gene, which is abundantly expressed during infection of insect cells, fails to function when the spheroidin gene is cloned into VV (13).

One possible explanation for the abortive infection of vertebrate cells by AmEPV prior to late gene expression relates to the nature of the uncoating of the input virus, which is a two-step process. The first step of uncoating is cell mediated, whereas the second step requires either a cellular component induced by viral infection or a virus-encoded early protein (25). The factor(s) provided by vaccinia virus then might influence the AmEPV uncoating process or, alternatively, might influence DNA replication and aspects of late gene transcription directly.

Coinfection of CV-1 cells with AmEPV and VV, however, allows at least partial rescue of AmEPV late gene expression (Fig. 2). Therefore, it is quite likely that at least some of the factors necessary for AmEPV growth in CV-1 cells are virus (vaccinia) encoded and are either missing or not functional in AmEPV.

Although the efficiency diminishes with time, rescue of late-promoter-regulated *lacZ* within AmEPV can occur for at least 4 days after the initial AmEPV infection, suggesting that the AmEPV DNA remains cytoplasmic and in a state resistant to degradation for extended periods of time after early gene expression has ceased. Interestingly, mixed VV and AmEPV infections of vertebrate cells do not result in any net production of infectious AmEPV particles. When CV-1 cells were infected with both an AmEPV β-galactosidase recombinant virus and VV, incubated for 48 h, and harvested and the presence of AmEPV particles was assayed by titration on LD cells and scoring for β-galactosidase-positive plaques, the level of infectious AmEPV never exceeded one particle per cell. However, the levels of infectious AmEPV were somewhat higher than those observed for CV-1 cells infected with AmEPV alone, where the levels of released virus were approximately 0.1 infectious AmEPV particle per cell (17b). The failure of mixed infections in vertebrate cells to result in the net production of AmEPV despite at least some activation of late AmEPV promoters suggests that interruption of AmEPV development can occur at steps both prior to and after the initiation of late AmEPV gene expression.

Even though AmEPV-mediated gene expression in vertebrate cells is limited to genes under the control of early promoters, the expression continues for an extended period of time, up to 20 to 24 h (Fig. 3). This is in contrast to vaccinia-mediated early gene expression, which is quite limited and usually ceases at the onset of DNA replication (3 to 4 h). The kinetics of AmEPV-directed early gene expression are quite similar in vertebrate and insect cells and may be solely mediated by the virus.

We observe no cytopathology in AmEPV-infected vertebrate cells, which continue to exclude trypan blue for at least 48 h postinfection (17b). The recent availability of the jellyfish GFP (4) as a reporter protein and the subsequent "humanization" and other modifications to increase sensitivity in higher eukaryotic environments (21, 32) have allowed us to examine the fate of AmEPV-infected cells. The advantage of GFP is that quantitation is simple and nondestructive so that cells can be monitored over a period of time. Vertebrate CV-1 cells infected with an AmEPV *esp-gfp* recombinant gave rise to single fluorescent cells, which after time proliferated into a microcolony of infected cells (Fig. 7). These results clearly indicate that the cells survive the infection and continue to divide.

The nature of the abortive AmEPV infection in vertebrate cells is strikingly similar to that recently reported for baculoviruses, as is the bias for high levels of expression in hepatic cells and poor expression in lymphoid cells (2, 15). Baculovirus infections of vertebrate cells are also abortive and result in only limited gene expression. These similarities are somewhat surprising because baculoviruses, which develop in the nucleus, provide a sharp contrast in developmental strategy to the poxviruses, which develop in the cytoplasm. It is also noteworthy that abortive infections have also been observed among the vertebrate poxviruses. For example, when an avipoxvirus is used to infect mammalian host cells the infection is abortive, yet significant gene expression occurs (8, 16, 24, 27, 28). Indeed, these properties have been cited as major safety factors for the development of avipoxvirus vectors for use as mammalian vaccine vectors.

Molecular insight into EPV development, though not nearly as extensive as that for baculoviruses, can in large part be inferred from the much more extensively studied vertebrate poxviruses, such as vaccinia, even though there are facets of the infection which are clearly virus specific. Poxviruses, including AmEPV, also possess a very large capacity for foreign genes (26). These advantages, plus their ability to infect vertebrate cells with little or no cell death, make recombinant AmEPV viruses excellent candidates to develop for delivery of genes for transient expression in vertebrates.

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