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Expression of Vaccinia E3L and K3L Genes by a Novel Recombinant Canarypox HIV Vaccine Vector Enhances HIV-1 Pseudovirion Production and Inhibits Apoptosis in Human Cells

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Poxviruses that are attenuated for growth in human cells provide a safe means of HIV antigen expression and are capable of eliciting HIV-specific immune responses, including $CD8^+$ cytotoxic T-lymphocyte (CTL) responses. HIV-1 antigen expression in human cells by attenuated poxvirus vectors may be limited by interferon-mediated host defense mechanisms. To enhance HIV antigen expression in human cells, the vaccinia virus E3L and K3L genes were inserted into a canarypox vector that expresses HIV-1 Gag, Env, and a Nef/Pol polyepitope string. E3L and K3L markedly reduced the activation of the double-stranded RNA-dependent protein kinase, PKR, and led to a significant reduction in apoptosis in HeLa cells. Production and release of HIV-1 antigen in the form of pseudovirions was enhanced in both duration and magnitude by this vector modification. The addition of immunomodulatory genes to attenuated poxviruses represents a novel strategy for enhancing antigen production by live vector HIV vaccine candidates.

Key Words: HIV-1; pseudovirion; HIV vaccine; canarypox virus; PKR; E3L; K3L.

INTRODUCTION

Live virus vectors expressing human immunodeficiency virus (HIV) antigens are essential components of many HIV vaccine regimens currently under evaluation in human trials. One reason that live vector HIV vaccine candidates hold a prominent position in current trials is because they have proven much more effective at generating HIV-specific cytotoxic T-lymphocyte (CTL) responses than have subunit protein approaches (Letvin, 1998). Recent studies using the SIV/macaque model of AIDS pathogenesis have established the importance of CTL in controlling viremia (Schmitz *et al.,* 1999) and have indicated that induction of a $CD8^+$ CTL response by a vaccination regimen can control viral replication and protect animals from disease (Barouch *et al.,* 2000; Letvin *et al.,* 1999). Recombinant poxviruses have been established as some of the leading candidate products for inducing HIV-specific CTL responses in humans.

Recombinant vaccinia viruses achieve high levels of antigen expression and replicate well within human cells. However, concerns with the potential toxicity of wild-type vaccinia viruses in humans have led to the advancement of highly attenuated vaccinia strains that are unable to replicate in human cells (Moss *et al.,* 1996). Modified vaccinia Ankara (MVA) is an attenuated vaccinia strain that was derived by greater than 500 serial passages of the Ankara vaccinia strain on chicken embryo fibroblasts. MVA harbors multiple genomic deletions that alter the host range of the virus, rendering it replication-deficient in most but not all mammalian cell lines (Carroll and Moss, 1997; Meyer *et al.*, 1991). NYVAC is an attenuated vaccinia strain that was created by engineering deletions of virulence genes from the Copenhagen strain of vaccinia virus (Tartaglia *et al.,* 1992b). Canarypox viruses are members of the *avipoxvirus* genus that are naturally restricted to replication in avian cells (Taylor *et al.,* 1995).

Recombinant canarypox vaccine vectors have been evaluated in phase I and phase II clinical trials in humans, where they have generated significant immune responses. ALVAC is the designation given to a canarypox virus vector system derived from a plaque-purified isolate from the attenuated canarypox strain Kanapox (Tartaglia *et al.,* 1992a). When combined with a subunit envelope glycoprotein boost, ALVAC-HIV vaccines have been shown to successfully prime neutralizing antibody responses directed against HIV-1 laboratory strains and against a limited number of primary isolate viruses (Mulligan and Weber, 1999; Verrier *et al.,* 2000). $CD8^+$ cytotoxic T-lymphocyte responses have been elicited in 56–64% of volunteers receiving ALVAC-HIV vectors, and CTL responses in some volunteers have been detected up to 2 years following vaccination (Evans *et al.,* 1999; Fleury

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et al., 1996; Mulligan and Weber, 1999). An important finding from recent trials was the finding that ALVAC-based vaccine regimens elicit CTL responses that can kill target cells infected with HIV from multiple clades (Ferrari *et al.,* 1997). Despite these encouraging results, improvements to ALVAC-HIV vectors are warranted to enhance HIV-specific responses. CTL responses in vaccines are often detectable at only one or a few time points during a trial, suggesting that the magnitude or durability of the response in most vaccinees is limited (Tartaglia *et al.,* 1998b). Although the magnitude of the CTL response required for vaccine efficacy is not known, it would be desirable to enhance the current ALVAC-HIV vectors to elicit cellular immune responses that are more sustained, more frequently detected, and present in a higher proportion of vaccine recipients. One potential method to achieve this goal is by engineering vectors that generate more HIV-1 antigen in human cells and sustain their production for a longer period of time.

We recently characterized HIV-1 antigen expression by ALVAC vector vCP205 in avian, simian, and human cell lines (Fang *et al.*, 1999). This vector, which encodes HIV-1 Gag and protease proteins and a membrane-anchored gp120 molecule, was found to be capable of eliciting the formation of Gag-Env pseudovirions in a wide variety of cell lines. However, the amount of Gag antigen expressed by vCP205 was strictly cell-type dependent, with some cell lines (BSC-40, Vero, C2C12) capable of producing large amounts of released pseudovirion particles, while others generated very low amounts of released particles. Although there were some differences in the efficiency of particle release between individual cell lines, most of the restriction in pseudovirion production in low-producing cell lines was due to poor intracellular antigen expression (Fang *et al.*, 1999). Especially striking was the poor production of pseudovirions by vCP205 in human cell lines. Given the safety profile and proven but limited success of ALVAC-HIV vectors as immunogens in humans, it is logical to pursue methods of enhancing the expression of HIV antigens by these vectors in human cells. One method of enhancing antigen expression is through inhibition of IFN-stimulated host defense mechanisms that limit viral antigen expression in human cells.

Most viral infections generate double-stranded RNA (dsRNA) as a replication intermediate of the viral life-cycle. PKR is a well-characterized IFN-induced gene product that binds to dsRNA and plays a key role in the host response to viral infection. PKR binds to dsRNA through binding motifs in its amino terminus; this binding promotes PKR dimerization and autophosphorylation (Wu and Kaufman, 1997). Phosphorylated, dimeric PKR represents the active form of the enzyme that can recognize and phosphorylate the eukaryotic translation initiation factor eIF-2 on its α-subunit (Wek, 1994). Phosphorylated eIF-2 complexed with GDP then forms a stable complex with eIF-2B, sequestering this translation factor from participating in subsequent translation initiation events. In this manner, PKR activation by viral infections restricts viral as well as cellular protein synthesis. To overcome this IFN-induced antiviral defense mechanism, numerous viruses have evolved methods to inhibit PKR function (reviewed in Gale and Katze, 1998). The interferon resistance of vaccinia virus has been mapped to two ORFs, E3L and K3L (Beattie *et al.,* 1995a,b). The E3L gene product is a dsRNA-binding protein that sequesters the dsRNA normally produced during viral replication, preventing activation of PKR (Davies *et al.,* 1993). K3L is a homolog of EIF-2α and downregulates PKR activity by acting as a competitor substrate for PKR-mediated phosphorylation (Davies *et al.,* 1993, 1992; Jagus and Gray, 1994). PKR activation following viral infection is also a potent stimulus for apoptosis, and E3L deletion from vaccinia virus results in greatly enhanced apoptosis in infected HeLa cells (Lee and Esteban, 1994).

We sought to test the hypothesis that inhibition of PKR by coexpression of the vaccinia virus E3L and K3L gene products would remove the relative restriction to antigen production in human cells observed with recombinant canarypox viruses, thus stimulating enhanced pseudovirion production and the potential for greater immunogenicity. The data supporting this approach included the following: (1) deletion of K3L and/or E3L from vaccinia reduced viral gene expression in human cells (Tartaglia *et al.,* 1998a); (2) deletion of E3L was found to reduce vaccinia virus propagation in human cells (Beattie *et al.,* 1996); (3) deletion of E3L and K3L was not found to debilitate vaccinia virus propagation on avian-derived cells (Beattie *et al.,* 1996); and (4) canarypox virus does not encode gene products with homologous functions to E3L and K3L (Tartaglia *et al.,* 1998a and unpublished observations). In this study, we demonstrate that the addition of the vaccinia virus E3L and K3L genes to the genome of a recombinant ALVAC-HIV vaccine candidate vector inhibited activation of PKR following viral infection. The modified vector, vCP1452, demonstrated markedly reduced apoptosis in HeLa cells. The magnitude and duration of HIV-1 pseudovirion production following infection with vCP1452 was greater than that elicited by vCP205 in each of four human cell lines tested. These results may have bearing on vector choices for future poxvirus-based HIV vaccine trials. The addition of immunomodulatory genes to a naturally attenuated poxvirus vaccine represents a promising approach to HIV vaccine design.

RESULTS

Generation of recombinant ALVAC vCP1452

The ALVAC-HIV vector that has been best characterized in studies *in vitro* and in human trials is vCP205. This recombinant vector includes an HIV_{LAI}*gag-pro* expres-

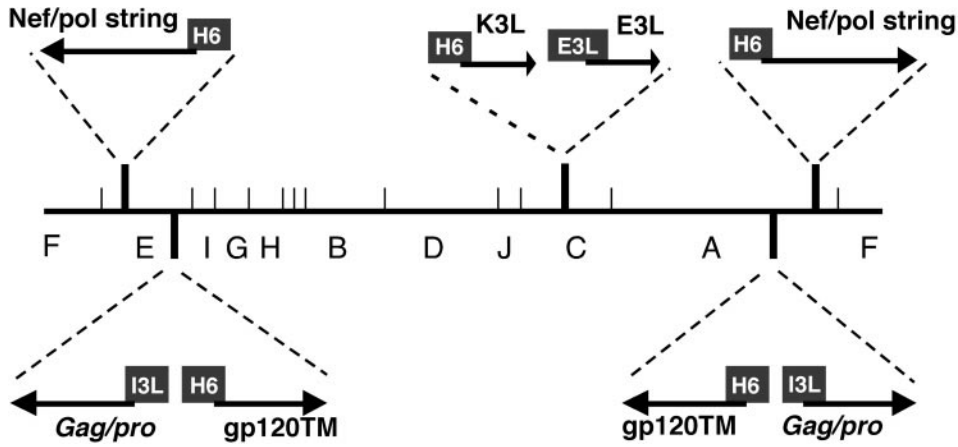

FIG. 1. Genome map of vCP1452. Insertion of HIV-1 genes and of vaccinia E3L, K3L genes are displayed on a schematic *Xho*I restriction map of the ALVAC genome. Solid boxes represent poxvirus promoter elements. Arrows indicate transcriptional orientation.

sion cassette and an envelope expression cassette that creates a fusion of the HIV-1_{MN} gp120 protein to the 18 amino acid transmembrane region from gp41 of HIV-1_{LAI}. To enhance the breadth of the CTL response elicited by this vector, a polyepitope string encoding Pol and Nef MHC class I restricted CTL epitopes was inserted into vCP205 to create vector vCP1433. The design and construction of both of these vectors has been previously described (Tartaglia *et al.,* 1998a,b). To achieve enhanced antigen expression from previous ALVAC-based HIV vaccine vectors, expression cassettes for the vaccinia virus E3L and K3L genes were created and inserted into the existing ALVAC-HIV vector vCP1433 to create recombinant virus vCP1452. A schematic diagram of the genome of vCP1452 is illustrated in Fig. 1. This recombinant virus contains three engineered HIV gene expression cassettes (*gag-pro*, gp120/TM, and a Nef/Pol polyepitope string) and expression cassettes for two vaccinia virus genes (E3L, K3L) (Fig. 1). The *gag/pro/env* expression cassette was inserted in the C3 locus, the *nef/pol* string was inserted in the C5 locus, and the E3L, K3L expression cassette was inserted in the C6 locus. Because the C3 and C5 loci are located within the inverted terminal repeat region, the Env/Gag and Nef/Pol cassettes are present in two copies in ALVAC genome. This vaccine vector differs from the best studied ALVAC vector, vCP205, only in the addition of the Nef/Pol polyepitope string and the E3L and K3L expression cassettes. The correct insertion of E3L and K3L gene expression cassettes in vCP1452 was verified by Southern blotting (data not shown).

Expression of E3L, K3L gene products

To demonstrate production of the E3L and K3L gene products by vCP1452, CEFs were infected with parental ALVAC (lacking any foreign insert) or with vCP1452. Expression of the E3L gene product was detected in CEFs infected with vCP1452 but not in cells infected with parental ALVAC or in uninfected cells by metabolic labeling and immunoprecipitation (Fig. 2A). The E3L gene product was present as both a 25-Kd and a 20-Kd species, as described previously in vaccinia virus-infected cells (Beattie *et al.*, 1995b). Expression of the 10-Kd K3L gene product was clearly demonstrated in cells infected by vCP1452 and not in control cells (Fig. 2B). These results demonstrate that vCP1452 expresses the vaccinia virus E3L and K3L gene products as designed.

Differential activation of PKR phosphorylation by vCP1452 and vCP205

The vaccinia virus E3L and K3L genes were included in the design of vCP1452 to enhance HIV antigen expression through suppression of the IFN-inducible, double-stranded RNA-dependent protein kinase (PKR). Activation of PKR is accompanied by autophosphorylation on serine and threonine residues (Taylor *et al.,* 1996). To directly assess the effects of canarypox-encoded E3L and K3L on activation of PKR, we compared the induction of the active, phosphorylated form of PKR in HeLa cells infected with vCP205 or vCP1452. vCP205 was included as the comparator virus because it contains the identical Gag-pro and gp120 expression cassettes and because this vector has been evaluated extensively in human trials (Mulligan and Weber, 1999) and in cell-based assays of antigen production (Fang *et al.*, 1999). HeLa cells were infected with either poxvirus vector at a multiplicity of infection (m.o.i.) of 10 PFU/cell and were preincubated where indicated with β-IFN to enhance PKR expression prior to viral infection. The addition of β-IFN to HeLa cells resulted in a small increase in active PKR due to enhanced expression of PKR (Fig. 3A, lanes 1 and 2). vCP205 infection of HeLa cells resulted in a significant increase in phosphorylated PKR either following β-IFN treatment (Fig. 3A, lane 5) or in the absence of β-IFN treatment (lane 8). Activation of PKR was detected in cells infected by vCP1452 only following pretreatment

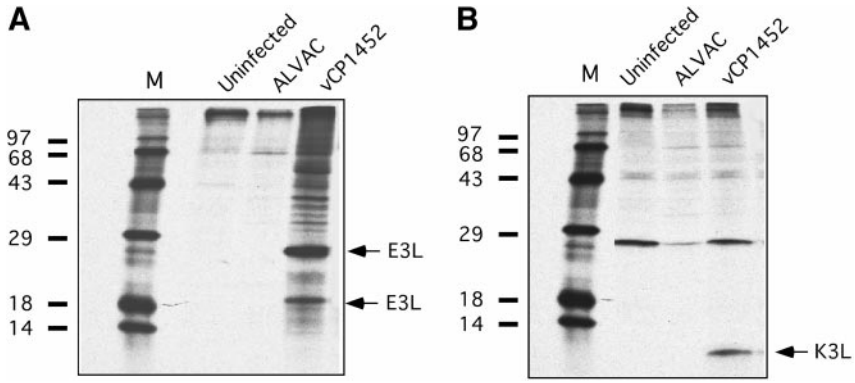

FIG. 2. Expression of E3L, K3L gene products by vCP1452. (A) Chicken embryo fibroblast (CEF) cells were mock infected or infected with ALVAC or vCP1452 at an m.o.i. of 10 PFU/cell in methionine- and cysteine-free media supplemented with 200 μCi/ml [^{35}S]methionine and [^{35}S]cysteine. The cells were lysed 18 h postinfection and the lysates immunoprecipitated with rabbit antisera against E3L (Watson *et al.,* 1991). Samples were fractionated on a 15% SDS–polyacrylamide gel and visualized by fluorography. M = molecular mass marker; mass in kilodaltons is indicated at the left of the gel. (B) CEF cells were mock infected or infected with ALVAC or vCP1452 at an m.o.i. of 10 PFU/cell in methionine- and cysteine-free media supplemented with [^{35}S]methionine and [^{35}S]cysteine. The cells were lysed 18 h postinfection and lysates immunoprecipitated with rabbit antisera against K3L (Beattie *et al.,* 1995). Samples were fractionated on a 15% SDS–polyacrylamide gel and visualized by fluorography. M = molecular mass marker; mass in kilodaltons is indicated at the left of the gel.

with IFN (lane 4, compare to lane 7). The total cellular levels of PKR were increased by β-IFN treatment, but did not differ substantially following infection with vCP205 or vCP1452 (Fig. 3A, Western blot shown below autoradiogram). The prominence of the band seen in lane 5 of the immunoblot may be due partially to the presence of phosphorylated and nonphosphorylated PKR, as the separation on this blot did not fully resolve the bands but resulted in a wider band. In the β-IFN-pretreated cells, the amount of activated PKR induced following vCP1452 infection was substantially less than that induced by vCP205 (Fig. 3A, compare lanes 4 and 5). Densitometric analysis of the PKR bands in Fig. 3A revealed that the activated form of PKR was induced fourfold by vCP1452 (lane 4) as compared to β-IFN alone (lane 2), while vCP205 caused a ninefold induction (lane 5). This difference was even more dramatic when β-IFN pretreatment was not performed. In this case, vCP1452 induced a 2.5-fold increase in activated PKR (lane 7 vs lane 6), while vCP205 induced a 36-fold activation (lane 8). These results indicate that the activation of PKR by vCP1452 is reduced as compared to that of vCP205.

We next compared the activation of PKR by vCP205 and vCP1452 at three different time points postinfection in cells that had not been pretreated with IFN. At 2 h postinfection, a faint band representing phosphorylated PKR was apparent by Western blotting in cells infected with vCP205 but not vCP1452 (Fig. 3B). The differential activation of PKR by vCP205 was more apparent by 4 h postinfection and persisted at 8 h postinfection. At none of the times tested was the activated form of PKR observed in lysates from vCP1452-infected HeLa cells that had not been pretreated with IFN. We conclude that the induction of PKR activation by vCP205 is apparent at multiple time points postinfection, and the suppression

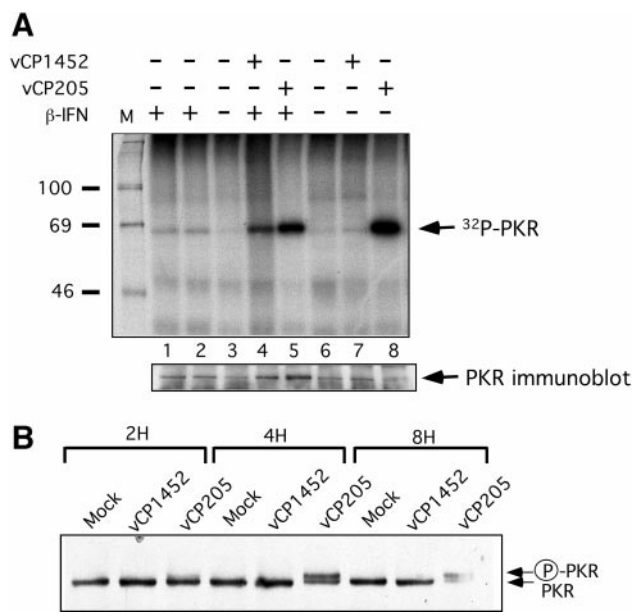

FIG. 3. Activation of PKR following infection with vCP205 or vCP1452. (A) Detection of [^{32}P]PKR. HeLa cells were infected with vCP205, vCP1452, or mock infected as indicated at the top of the gel. Pretreatment of cells with 50 units/ml β-IFN was performed in some experiments as indicated (+, with; −, without). Four hours following infection, cells were labeled with [^{32}P]orthophosphate for 1 h, followed by cell lysis, immunoprecipitation with rabbit polyclonal anti-PKR antibody, and analysis via SDS–PAGE. The cell lysates were also analyzed by Western blotting with a murine anti-PKR antibody, as shown immediately below the autoradiogram. (B) Time course of PKR activation as indicated by immunoblotting. HeLa cells were infected with vCP205, vCP1452, or mock-infected. Cells were harvested at the time points indicated above the blot. Cell lysates were immunoprecipitated with rabbit polyclonal antibody directed against PKR and detected with a murine monoclonal anti-PKR antibody. Phosphorylated (activated) PKR is indicated by the appearance of a slower migrating band in the vCP205 lanes.

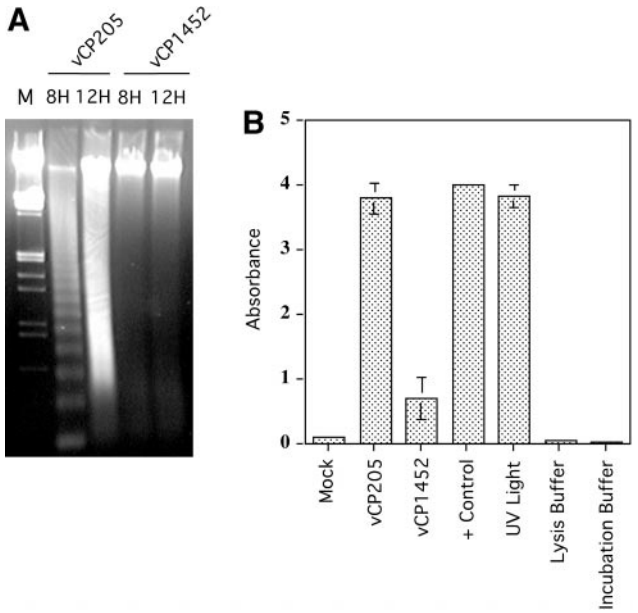

FIG. 4. Induction of apoptosis by recombinant canarypox virus. (A) HeLa cell monolayers were infected with vCP205 or vCP1452 at an m.o.i. of 10 PFU/cell. Cells were harvested at the time points indicated above the gel and cellular DNA isolated for analysis by gel electrophoresis. M = lambda DNA marker, digested with *Eco*RI and *Hind*III. (B) Detection of apoptosis by ELISA. HeLa cells were infected with vCP205, vCP1452, mock-infected, subjected to UV irradiation, or subjected to treatment with two of the buffers from the Cell Death Detection ELISA Kit (Roche). The positive control lane represents DNA–histone complexes included with the kit. Mono- and oligonucleosomes present in the cytoplasmic fraction of cells were detected using a sandwich ELISA technique according to the manufacturer's instructions. Results shown represent the mean ± standard deviation of triplicate assays.

of PKR activation by E3L and K3L results in no detectable PKR activation at up to 8 h postinfection.

Reduced apoptosis in HeLa cells infected with vCP1452 as compared with vCP205

The activation of PKR by viral infection is a potent stimulus for apoptosis (Lee and Esteban, 1994). Apoptosis of cells infected with live vaccine vectors such as recombinant canarypox may limit the duration of antigen expression and the total amount of antigen produced. To determine whether the expression of the vaccinia virus E3L and K3L gene products inhibited apoptosis, we compared the induction of apoptosis in HeLa cells following infection with vCP205 or vCP1452. We first performed electrophoretic analysis of chromosomal DNA from cells infected with the two vectors. vCP205 induced fragmentation of chromosomal DNA into nucleosomal-size fragments (DNA ladder) characteristic of apoptosis (Fig. 4A). This fragmentation was most apparent at 8 h postinfection and had progressed to produce a smear of chromosomal DNA by 12 h (Fig. 4A). In contrast, no fragmentation of chromosomal DNA was apparent in cells infected with vCP1452 at either the 8 or 12 h time points postinfection (Fig. 4A, vCP1452 lanes). To confirm the results of DNA fragmentation analysis, an ELISA measuring the cytosolic appearance of mono- and oligonucleosomes as a measure of apoptosis was next employed. Infection of HeLa cells with vCP205 resulted in significantly higher degrees of apoptosis than vCP1452. Figure 4B illustrates the results of this assay performed on lysates harvested 24 h postinfection. Apoptosis due to infection with vCP1452 was higher than that of mock-infected cells, but remained significantly below that of vCP205 (Fig. 4B). These results support the ability of E3L and K3L present in vCP1452 to inhibit poxvirus-induced apoptosis through downmodulation of PKR activation.

HIV antigen production and release is enhanced by expression of E3L, K3L

Virus-specific antigen expression is inhibited by the actions of activated PKR. We therefore asked whether PKR inhibition mediated by E3L and K3L would enhance HIV gene expression by recombinant ALVAC vectors. We have previously demonstrated that p24 antigen release by vCP205 in the form of HIV-1 pseudovirions correlates well with intracellular antigen production by this vector in mammalian cells (Fang *et al.*, 1999). To determine whether the inclusion of the vaccinia E3L and K3L genes enhanced HIV-specific antigen expression, we measured p24 antigen release in the supernatant of avian, simian, and human cell lines following infection with vCP205 or vCP1452. Data shown are representative of at least three experiments comparing the vectors in each cell line. Both vectors produced only low amounts of p24 antigen release from CEFs (Fig. 5A). The low production of HIV Gag antigen from CEFs was reported previously and appears to be due not to defects in antigen production but to defective particle production and release (Fang *et al.*, 1999). In contrast, both vectors produced significant amounts of pseudovirions from the African green monkey kidney cell line BSC-40 (Fig. 5B). However, vCP1452 infection resulted in an approximately threefold enhancement of particle release in this cell line. Next, we compared HIV-1 pseudovirion release from four human cell lines. HeLa cells infected with vCP205 produced low amounts of pseudovirions and did not demonstrate antigen release after the first 24 h (Fig. 5C, open squares). In contrast, p24 release from HeLa cells infected with vCP1452 continued for a longer time period and achieved significantly higher levels (Fig. 5C, filled circles). At the end of 72 h, total p24 release from HeLa cells reached 30 ng/million infected cells, a level consistent with the subset of high-particle producing mammalian cell lines in our previous study of vCP205 (Fang *et al.*, 1999). A similar pattern of enhanced particle release by vCP1452 was seen in three additional human cell lines: Hep-2, a human epidermoid carcinoma cell line

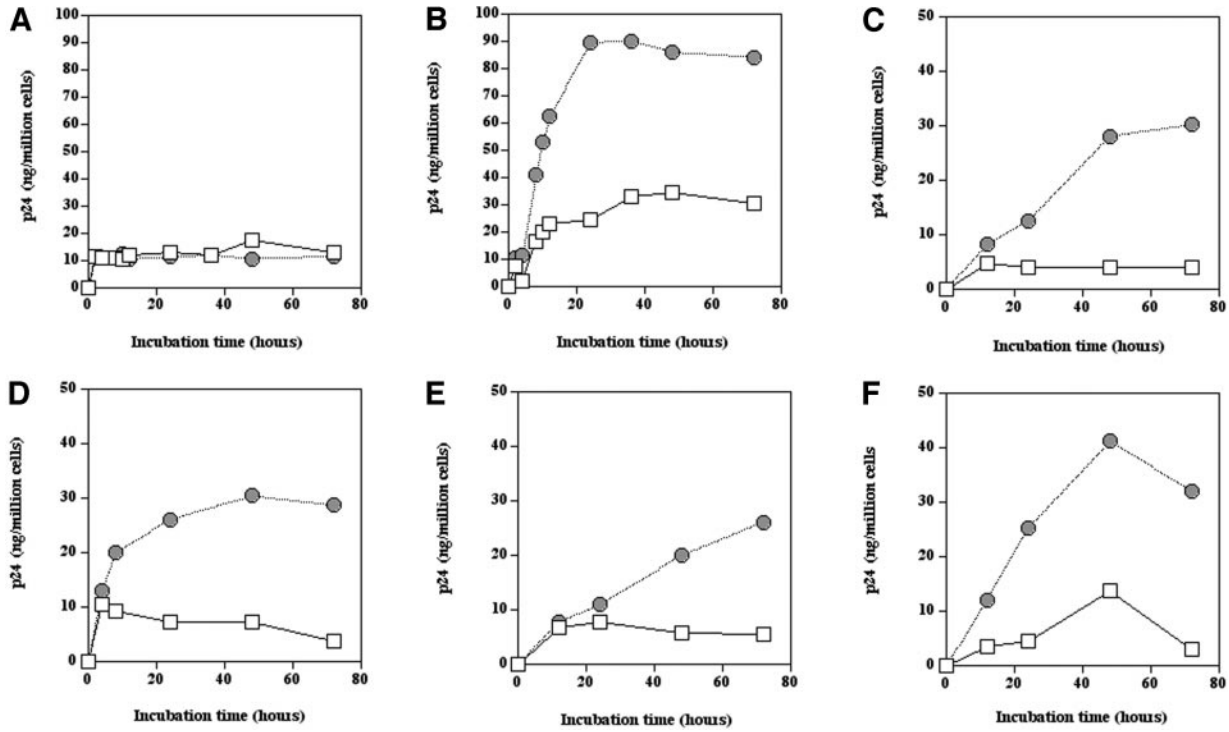

FIG. 5. p24 antigen release from CEFs and human cell lines. Cells were infected with vCP205 (open squares) or vCP1452 (filled circles) at an m.o.i. of 10 PFU/cell, and cellular supernatants harvested at the indicated times following infection. p24 antigen in the cellular supernatants was quantitated by p24 capture ELISA and reported as nanograms of p24 released per 1×10^6 cells. (A) p24 release from CEFs. (B) p24 release from BSC-40. (C) p24 release from HeLa. (D) p24 release from Hep-2. (E) p24 release from MRC-5. (F) p24 release from TE671. Note that the scale used for presentation of results in CEF and BSC-40 differs from that of the other four cell lines.

(Fig. 5D); MRC-5, a human lung fibroblast cell line (Fig. 5E); and TE671, a human rhabdomyosarcoma cell line (Fig. 5F). vCP1452 therefore demonstrated superior HIV-1 pseudovirion release from each of the human cell lines tested. TE671, derived from human muscle, generated the highest levels of particle release from human cells in repeated experiments (Fig. 5F and data not shown).

vCP1452 elicits HIV-1 Gag-Env pseudovirion formation from human cells

To confirm that Gag antigen was released from human cells infected with vCP1452 in the form of HIV-1 pseudovirions, supernatants from ten 100-mm^2 plates of three cell types were harvested, pelleted through a 20% sucrose cushion, and subjected to analysis by equilibrium density gradient centrifugation. CEFs, despite releasing low total amounts of antigen, generated particles of a peak equilibrium density of 1.16–1.17 g/ml (Fig. 6A). Higher amounts of pseudovirions of the same density were detected from infected BSC-40 cells (Fig. 6B). Pseudovirion release from TE 671 cells also peaked at a similar particle density of 1.17–1.18 g/ml (Fig. 6C). These data demonstrate that vCP1452 elicits the production and release of particulate Gag antigen of a density identical to that of retroviral particles from avian, simian, and human cell lines. To determine whether the released pseudovirions incorporated the membrane-bound gp120 molecule, supernatants from vCP1452-infected TE671 cells labeled with [^{35}S]cysteine/methionine were subjected to centrifugation through a 20% sucrose cushion. Analysis was then performed by immunoprecipitation with HIV patient sera. Figure 6D shows that Gag and Env were produced within the infected cells and that both components sedimented efficiently through sucrose. As expected, most of the intracellular Gag produced by vCP1452 infection was immature ($Pr55^{Gag}$), while most of the released Gag was completely cleaved (p24, Fig. 6D). Next, we sought to determine the amount of the pelleted Env protein that was truly incorporated into the released pseudovirions. For this analysis, the released particles from metabolically labeled TE671 cells were pelleted through sucrose, resuspended, and examined by equilibrium density centrifugation on a linear sucrose gradient. Surprisingly, two separate peaks were seen in this analysis (Fig. 6E). The completely cleaved Gag protein was found at a peak density of 1.18 g/ml, consistent with the density of retrovirus-like particles and with the gradient data presented in Fig. 6C. However, there was little Env found in these peak fractions. Instead, the Env protein was found in a peak primarily at a density of 1.15 g/ml (Fig. 6E, gp120/TM). This density corresponded with the peak fraction for the uncleaved Gag protein (Fig. 6E,

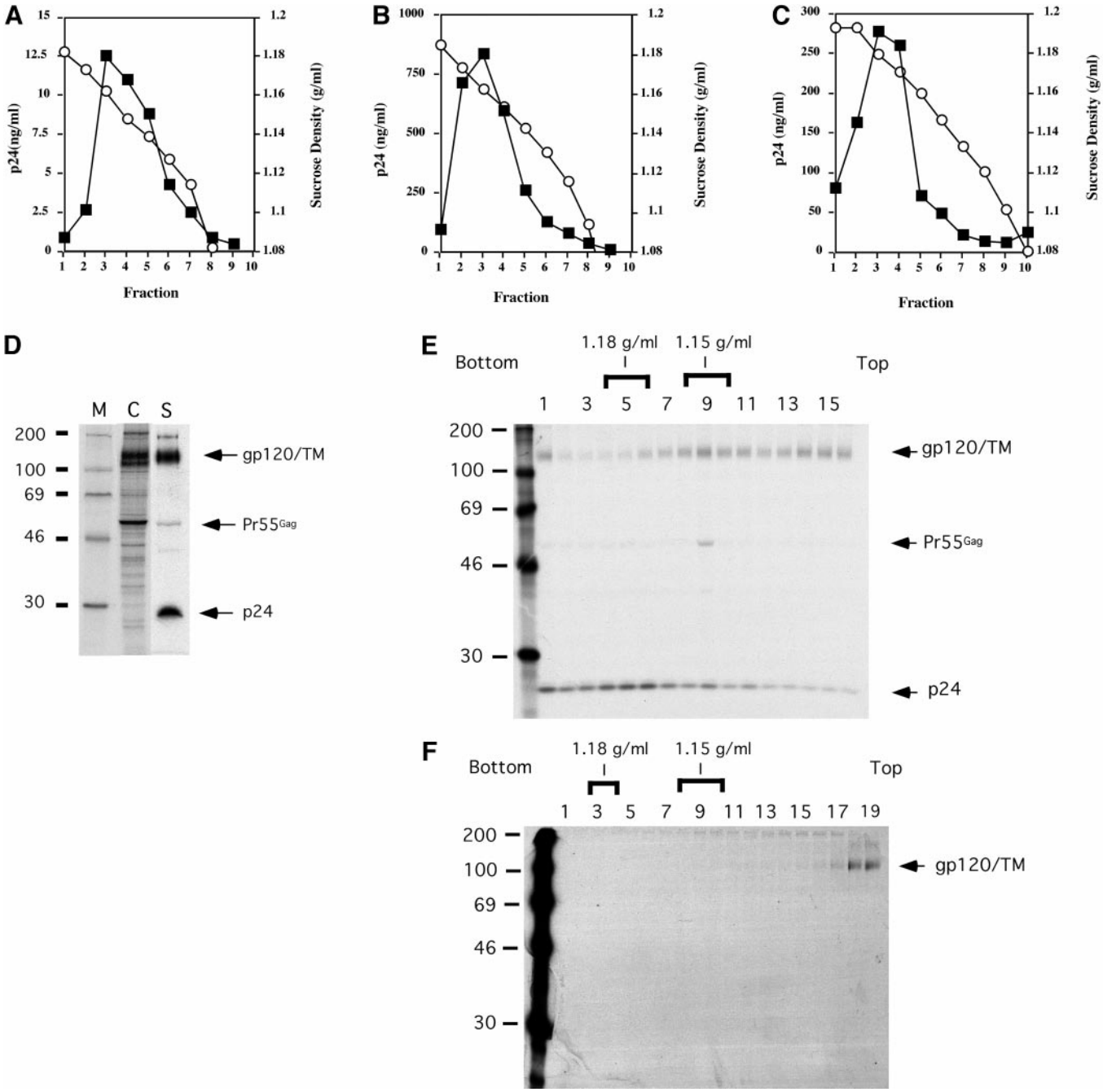

FIG. 6. HIV-1 pseudovirion formation elicited by vCP1452. (A) Supernatants from CEFs infected with vCP1452 were harvested, filtered, purified through a sucrose cushion, and analyzed by equilibrium sedimentation centrifugation on 20–60% linear sucrose gradients. p24 is indicated by filled squares; the density of each fraction is indicated by open circles. (B) HIV-1 pseudovirion formation by vCP1452 infection of simian BSC-40 cells. Processing was performed in an identical manner to that of (A). (C) Pseudovirion formation from the human muscle cell line TE671. Processing was identical to that of (A) and (B). (D) Gag and Env protein content of cells and pseudovirion pellet. Cell lysates and supernatant were harvested from vCP1452-infected TE671 cells following metabolic labeling with 100 μCi/ml [^{35}S]cysteine/methionine. Lysates were directly immunoprecipitated with HIV patient sera; supernatants were centrifuged through a 20% sucrose cushion prior to immunoprecipitation with HIV patient sera. The positions of Pr55Gag, p24, and gp120 are indicated, and molecular mass markers in kilodaltons given at the left of the figure. C = cell lysate, S = supernatant, M = marker lane. (E) Gradient analysis of released pseudovirion particles. Radiolabeled pseudovirion particles were pelleted through 20% sucrose, resuspended, and separated on a 20–60% sucrose gradient to their equilibrium density. Fractions were immunoprecipitated using HIV patient sera. The positions of the 1.18 g/ml p24 peak and the 1.15 g/ml Env peaks are indicated above the autoradiogram. Molecular mass markers are indicated at the left in kilodaltons. (F) Gradient analysis of the gp120/TM molecule. Radiolabeled supernatants from cells infected with vCP124 were pelleted through a 20% sucrose cushion and treated in an identical manner to that of (E) above.

fraction 9). These results suggest that the gp120/TM molecule is incorporated onto a subset of Gag pseudovirions. Alternatively, the gp120/TM molecule may have been incorporated into both fractions but selectively lost from the denser fractions containing fully cleaved Gag protein during processing. To test whether the gp120 seen in this gradient was due to its presence on pseudovirions and not on microvesicles, we repeated this experiment using a recombinant canarypox that expressed the identical gp120 construct in the absence of Gag (vCP124). Env produced by this vector was found to pellet through a 20% sucrose cushion, but remained entirely in the top (lightest) fractions of the 20–60% sucrose gradient (Fig. 6F). We conclude that the HIV Env in the denser fractions of the gradient shown in Fig. 6E is present in association with Gag on pseudovirions.

Electron microscopic analysis of HIV-1 pseudovirions produced by vCP1452

We have previously demonstrated HIV-1 pseudovirion production by an earlier generation canarypox vector, vCP205, from avian and mammalian cells (Fang *et al.*, 1999). However, production of pseudovirions by vCP205 in human cells was extremely low, and HIV-1 pseudovirions were rare upon examination of infected human cells by electron microscopy (data not shown). To prove that the Gag antigen detected in the experiments described above was released as HIV pseudovirions, we performed transmission electron microscopic analysis of vCP1452-infected cells. HIV-1 pseudovirions were readily detected budding from human cells (Figs. 7A, 7B, and 7D). The diameter (110–130 nm) and appearance of particles was identical to that of immature HIV-1 virions. The appearance of HIV-1 pseudovirions produced by vCP1452 was identical when released from the African green monkey kidney cell line BSC-40 (Fig. 7C). Notably, all observed pseudovirions had an immature morphology (Figs. 7C and 7D), despite the apparent processing of the Gag precursor (Fig. 6). We conclude that vCP1452 readily generates HIV-1 pseudovirion formation, that released pseudovirions are predominantly immature in morphology, and that pseudovirion formation and release in human cells by vCP1452 is superior to that of vCP205.

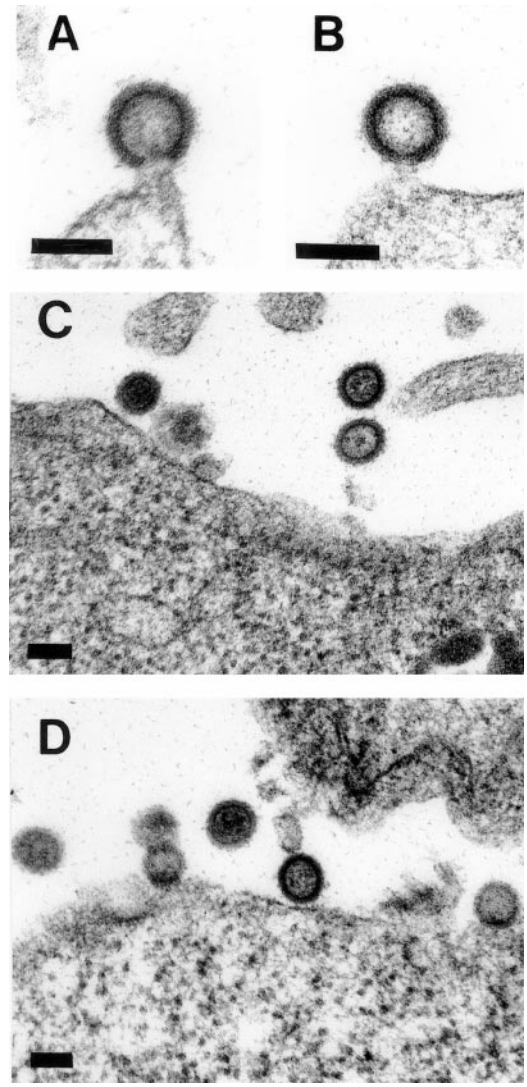

FIG. 7. Electron micrographs of HIV-1 pseudovirions produced by infection with vCP1452. (A and B) HIV-1 pseudovirions budding from the surface of the human muscle cell line TE671. Magnification, ×130,520; bar = 100 nm. (C) HIV-1 pseudovirions released by BSC-40 cells infected with vCP1452. Magnification, ×62,750, bar = 100 nm. (D) HIV-1 pseudovirions released by TE671 cells infected with vCP1452. Magnification, ×62,750, bar = 100 nm.

DISCUSSION

The development of a successful vaccine capable of providing protection against HIV infection or disease is likely to be an iterative process, in which lessons learned from early trials contribute to the development of improved vaccine candidate vectors. ALVAC vCP1452 is a second-generation canarypox HIV vaccine candidate that is designed to enhance antigen expression in human cells. Based upon the hypothesis that PKR activation represents an important factor that has limited antigen expression by earlier ALVAC-HIV vectors in human cells, two vaccinia ORFs that inhibit PKR activation were included in the vector design. The addition of vaccinia E3L and K3L genes to this vector thus represents a novel attempt to increase antigen expression through the addition of modulatory genes to an attenuated poxvirus vaccine vector. This approach was based on studies in which E3L- and K3L-deleted vaccinia viruses were found to be exquisitely sensitive to interferon (Beattie *et al.,* 1995b, 1991). The IFN-resistant phenotype of K3L-deleted vaccinia virus was restored by reinsertion of the K3L ORF at a distinct genetic locus or by insertion of an expression cassette for the heterologous dsRNA-binding protein reovirus $\sigma 3$ (Beattie *et al.,* 1995a,b), suggesting that such manipulations could be applied to vectors derived

from viruses lacking natural IFN resistance in mammalian cell systems. Consistent with this, in the present study the addition of E3L and K3L resulted in significant downmodulation of PKR activation in vCP1452-infected cells. These results demonstrate that the inhibitory effects of E3L and K3L on PKR activation are active in a heterologous viral context and establish the utility of this approach in manipulating the host response directed toward vaccine vectors. We note that the addition of immunomodulatory genes to attenuated poxvirus vaccine vectors is not unique to this study. Attenuated poxviruses expressing human tumor antigens combined with B7.1 and interleukin-2 (Sivanandham *et al.,* 1998) or with granulocyte/macrophage-colony stimulating factor (Kass *et al.,* 2001) as immunoadjuvants have demonstrated promising results in mice.

It is interesting to note that the release of Gag-Env pseudovirions by vCP1452-infected human cells persisted much longer than that seen in cells infected with vCP205. There are two likely contributing factors to explain this finding. First, the lack of PKR-mediated inhibition of translation may simply have allowed for continued translation of viral mRNA over the 72-h period examined. Second, the reduced cell death attributable to inhibition of apoptosis by the actions of E3L and K3L is likely to have contributed to ongoing expression of poxvirus-encoded HIV genes. Recently, Yeung and co-workers reported that inhibition of PKR using an antisense strategy led to the conversion of a normally cytolytic infection by encephalomyocarditis virus to a persistent infection (Yeung *et al.,* 1999). Their study supports the idea that viral pathogenesis may be profoundly altered through inhibition of PKR-mediated apoptosis. Our findings with modifications of gene expression by canarypox are in agreement with this idea and suggest that such modifications may be a feasible approach in the creation of more potent vaccine immunogens. The immunological consequences of this vector modification remain to be examined. We note also that the addition of vaccinia E3L and K3L genes to canarypox did not relieve the host–cell restriction on viral growth, as determined by growth curve experiments in HeLa cells (data not shown).

The production of Gag-Env pseudovirions in human cell lines by vCP1452 indicates that such particles may be produced from cells of vaccinees receiving this vector. The efficient formation of Gag-Env pseudovirions by a human muscle cell line (TE671) in particular suggests that vaccinees receiving im injections of vCP1452 should generate pseudovirions *in vivo*. Gag-Env pseudovirion particles have been shown themselves to elicit CTL responses in small animals and can also act as a boost for live vector-induced cellular immunity (Arp *et al.,* 1999). Furthermore, macaque immunizations with pseudovirions have resulted in strong, long-lived Gag-specific $CD8^+$ CTL responses (Paliard *et al.,* 2000). It is possible that pseudovirion particles released from poxvirus-infected muscle cells may be taken up by professional antigen-presenting cells and processed for presentation by MHC class I molecules, thus enhancing the cellular immune response beyond that which would be induced by intracellular expression of antigen in the absence of particle formation. This hypothesis remains to be tested and will require a comparison of vectors encoding assembly-competent and assembly-deficient Gag molecules.

The pseudovirions produced by vCP1452 demonstrated two unexplained findings. First, electron microscopic analysis showed that all of the released particles had an immature core morphology. This suggests that processing of Gag occurred in an aberrant fashion that prevented the formation of a mature core. Although this may be attributable to inefficient proteolytic processing, the majority of the Gag protein (as shown in Fig. 6D) was completely processed. This result is consistent with our previous examination of pseudovirion particle morphology elicited by vCP205 (Fang *et al.*, 1999). A second unexplained finding was the presence of two apparent populations of pseudovirions: one that incorporated the truncated envelope product and peaked at 1.15 g/ml, and one that incorporated less envelope glycoprotein and peaked at 1.18 g/ml. Notably, some uncleaved Gag protein was associated with the lighter particle peak. While it is possible that the immature pseudovirions seen by electron microscopy represent only the product of the minority, uncleaved Gag protein present in the 1.15 g/ml peak, this seems unlikely given the abundance of particles seen and the relative paucity of the uncleaved Gag protein. The low amount of envelope protein incorporation in the denser pseudovirion peak (1.18 g/ml) could result from selective loss of gp120/TM from this peak during processing or could represent a separate population of particles that incorporate gp120/TM with lower efficiency. Overall, the data support the ability of vCP1452 to elicit the formation of HIV pseudovirion particles and establish that at least a subpopulation of these particles incorporate the truncated envelope glycoprotein.

We anticipate that an increase in antigen production by ALVAC-HIV vectors will yield enhanced immunogenicity. However, it is not certain that a reduction in apoptosis itself is a beneficial property for a vaccine construct. There is now considerable evidence that apoptosis may enhance antigen uptake and processing by dendritic cells, resulting in presentation by the MHC class I pathway (Bellone *et al.,* 1997; Ronchetti *et al.,* 1999). In fact, it has been proposed that the enhanced immunogenicity of recombinant alphavirus replicon particles depends upon their ability to induce caspase-dependent apoptotic cell death rather than upon the production of large amounts of antigen (Leitner *et al.*, 1999). Infection of immature dendritic cells by first-generation ALVAC vectors has recently been shown to contribute to dendritic cell maturation and antigen uptake by dendritic cells in a tissue

culture model system (Ignatius *et al.,* 2000). Therefore, in designing the optimal poxvirus-based HIV vaccine immunogen, it may be necessary to achieve a balance between enhancing the production of antigen in infected cells and allowing sufficient apoptosis to promote dendritic cell maturation and antigen uptake. It will be interesting to determine in ongoing studies of the immunogenicity of this second-generation ALVAC vector whether the enhanced magnitude and duration of HIV-1 antigen production leads to an enhanced cellular immune response. This question is currently being addressed in comparative trials performed by the NIH-sponsored HIV Vaccine Trials Network.

MATERIALS AND METHODS

Generation of recombinant canarypox viruses

The construction of recombinant vaccinia virus vCP205 has been previously described (Fang *et al.*, 1999; Tartaglia *et al.,* 1998b). This vector contains expression cassettes for the HIV-1_{LAI} gag and protease genes under the control of the vaccinia I3L promoter, and an env expression cassette consisting of gp120 (SU) from HIV-1MN fused to the membrane-spanning domain of the gp41 (TM) from HIV-1_{LAI}. The env expression cassette is under the control of the vaccinia virus H6 promoter. Addition of a nef/pol polyepitope string under the control of the H6 promoter to vCP205 resulted in the vCP1433 (Tartaglia *et al.,* 1998a,b). vCP1452 was created by introducing vaccinia virus E3L and K3L gene expression cassettes into vCP1433 as described below.

Insertion of vaccinia virus E3L and K3L expression cassettes

The vaccinia virus K3L gene was synthesized by PCR, using a plasmid containing the Copenhagen vaccinia virus *Hin*dIII K fragment (pSD407) as template and the oligonucleotides, MPSYN763 (5′-CCCTCTAGATCGCGATATCCGTTAAGTTTGTATCGTAATGCTTGCATTTTGTTATTCGT-3′) and MPSYN764 (5′-CCCGAATTCATAAAAATTATTGATGTCTACA-3′), as primers. This PCR fragment was digested with *Xba*I and *Eco*RI and the resulting 315-bp fragment, containing the K3L gene, was cloned into pBS SK^+ (Stratagene). The plasmid generated by this manipulation was designated pBS763/764. The K3L gene was then cloned downstream from the vaccinia virus early/late H6 promoter (Perkus *et al.*, 1989). This was accomplished by cloning the 340-bp *Nru*I–*Xho*I fragment from pBS763/764, containing the K3L gene, into a plasmid containing the H6 promoter (pMM154). The plasmid generated by this manipulation was designated pMPTKH6K3L. A selectable marker was then cloned into pMPTKH6K3L. This was accomplished by cloning a 700-bp *Sma*I–*Bam*HI fragment from pMP42GPT, containing an *Escherichia coli* gpt expression cassette, into pMPTKH6K3L. The plasmid generated by this manipulation was designated pMPTKH6K3Lgpt. The K3L and gpt expression cassettes were then cloned into an ALVAC insertion plasmid. This was accomplished by cloning the 1.2-kb *Xho*I fragment from pMPTKH6K3Lgpt, containing the K3L and *E. coli* gpt expression cassettes, into the ALVAC insertion plasmid, pC6L. The plasmid generated by this manipulation was designated pMPC6H6K3Lgpt. The vaccinia virus E3L gene was then cloned into pMPC6H6K3Lgpt. This was accomplished by cloning the 2.3-kb *Eco*RI fragment from pSD401VC, containing the Copenhagen vaccinia virus E3L gene (and the E3L early promoter), into pMPC6H6K3Lgpt. The plasmid generated by this manipulation was called pMPC6H6K3E3gpt. The K3L and *E. coli* gpt expression cassettes were then removed by digesting pMPC6H6K3E3gpt with *Xho*I and self-ligating the resulting 6.8-kb fragment. The plasmid generated by this manipulation was called pMPC6E3. The K3L expression cassette was then cloned into pMPC6E3. This was accomplished by cloning the 560-bp *Psp*AI fragment from pMPTKH6K3L, containing the H6-promoted K3L expression cassette, into pMPC6E3. The plasmid generated by this manipulation, pMPC6H6K3E3, was transfected into CEFs that were simultaneously infected with vCP1433 to generate viruses incorporating E3L and K3L genes at the C6 locus by homologous recombination. Recombinant viruses were identified by Southern blotting using probes specific for E3L and K3L and were purified by multiple rounds of plaque purification. The resulting recombinant virus was designated vCP1452. The nucleotide sequence of the regions of the canarypox genome used for the generation of transfer plasmids is available upon request.

One experiment depicted in Fig. 3 was performed with recombinant canarypox vCP124. This virus contains the same gp120/TM cassette as that of vCP205 and vCP1452, but lacks the *gag/pro* expression cassette. In this construct the gp120/TM cassette was cloned into the C5 locus using the same procedures as described above.

Detection of E3L, K3L expression in human cells

Chicken embryo fibroblasts (CEFs) were mock infected or infected with parental ALVAC (containing no heterologous genes) or vCP1452 at an m.o.i. of 10 PFU/cell in methionine- and cysteine-free media supplemented with 100 μCi/ml [^{35}S]cysteine/methionine. The cells were lysed 18 h postinfection, and the lysates immunoprecipitated with rabbit antisera against E3L (Watson *et al.*, 1991) or rabbit antisera against K3L (Beattie *et al.*, 1995b).

Preparation of virus stocks

Primary chicken embryo fibroblasts were prepared from 8- to 10-day-old chicken embryos and were main-

tained in Media 199 with 9% fetal calf serum (FCS), 2% HI-Chick serum, 10% tryptose-phosphate broth, 0.056% sodium bicarbonate, 100 U/ml penicillin, and 100 μg/ml streptomycin. Viral stocks were prepared in primary chicken embryo fibroblasts as previously described (Fang *et al.*, 1999). CEFs were infected at an m.o.i. of 0.1 using a vaccine seed stock prepared at Virogenetics Corp. (Troy, NY). Infected flasks were incubated at 37°C for 3–5 days until maximum cytopathic effect was apparent. Working stocks were generated by freeze-thawing, sonicating, and clarification of the sonicate by centrifugation at 1000 *g* for 10 min at 4°C. Plaque titering of canarypox stocks was performed as described (Fang *et al.*, 1999). Resulting virus stocks were stored at −70°C until needed.

Analysis of PKR phosphorylation

Tissue culture dishes (100 mm^2) were seeded with HeLa cells in sufficient number to achieve 40% confluence on the night prior to treatment. The cells were incubated with or without β-interferon (50 units/ml, Calbiochem) for 18 h prior to viral infection. The cells were then infected with vCP1452 or vCP205 at an m.o.i. of 10 in 2 ml of complete nutrient media. Control plates were mock infected with an equivalent volume of phosphate-buffered saline (PBS). Four hours postinfection, the cells were washed with phosphate-free DMEM with 0.1% FCS and incubated in the same phosphate-free medium containing 200 μCi/ml [^{32}P]orthophosphate (Amersham) for 1 h. The cells were then harvested in 0.5 ml NP-40 lysis buffer (150 mM NaCl, 50 mM Tris–HCl pH 8.0, and 1% NP-40) and lysed by three cycles of freeze-thawing, and the lysates clarified by centrifugation at 13,000 *g* for 15 min at 4 °C. ^{32}P-labeled PKR was immunoprecipitated using rabbit polyclonal anti-PKR antiserum (Santa Cruz Biotechnology) and analyzed via SDS–PAGE and autoradiography. Western blotting was carried out by transferring an aliquot of each immunoprecipitated sample to nitrocellulose and immunoblotting with a murine monoclonal antibody directed against PKR (Santa Cruz Biotechnology). Quantitation of ^{32}P-labeled PKR was performed by scanning of autoradiographs on a flatbed scanner, followed by quantitation of the p68 bands using the program NIH Image (version 1.61).

Measurement of virus-mediated apoptosis

DNA fragmentation was assessed in HeLa cells following infection with vCP205 or vCP1452. Monolayers of HeLa cells grown in 100-mm^2 plates (4×10^7 cells/plate) were infected at an m.o.i. of 10 with vCP205 or vCP1452. Cells were scraped and collected in media at 8 and 12 h postinfection, washed once with PBS, and lysed in binding/lysis buffer (6 M guanidine–HCl, 10 mM urea, 10 mM Tris–HCl, 20% Triton X-100, pH 4.4). After adding isopropanol to 20% (v/v) and shaking, the lysate was applied to polypropylene tubes containing glass fiber fleece. Cellular DNA was captured on the glass fibers, washed, and eluted with 10 mM Tris–HCl, pH 8.5. Samples were analyzed on a 1.5% agarose gel containing 0.1 μg/ml of ethidium bromide. A second assay for apoptosis employed a commercial kit for measurement of cytoplasmic histone-associated DNA fragments (Cell Death Detection ELISA, Roche Molecular Biochemicals). Detection of mono- and oligonucleosomes was performed according to the manufacturer's instructions using cytoplasmic extracts from HeLa cells harvested 24 h postinfection with vCP205 or vCP1452. Experiments were performed in triplicate.

p24 antigen release in human cell lines

p24 antigen production and release was measured using a p24 capture ELISA (Organon-Teknika, Raleigh, NC). BSC-40 cells were obtained through Bernard Moss (NIH, Bethesda, MD). The following cell lines were obtained through the American Type Culture Collection (ATCC): HeLa, derived from a human cervical carcinoma (ATCC CCL-2); Hep-2, derived from a human epidermoid carcinoma (ATCC CCL-23); TE671, a human rhabdomyosarcoma cell line (ATCC CRL-8805); and MRC-5, a human lung fibroblast cell line (ATCC CCL-171). Cells were plated in the evening in 100 mm^2 dishes at a density that achieved 70% confluence by the next morning. Precise cell numbers were obtained by counting the cells at this time to normalize the viral m.o.i.; the total number of cells varied by the particular cell type. Cells were infected at an m.o.i. of 10 with vCP205 or vCP1452. Supernatants were harvested at multiple time points up to 72 h postinfection and assayed for p24 content. The number of cells present at the time of infection was used to normalize the p24 release data to facilitate comparisons of the efficiency of release from individual cell lines (reported as nanograms of p24 released per 1×10^6 cells).

Analysis of HIV pseudovirion formation by equilibrium density centrifugation

Pseudovirion formation and release from cells was analyzed by equilibrium density centrifugation on linear sucrose gradients. Cells were infected with vCP1452 at an m.o.i. of 10. Seventy-two hours postinfection, cellular supernatants were harvested, filtered through a 0.45-μm filter, layered over a 20% sucrose gradient, and subjected to centrifugation at 100,000 *g* for 3 h. Particulate matter that sedimented through the sucrose cushion was resuspended in PBS and layered on the top of a 20–60% linear sucrose gradient. Equilibrium density centrifugation was carried out by centrifugation at 100,000 *g* for 16 h in an SW41ti rotor (Beckman). One milliliter fractions were collected from the bottom of the gradient. p24 antigen content in each fraction was quantified using p24 antigen-capture ELISA. For the detection of Gag and Env anti-

gens by radiolabeling, TE 671 cells were infected with vCP205 or vCP1452, and complete DMEM replaced with media deficient in methionine and cysteine supplemented with 100 μCi/ml [^{35}S]cysteine/methionine. Cells and supernatants were collected 16 h later; cells were lysed in RIPA buffer (1% NP-40, 0.1% SDS in PBS) and subjected to immunoprecipitation with pooled HIV patient sera. Supernatants were subjected to pelleting and centrifugation on 20–60% sucrose gradients as described above; gradient fractions were then immunoprecipitated with pooled HIV patient sera. Analysis was performed via SDS–PAGE and fluorography.

Detection of Gag-Env pseudovirions by electron microscopy

Cells were harvested 24 h following infection with vCP205 or vCP1452, washed in PBS, and fixed in 2% glutaraldehyde in PBS. Dehydration, embedding, and sectioning were performed as described previously (Fang *et al.*, 1999). Sections were examined and photographed using a Phillips Model 3000 electron microscope.

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REFERENCES

Arp, J., Rovinski, B., Sambhara, S., Tartaglia, J., and Dekaban, G. (1999). Human immunodeficiency virus type 1 envelope-specific cytotoxic T lymphocytes response dynamics after prime-boost vaccine regimens with human immunodeficiency virus type 1 canarypox and pseudovirions. *Viral Immunol.* **12,** 281–296.

Barouch, D. H., Santra, S., Schmitz, J. E., Kuroda, M. J., Fu, T. M., Wagner, W., Bilska, M., Craiu, A., Zheng, X. X., Krivulka, G. R., Beaudry, K., Lifton, M. A., Nickerson, C. E., Trigona, W. L., Punt, K., Freed, D. C., Guan, L., Dubey, S., Casimiro, D., Simon, A., Davies, M. E., Chastain, M., Strom, T. B., Gelman, R. S., Montefiori, D. C., and Lewis, M. G. (2000). Control of viremia and prevention of clinical AIDS in rhesus monkeys by cytokine-augmented DNA vaccination. *Science* **290**(5491), 486–492.

Beattie, E., Denzler, K. L., Tartaglia, J., Perkus, M. E., Paoletti, E., and Jacobs, B. L. (1995a). Reversal of the interferon-sensitive phenotype of a vaccinia virus lacking E3L by expression of the reovirus S4 gene. *J. Virol.* **69,** 499–505.

Beattie, E., Kauffman, E. B., Martinez, H., Perkus, M. E., Jacobs, B. L., Paoletti, E., and Tartaglia, J. (1996). Host-range restriction of vaccinia virus E3L-specific deletion mutants. *Virus Genes* **12,** 89–94.

Beattie, E., Paoletti, E., and Tartaglia, J. (1995b). Distinct patterns of IFN sensitivity observed in cells infected with vaccinia K3L- and E3L-mutant viruses. *Virology* **210,** 254–263.

Beattie, E., Tartaglia, J., and Paoletti, E. (1991). Vaccinia virus-encoded eIF-2 alpha homolog abrogates the antiviral effect of interferon. *Virology* **183,** 419–422.

Bellone, M., Iezzi, G., Rovere, P., Galati, G., Ronchetti, A., Protti, M. P., Davoust, J., Rugarli, C., and Manfredi, A. A. (1997). Processing of engulfed apoptotic bodies yields T cell epitopes. *J. Immunol.* **159,** 5391–5399.

Carroll, M. W., and Moss, B. (1997). Host range and cytopathogenicity of the highly attenuated MVA strain of vaccinia virus: Propagation and generation of recombinant viruses in a nonhuman mammalian cell line. *Virology* **238,** 198–211.

Davies, M. V., Chang, H. W., Jacobs, B. L., and Kaufman, R. J. (1993). The E3L and K3L vaccinia virus gene products stimulate translation through inhibition of the double-stranded RNA-dependent protein kinase by different mechanisms. *J. Virol.* **67,** 1688–1692.

Davies, M. V., Elroy-Stein, O., Jagus, R., Moss, B., and Kaufman, R. J. (1992). The vaccinia virus K3L gene product potentiates translation by inhibiting double-stranded-RNA-activated protein kinase and phosphorylation of the alpha subunit of eukaryotic initiation factor 2. *J. Virol.* **66,** 1943–1950.

Evans, T. G., Keefer, M. C., Weinhold, K. J., Wolff, M., Montefiori, D., Gorse, G. J., Graham, B. S., McElrath, M. J., Clements-Mann, M. L., Mulligan, M. J., Fast, P., Walker, M. C., Excler, J. L., Duliege, A. M., and Tartaglia, J. (1999). A canarypox vaccine expressing multiple human immunodeficiency virus type 1 genes given alone or with rgp120 elicits broad and durable CD8$^+$ cytotoxic T lymphocyte responses in seronegative volunteers. *J. Infect. Dis.* **180,** 290–298.

Fang, Z. Y., Kuli-Zade, I., and Spearman, P. (1999). Efficient human immunodeficiency virus (HIV)-1 Gag-Env pseudovirion formation elicited from mammalian cells by a canarypox HIV vaccine candidate. *J. Infect. Dis.* **180,** 1122–1132.

Ferrari, G., Humphrey, W., McElrath, M. J., Excler, J. L., Duliege, A. M., Clements, M. L., Corey, L. C., Bolognesi, D. P., and Weinhold, K. J. (1997). Clade B-based HIV-1 vaccines elicit cross-clade cytotoxic T lymphocyte reactivities in uninfected volunteers. *Proc. Natl. Acad. Sci. USA* **94,** 1396–1401.

Fleury, B., Janvier, G., Pialoux, G., Buseyne, F., Robertson, M. N., Tartaglia, J., Paoletti, E., Kieny, M. P., Excler, J. L., and Riviere, Y. (1996). Memory cytotoxic T lymphocyte responses in human immunodeficiency virus type 1 (HIV-1)-negative volunteers immunized with a recombinant canarypox expressing gp 160 of HIV-1 and boosted with a recombinant gp160. *J. Infect. Dis.* **174,** 734–738.

Gale, M., and Katze, M. G. (1998). Molecular mechanisms of interferon resistance mediated by viral-directed inhibition of PKR, the interferon-induced protein kinase. *Pharmacol. Ther.* **78,** 29–46.

Ignatius, R., Marovich, M., Mehlhop, E., Villamide, L., Mahnke, K., Cox, W. I., Isdell, F., Frankel, S. S., Mascola, J. R., Steinman, R. M., and Pope, M. (2000). Canarypox virus-induced maturation of dendritic cells is mediated by apoptotic cell death and tumor necrosis factor alpha secretion. *J. Virol.* **74,** 11329–11338.

Jagus, R., and Gray, M. M. (1994). Proteins that interact with PKR. *Biochimie (Paris)* **76,** 779–791.

Kass, E., Panicali, D. L., Mazzara, G., Schlom, J., and Greiner, J. W. (2001). Granulocyte/macrophage-colony stimulating factor produced by recombinant avian poxviruses enriches the regional lymph nodes with antigen-presenting cells and acts as an immunoadjuvant. *Cancer Res.* **61,** 206–214.

Lee, S. B., and Esteban, M. (1994). The interferon-induced double-stranded RNA-activated protein kinase induces apoptosis. *Virology* **199,** 491–496.

Leitner, W. W., Ying, H., and Restifo, N. P. (1999). DNA and RNA-based vaccines: Principles, progress and prospects. *Vaccine* **18**(9–10), 765–777.

Letvin, N. L. (1998). Progress in the development of an HIV-1 vaccine. *Science* **280**(5371), 1875–1880.

Letvin, N. L., Schmitz, J. E., Jordan, H. L., Seth, A., Hirsch, V. M., Reimann, K. A., and Kuroda, M. J. (1999). Cytotoxic T lymphocytes specific for the simian immunodeficiency virus. *Immunol. Rev.* **170,** 127–134.

Meyer, H., Sutter, G., and Mayr, A. (1991). Mapping of deletions in the genome of the highly attenuated vaccinia virus MVA and their influence on virulence. *J. Gen. Virol.* **72**(Pt. 5), 1031–1038.

Moss, B., Carroll, M. W., Wyatt, L. S., Bennink, J. R., Hirsch, V. M., Goldstein, S., Elkins, W. R., Fuerst, T. R., Lifson, J. D., Piatak, M., Restifo, N. P., Overwijk, W., Chamberlain, R., Rosenberg, S. A., and Sutter, G. (1996). Host range restricted, non-replicating vaccinia virus vectors as vaccine candidates. *Adv. Exp. Med. Biol.* **397,** 7–13.

Mulligan, M. J., and Weber, J. (1999). Human trials of HIV-1 vaccines. *AIDS* **13**(Suppl. A), S105–112.

Paliard, X., Liu, Y., Wagner, R., Wolf, H., Baenziger, J., and Walker, C. M. (2000). Priming of strong, broad, and long-lived HIV type 1 p55gag-specific $CD8^+$ cytotoxic T cells after administration of a virus-like particle vaccine in rhesus macaques. *AIDS Res. Hum. Retroviruses* **16,** 273–282.

Perkus, M. E., Limbach, K., and Paoletti, E. (1989). Cloning and expression of foreign genes in vaccinia virus, using a host range selection system. *J. Virol.* **63,** 3829–3836.

Ronchetti, A., Rovere, P., Iezzi, G., Galati, G., Heltai, S., Protti, M. P., Garancini, M. P., Manfredi, A. A., Rugarli, C., and Bellone, M. (1999). Immunogenicity of apoptotic cells in vivo: Role of antigen load, antigen-presenting cells, and cytokines. *J. Immunol.* **163,** 130–136.

Schmitz, J. E., Kuroda, M. J., Santra, S., Sasseville, V. G., Simon, M. A., Lifton, M. A., Racz, P., Tenner-Racz, K., Dalesandro, M., Scallon, B. J., Ghrayeb, J., Forman, M. A., Montefiori, D. C., Rieber, E. P., Letvin, N. L., and Reimann, K. A. (1999). Control of viremia in simian immunodeficiency virus infection by $CD8^+$ lymphocytes. *Science* **283**(5403), 857–860.

Sivanandham, M., Shaw, P., Bernik, S. F., Paoletti, E., and Wallack, M. K. (1998). Colon cancer cell vaccine prepared with replication-deficient vaccinia viruses encoding B7.1 and interleukin-2 induce antitumor response in syngeneic mice. *Cancer Immunol. Immunother.* **46,** 261–267.

Tartaglia, J., Benson, J., Cornet, B., Cox, W. I., El Habib, R., Excler, J.-L., Franchini, G., Goebel, S., Jacobs, B. L., Klein, M., Limbach, K., Martinez, H., Meignier, B., Pincus, S., and Plotkin, S. (1998a). Retroviruses of Human AIDS and Related Animal Diseases. 11th Colloque des 'Cent Gardes' (M. Girard and B. Dodet, Eds.), Elsevier Science, Marnes-La-Coquette.

Tartaglia, J., Cox, W. I., Taylor, J., Perkus, M., Riviere, M., Meignier, B., and Paoletti, E. (1992a). Highly attenuated poxvirus vectors. *AIDS Res. Hum. Retroviruses* **8,** 1445–1447.

Tartaglia, J., Excler, J. L., El Habib, R., Limbach, K., Meignier, B., Plotkin, S., and Klein, M. (1998b). Canarypox virus-based vaccines: Prime-boost strategies to induce cell-mediated and humoral immunity against HIV. *AIDS Res. Hum. Retroviruses* **14**(Suppl. 3), S291–S298.

Tartaglia, J., Perkus, M. E., Taylor, J., Norton, E. K., Audonnet, J. C., Cox, W. I., Davis, S. W., van der Hoeven, J., Meignier, B., Riviere, M., *et al.* (1992b). NYVAC: A highly attenuated strain of vaccinia virus. *Virology* **188,** 217–232.

Taylor, D. R., Lee, S. B., Romano, P. R., Marshak, D. R., Hinnebusch, A. G., Esteban, M., and Mathews, M. B. (1996). Autophosphorylation sites participate in the activation of the double-stranded-RNA-activated protein kinase PKR. *Mol. Cell. Biol.* **16,** 6295–6302.

Taylor, J., Meignier, B., Tartaglia, J., Languet, B., VanderHoeven, J., Franchini, G., Trimarchi, C., and Paoletti, E. (1995). Biological and immunogenic properties of a canarypox-rabies recombinant, ALVAC-RG (vCP65) in non-avian species. *Vaccine* **13,** 539–549.

Verrier, F., Burda, S., Belshe, R., Duliege, A. M., Excler, J. L., Klein, M., and Zolla-Pazner, S. (2000). A human immunodeficiency virus prime-boost immunization regimen in humans induces antibodies that show interclade cross-reactivity and neutralize several X4-, R5-, and dualtropic clade B and C primary isolates. *J. Virol.* **74,** 10025–10033.

Watson, J. C., Chang, H. W., and Jacobs, B. L. (1991). Characterization of a vaccinia virus-encoded double-stranded RNA-binding protein that may be involved in inhibition of the double-stranded RNA-dependent protein kinase. *Virology* **185,** 206–216.

Wek, R. C. (1994). eIF-2 kinases: Regulators of general and gene-specific translation initiation. *Trends Biochem. Sci.* **19,** 491–496.

Wu, S., and Kaufman, R. J. (1997). A model for the double-stranded RNA (dsRNA)-dependent dimerization and activation of the dsRNA-activated protein kinase PKR. *J. Biol. Chem.* **272,** 1291–1296.

Yeung, M. C., Chang, D. L., Camantigue, R. E., and Lau, A. S. (1999). Inhibitory role of the host apoptogenic gene PKR in the establishment of persistent infection by encephalomyocarditis virus in U937 cells. *Proc. Natl. Acad. Sci. USA* **96,** 11860–11865.