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Replication of Modified Vaccinia Virus Ankara in Primary Chicken Embryo Fibroblasts Requires Expression of the Interferon Resistance Gene E3L

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Highly attenuated modified vaccinia virus Ankara (MVA) serves as a candidate vaccine to immunize against infectious diseases and cancer. MVA was randomly obtained by serial growth in cultures of chicken embryo fibroblasts (CEF), resulting in the loss of substantial genomic information including many genes regulating virus-host interactions. The vaccinia virus interferon (IFN) resistance gene E3L is among the few conserved open reading frames encoding viral immune defense proteins. To investigate the relevance of E3L in the MVA life cycle, we generated the deletion mutant MVA-ΔE3L. Surprisingly, we found that MVA-ΔE3L had lost the ability to grow in CEF, which is the first finding of a vaccinia virus host range phenotype in this otherwise highly permissive cell culture. Reinsertion of E3L led to the generation of revertant virus MVA-E3rev and rescued productive replication in CEF. Nonproductive infection of CEF with MVA-ΔE3L allowed viral DNA replication to occur but resulted in an abrupt inhibition of viral protein synthesis at late times. Under these nonpermissive conditions, CEF underwent apoptosis starting as early as 6 h after infection, as shown by DNA fragmentation, Hoechst staining, and caspase activation. Moreover, we detected high levels of active chicken alpha/beta IFN (IFN-α/β) in supernatants of MVA-ΔE3L-infected CEF, while moderate IFN quantities were found after MVA or MVA-E3rev infection and no IFN activity was present upon infection with wild-type vaccinia viruses. Interestingly, pretreatment of CEF with similar amounts of recombinant chicken IFN-α inhibited growth of vaccinia viruses, including MVA. We conclude that efficient propagation of MVA in CEF, the tissue culture system used for production of MVA-based vaccines, essentially requires conserved E3L gene function as an inhibitor of apoptosis and/or IFN induction.

Modified vaccinia virus Ankara (MVA) was developed by long-term serial passage on primary chicken embryo fibroblasts (CEF) for use as a safe vaccine against human smallpox (34, 57). After more than 500 passages in CEF, the attenuated MVA had lost a substantial part of coding genome sequences and demonstrated a severe restriction of replication on mammalian cells (11, 18, 35). The latter phenotype may be the likely basis for the particular avirulence of MVA also shown upon inoculation of immunodeficient animals with high-level doses (59, 67). As MVA can be easily grown to high titers in CEF, an approved tissue culture source for the production of human vaccines, considerable renewed interest in MVA originated from its usefulness as a safe yet efficacious viral vector (36, 60, 63). When tested in animal models, recombinant MVA vaccines protected against infectious disease or cancer (2, 12, 20, 25, 49, 58, 61, 66, 69, 70), and first-candidate vaccines producing melanoma-associated tyrosinase (19) or human immunodeficiency virus antigen (24) are undergoing clinical testing. Moreover, use of MVA appeared advantageous when recombinant MVA and fully replication-competent vaccinia virus vectors were compared for protective capacities and immune responses elicited against foreign antigens (25, 49, 61). This at

first rather surprising observation may be explained by the recent finding that immunizations with MVA appear to be less affected by anti-vector immunity which may allow for higher immunogenicity of MVA-delivered antigens (42).

Alternatively, it is known that multiple vaccinia virus genes with ascribed immunomodulatory function, i.e., the coding sequences for the two viral proteins that function as soluble receptors for gamma interferon (IFN-γ) and IFN-α/β, respectively, are absent from or defective in the MVA genome (3, 8). This offers the appealing possibility that MVA vector vaccine immunogenicity is enhanced because of reduced vaccinia virus immune interference. Interestingly, several other regulatory gene sequences of vaccinia virus are conserved within the MVA genome, including those of genes for two proteins (K3L and E3L) that function within the infected cell to block the activity of IFN-induced antiviral proteins (3; for a review, see reference 37). The vaccinia virus E3L gene encodes as its main product a 25-kDa polypeptide which is made early during viral infection and localizes to both the cytoplasm and nuclei of infected cells (13, 73). E3L possesses binding activity for double-stranded RNA (dsRNA) and has been shown to inhibit the activity of the dsRNA-dependent enzymes protein kinase PKR, 2'-5'-oligoadenylate synthetase, and RNA-specific adenosine deaminase (13, 14, 33, 45). Also, direct interactions between E3L and PKR have been described, suggesting the pos-

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sibility of additional mechanisms for inhibition besides the sequestering of dsRNA (46, 52).

Vaccinia virus with inactivated E3L gene function was found to be highly sensitive to the antiviral activity of IFN- α/β and restricted in growth in certain tissue cultures, including Vero and HeLa cell cultures, while maintaining full replicative capacity in CEF, hamster BHK, and rabbit RK13 cells (4, 6, 7, 15, 30). Restoration of IFN resistance is possible through transient expression of E3L (15), by reinsertion of E3L gene sequences with functional dsRNA binding activity into the mutant virus genome (53), or by provision of a heterologous dsRNA-binding protein such as reovirus σ_3 protein to complement the deletion of E3L (5). Importantly, the E3L polypeptide can function as a pathogenesis factor in vivo, as demonstrated after vaccinia virus infection in a mouse model (9), which suggests that removal of E3L gene sequences from the genome of viruses to be used as vector vaccines can be desirable. On the other hand, the construction of avipoxvirus vectors that coexpress vaccinia virus E3L resulted in more efficient production of recombinant antigen in human cells (23).

These findings make further evaluation of E3L function even more relevant for the genetic engineering of vaccinia virus-based vectors. Our interest is the investigation of the role of the conserved E3L gene within the genome of the highly attenuated vaccinia virus strain MVA. Here, we report the generation of MVA- Δ E3L deletion mutants which we found to be unable to replicate in CEF, the cell culture used for serial passage in MVA attenuation, while the growth capacity of the virus mutants was unimpaired in mammalian BHK-21 cells. This surprising vaccinia virus host range phenotype in CEF was associated with deficient viral protein and DNA synthesis, rapid induction of apoptosis, and enhanced production of chicken IFN- α/β . Our data demonstrate that vaccinia virus E3L has functional activity in CEF and suggest that the relevant IFN-mediated antipoxvirus pathways are conserved in avian hosts.

MATERIALS AND METHODS

Viruses and cells. Primary CEF, baby hamster kidney BHK-21 (ATCC CCL-10), and rabbit kidney RK-13 (ATCC CCL-37) cells were grown in minimal essential medium (MEM) or RPMI 1640 medium supplemented with 10% fetal calf serum (FCS). Cells were maintained in a humidified air-5% CO₂ atmosphere at 37°C. MVA (cloned isolate F6) was routinely propagated, and the titers of the virus were determined by vaccinia virus-specific immunostaining in CEF to determine the numbers of infectious units (IU) per milliliter. MVA from the 582nd CEF passage was used for this study. Vaccinia virus strains chorioallantois vaccinia virus Ankara (CVA) and Western Reserve (WR; kindly provided by Bernard Moss, National Institutes of Health, Bethesda, Md.) were propagated in RK-13 cells, and the titers of the viruses were determined by plaque assay.

Construction of vector plasmids. MVA DNA sequences flanking the E3L gene (MVA nucleotides 42697 to 43269; GenBank accession no. U94848) were amplified by PCR using genomic MVA DNA as template. Primers of the upstream flanking region of E3L were 5'-ATA TGA TTG GGC CCA CTA GCG GCA CCG AAA AAG AAT TCC-3' (*Apa*I site underlined) and 5'-GTC AAT AGC GGC CGC AGA CAT TTT TAG AGA GAA CTA AC-3' (*Not*I site underlined). Primers for the downstream region were 5'-TAC ATG AAA CGC GTT CTA TTG ATG ATA GTG ACT ATT TC-3' (*Mlu*I site underlined) and 5'-GTT ACG TCG GAT CCA GAT TCT GAT TCT AGT TAT C-3' (*Bam*HI site underlined). The amplified DNA fragments were digested with restriction endonucleases *Apa*I/*Not*I or *Mlu*I/*Bam*HI and inserted into the corresponding sites of plasmid pAK1L (55) to obtain the E3L deletion plasmid pAK1L-050L. For generation of the rescue plasmid pE3rev, we amplified the complete E3L gene sequence together with flanking regions by PCR using the following oligonucleotide primers: 5'-GTC AAT AGC GGC CGC AGA CAT TTT TAG AGA GAA

CTA AC-3' (*Not*I site underlined) and 5'-GTT ACG TCG GAT CCA GAT TCT GAT TCT AGT TAT C-3' (*Bam*HI site underlined). The obtained PCR product was digested with the restriction enzymes *Not*I and *Bam*HI and ligated into plasmid pAK1L.

Generation of E3L deletion mutant virus. Monolayers of 10⁶ confluent BHK-21 cells grown in 6-well tissue culture plates (Costar, Corning, N.Y.) were infected with MVA at a multiplicity of infection (MOI) of 0.01 IU per cell. Using calcium phosphate (CellPfect transfection kit; Amersham Pharmacia Biotech, Freiburg, Germany) as recommended by the manufacturer, cells from one well were transfected with 10 μ g of plasmid DNA at 90 min after infection. At 48 h after infection, the cells and medium were harvested, freeze-thawed three times, and homogenized in a cup sonicator (Sonopuls HD 200; Bandelin, Berlin, Germany). From this material, MVA mutant virus was isolated using a previously described methodology (55). Briefly, 10-fold serial dilutions (10⁻¹ to 10⁻⁴) of the harvested material in medium were used to infect subconfluent monolayers of RK-13 cells grown in 6-well tissue culture plates. After 3 days of incubation at 37°C, single foci of infected RK-13 cells were picked in a 20- μ l volume by aspiration with an air displacement pipette, transferred to microcentrifuge tubes containing 500 μ l of medium, and processed by freeze-thawing and sonication for another infection of RK-13 cell monolayers. After the elimination of parental MVA during passage on RK-13 cells, 10-fold serial dilutions (10⁻¹ to 10⁻⁶) of the recombinant viruses were used for infection of subconfluent BHK-21 cells grown in 6-well tissue culture plates (Costar). Well-separated foci of infected BHK-21 cells were harvested to isolate K1L-negative mutant viruses. Viral DNA from cloned MVA isolates was routinely analyzed by PCR as described previously (55). To monitor the E3L gene locus in the MVA genome, we used oligonucleotides from gene sequences adjacent to open reading frame (ORF) E3L: E3A (5'-ATA TGA TTG GGC CCA CTA GCG GCA CCG AAA AAG AAT TCC-3') and E3B (5'-TAC ATG AAA CGC GTT CTA TTG ATG ATA GTG ACT ATT TC-3').

Western blot analysis. Confluent BHK-21 cell monolayers grown in 6-well tissue culture plates were inoculated with 10 IU of virus/cell for 1 h. Infected cells were briefly washed with medium and incubated in the presence or absence of cytosine arabinoside (AraC; 40 μ g/ml). After 24 h, cell lysates were prepared and separated by sodium dodecyl sulfate-12% polyacrylamide gel electrophoresis (SDS-12% PAGE). Proteins were then electroblotted onto nitrocellulose membranes (Bio-Rad, Munich, Germany) for 2 h in a buffer containing 25 mM Tris, 19.2 mM glycine, and 20% methanol (pH 8.3). After blocking overnight in blocking buffer containing 2% (wt/vol) bovine serum albumin, 0.05% (vol/vol) Tween, 50 mM Tris, and 150 mM NaCl (pH 7.5), the blot was probed in blocking buffer for 1 h with 10-fold-diluted supernatants from hybridoma TW2.3, producing an anti-E3L mouse monoclonal antibody (73). After being washed, the blot was incubated for 1 h with peroxidase-conjugated polyclonal goat anti-mouse antibody (Dianova, Hamburg, Germany) diluted 5,000-fold in blocking buffer, washed again, and developed by visualization with an enhanced chemiluminescence procedure (Lumi-light; Roche, Mannheim, Germany) and Kodak X-Omat film.

Analysis of virus growth. To determine low- or high-multiplicity growth profiles, CEF or BHK-21 monolayers were infected with 0.05, 0.1, or 10 IU of MVA, MVA- Δ E3L, or MVA-E3rev per cell, respectively. For all infection experiments, confluent monolayers grown in 6-well tissue culture plates were used. After virus adsorption for 60 min at 37°C, the inoculum was removed. The infected cells were washed twice with RPMI 1640 medium and incubated with fresh RPMI 1640 medium containing 10% FCS at 37°C in a 5% CO₂ atmosphere. At multiple time points postinfection (p.i.), infected cells were harvested and virus was released by freeze-thawing and brief sonication. Serial dilutions of the resulting lysates were plated on confluent BHK-21 monolayers grown in 6-well plates as replicates of two. For vaccinia virus-specific immunostaining of virus-infected cells, media were removed at 48 h p.i. and cells were briefly fixed in acetone-methanol (1:1). After washing, cells were incubated for 60 min with polyclonal rabbit anti-vaccinia virus antibody (cat. no. 9503-2057; Biogenesis Ltd., Poole, England) (immunoglobulin G fraction; diluted 1:1,000 in phosphate-buffered saline [PBS]-3% FCS). After the cells were washed with PBS, horseradish-peroxidase-conjugated polyclonal goat anti-rabbit antibody (Dianova) (1:5,000 dilution in PBS-3% FCS) was added and the mixture was incubated for 45 min. After washing with PBS, antibody-labeled cells were developed using dianisidine (Sigma, Taufkirchen, Germany) substrate solution, foci of stained cells were counted, and virus titers were calculated (in international units per milliliter) as with the vaccinia virus plaque assay (21).

Analysis of viral DNA. Genomic viral DNA was isolated from infected cells as described previously (21). To assess viral DNA replication, total DNA was transferred with a slot blot apparatus to Hybond TM-N membranes (Amersham)

and hybridized to a ^{32}P -labeled MVA DNA probe. Radioactivity was quantitated with a phosphorimager analyzer (Bio-Rad).

Analysis of [^{35}S]methionine-labeled polypeptides. BHK and CEF cell monolayers in 12-well plates were either mock infected or infected with MVA, MVA- ΔE3L , or MVA-E3rev at an MOI of 20. Following 45 min of adsorption at 4°C , virus inocula were replaced by prewarmed tissue culture medium and incubated at 37°C in a 5% CO_2 atmosphere. At indicated time points p.i., cells were washed with methionine-free medium and incubated with methionine-free medium at 37°C for 10 min, 50 μCi of [^{35}S]methionine was added to each well, and the mixture was incubated for 30 min at 37°C . Cytoplasmic extracts of infected cell monolayers were prepared by adding 0.2 ml of 0.5% Nonidet P-40 lysis buffer (20 mM Tris-HCl, 10 mM NaCl [pH 8.0]) for 10 min at 37°C . Polypeptides from cell extracts were separated by SDS-10% PAGE and analyzed by autoradiography.

Analysis of DNA fragmentation. CEF were mock infected or infected with MVA, MVA- ΔE3L , or MVA-E3rev at an MOI of 20. Cells were harvested at 16 and 24 h p.i., and total DNA was extracted as described previously (55). Precipitated DNA was resuspended in 100 μl of H_2O , treated with RNase (final concentration, 1 mg/ml) for 15 min at 37°C , and resolved in a 1% agarose gel. DNA fragments were visualized by staining with ethidium bromide.

Hoechst staining. CEF cells grown to confluency in 12-well plates containing 12-mm-diameter glass coverslips were either left uninfected or infected with MVA, MVA- ΔE3L , or MVA-E3rev at an MOI of 5 or 20. Cells were stained at 16 or 24 h p.i. with Hoechst 3343 for 30 min at room temperature and photographed under a fluorescence microscope. The ratios of apoptotic cells to non-apoptotic cells were determined by counting and are presented as means plus or minus three times the standard errors of the means ($3\times\text{SEM}$).

Assay for caspase activity. Monolayers of 5×10^5 confluent CEF grown in 12-well tissue culture plates were either mock infected or infected with MVA, MVA- ΔE3L or MVA-E3rev at an MOI of 20. At 90 min after infection at 4°C , cells were washed twice with MEM containing 10% lactalbumin and 5% basal medium supplement (BMS; Biochrom, Berlin, Germany) and incubated for 16 h at 37°C . Cells were harvested, collected by centrifugation, washed once in PBS, and lysed by incubating 2×10^7 cells/ml of lysis buffer (1% Nonidet P-40, 50 mM Tris-HCl, 150 mM NaCl [pH 8.0]) for 10 min on ice followed by vigorous vortexing. Extracts were cleared by centrifugation for 5 min at $10,000\times g$ at 4°C and transferred to fresh vials. To determine DEVD-cleaving activity, extracts were diluted 1:10 in reaction buffer (31) containing 10 mM HEPES-KOH, 40 mM β -glycerophosphate, 50 mM NaCl, 2 mM MgCl_2 , 5 mM EGTA, and 1 mM dithiothreitol (pH 7.0) supplemented with 0.1% CHAPS {3'-(3'-cholamidopropyl)-dimethylammonio]-1-propanesulfonate}, 100 μg of bovine serum albumin, and acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (DEVD-AMC) (final concentration, 10 μM). Reactions were performed in triplicate in flat-bottomed 96-well plates at 37°C for 1 h. Free AMC was then measured by determining the fluorescence at 390 nm (excitation) and at 460 nm (emission) in a Millipore Cytofluor 96 reader. Values were calculated by subtracting the background fluorescence (buffer and substrate alone) and are presented as means plus or minus $3\times\text{SEM}$.

Measurement of apoptosis by ELISA. Confluent CEF monolayers with 10^5 cells were mock infected or infected with MVA, MVA- ΔE3L , or MVA-E3rev at an MOI of 5, 10, or 20, respectively. At 16 h p.i., the extent of apoptosis was analyzed using a Cell Death Detection enzyme-linked immunosorbent assay (ELISA) kit (Roche) according to the manufacturer's instructions. Briefly, at 16 h p.i., medium was removed and cells were incubated in lysis buffer. After lysis, intact nuclei were pelleted by centrifugation and aliquots of supernatant were transferred to streptavidin-coated wells of microtiter plates. The amount of apoptotic nucleosomes present in the sample was determined using mouse monoclonal antibodies directed against DNA and histones and spectrophotometrical analysis.

Propidium iodide staining. To determine nuclear fragmentation, monolayers of 10^6 confluent CEF grown in 6-well tissue culture plates were infected with MVA, MVA- ΔE3L , or MVA-E3rev at an MOI of 5 or 20. At 90 min after infection at 4°C , cells were washed twice with MEM containing 10% lactalbumin and 5% BMS. At various time points p.i., cells were trypsinized, collected, and stored in 70% ethanol at 4°C overnight. Cells were then washed twice in PBS and resuspended in PBS containing propidium iodide, with a final concentration of 50 $\mu\text{g}/\text{ml}$. Samples were stored in the dark for 1 h at 4°C and analyzed with a Becton Dickinson FACSCalibur apparatus as described previously (39).

Measurement of IFN activity in tissue culture supernatants. The antiviral activity in tissue culture supernatants was quantified by a cytopathic effect inhibition assay (47) of chicken CEC-32 cells (74). Briefly, CEC-32 cells were grown to a monolayer in 96-well flat-bottom plates and cultured in the presence of triplicate samples of twofold serial dilutions of supernatants or COS cell-expressed recombinant chicken IFN- α/β (reIFN- α/β) (50). After 24 h, cultures

were infected with vesicular stomatitis virus and maintained for another 24 h. Viable cells were visualized by uptake of neutral red dye, dried, and lysed by the addition of 3 M guanidine-HCl. Optical density was measured at 540 nm in a microplate reader.

Generation of recombinant MVA by E3L selection. Plasmid pIII-E3-P_{mH5} was constructed by inserting a 627-bp PCR-amplified DNA fragment (with the complete MVA E3L gene sequence, including its authentic promoter sequence) into the *Bam*HI restriction site of plasmid pLW9 (kindly provided by B. Moss, National Institutes of Health, Bethesda, Md.) (69). Using the restriction endonucleases *Bam*HI and *Not*I, a 723-bp DNA fragment containing the *gfp* ORF was excised from pEGFP-N1 (Clontech Laboratories GmbH, Heidelberg, Germany), treated with Klenow polymerase, and inserted into the restriction site *Sma*I of pIII-E3-P_{mH5}, generating pIII-E3-P_{mH5}-gfp. To obtain recombinant MVA-P_{mH5}-gfp, BHK-21 cells were infected with MVA- ΔE3L at an MOI of 0.01 and transfected (using calcium phosphate [CellPfect transfection kit; Amersham Pharmacia Biotech] as recommended by the manufacturer) with pIII-E3-P_{mH5}-gfp DNA. At 48 h after transfection, cells were harvested, freeze-thawed three times, and homogenized in a cup sonicator (Sonopuls HD 200). Serial dilutions (10-fold) of the harvested material in medium were used to infect subconfluent monolayers of CEF grown in 6-well tissue culture plates. After 3 days of incubation, single foci of MVA-infected CEF were picked in a 20- μl volume by aspiration with an air displacement pipette, transferred to microcentrifuge tubes containing 500 μl of medium, and processed by freeze-thawing and sonication for subsequent infection of CEF monolayers.

RESULTS

Generation of mutant virus MVA- ΔE3L . To evaluate the relevance of the key IFN resistance gene E3L in the MVA life cycle, we used homologous recombination to replace the complete promoter and coding sequences of the E3L ORF in the MVA genome with a vaccinia virus K1L gene expression cassette (Fig. 1A). This marker allows for stringent growth selection of mutated viruses upon infection of rabbit kidney RK-13 cells; in a second step in which nonselective growth conditions are used, it is simply removed again by further homologous recombination between flanking repetitive DNA sequences (55). Recombinant MVA from which E3L gene sequences had been deleted were isolated during several rounds of plaque purification on RK-13 and BHK-21 cell monolayers. During plaque cloning, the expected genome alterations were monitored by PCR (Fig. 1B). Primary virus stocks were amplified on BHK-21 cells, reassessed by Southern blot analysis of viral DNA (data not shown), and designated MVA- ΔE3L . Western blotting of cell lysates which were prepared in the presence or absence of AraC confirmed the synthesis of E3L protein in cells infected with wild-type MVA, whereas no E3L polypeptides were detected after infection with MVA- ΔE3L (Fig. 1C).

Replication of MVA- ΔE3L is inhibited in CEF. In an attempt to generate secondary virus stocks of MVA- ΔE3L , we infected confluent CEF monolayers at a low MOI, but to our surprise the virus failed to amplify. Suspecting a host range phenotype, we wished to comparatively analyze virus growth levels after infection of BHK-21 and CEF cells. A growth deficiency might result from a defect in virus replication or virus spread. First, we determined virus yields in multiple-step growth experiments, infecting cells with MVA or MVA- ΔE3L at 0.1 IU per cell (Fig. 2A and B). At this multiplicity, replication and spread of MVA and MVA- ΔE3L in BHK-21 cells were close to identical, with very similar peaks of infectivity titers reached between 20 and 48 h after infection (Fig. 2A). As expected, MVA replicated efficiently in CEF. In contrast, titers of MVA- ΔE3L in CEF steadily decreased in comparison to input infectivity, suggesting a virtual absence of productive

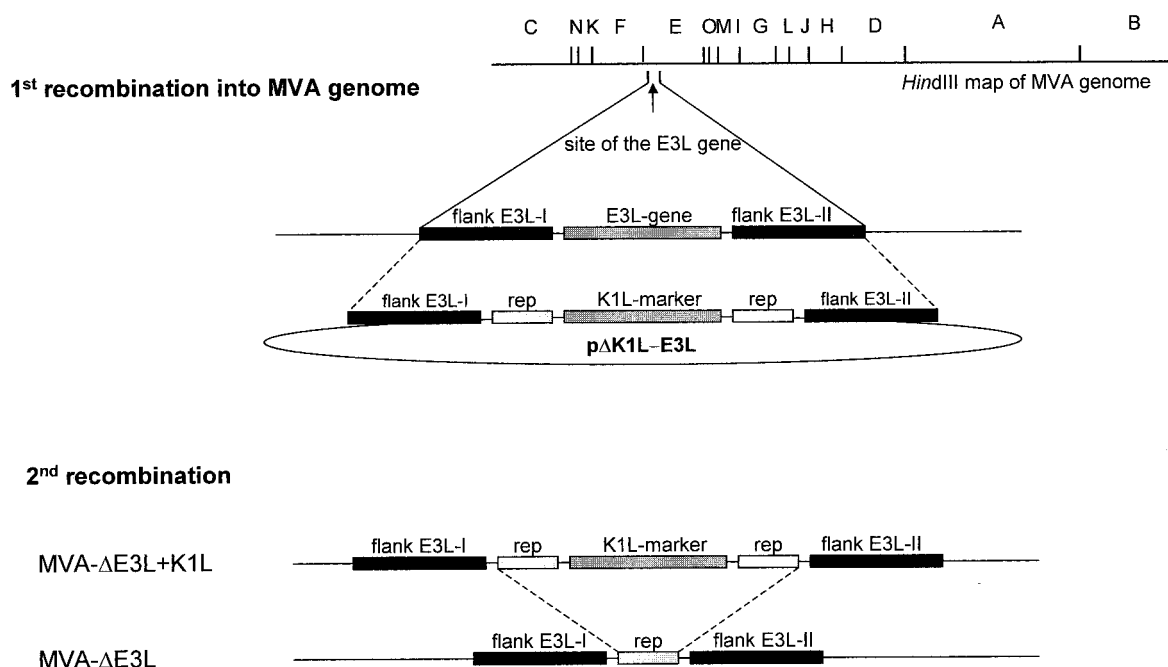
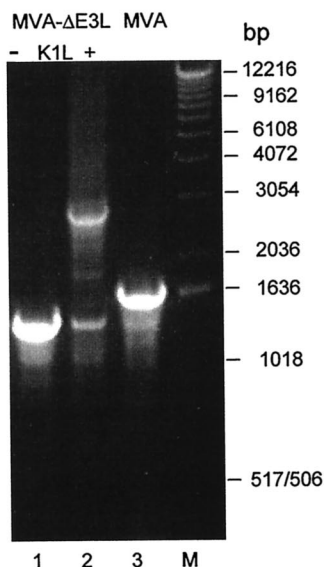
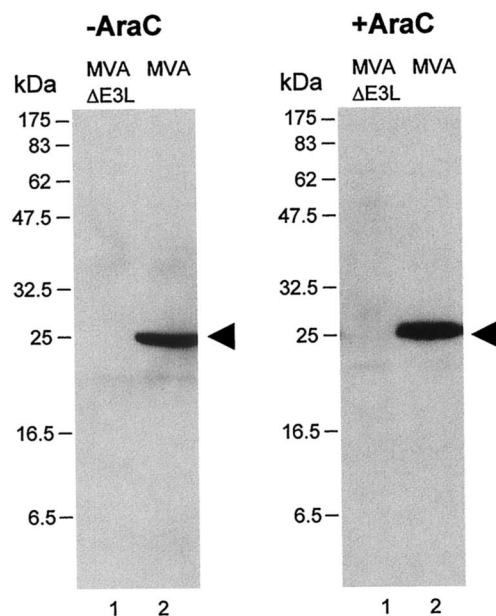
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FIG. 1. Construction and characterization of MVA- Δ E3L. (A) Schematic maps of the MVA genome and the plasmid p Δ K1L-E3L designed for deletion of the E3L gene sequences. The *Hind*III restriction endonuclease sites of the MVA genome are indicated at the top of the panel. MVA DNA sequences adjacent to the E3L gene (flank E3L-I and flank E3L-II) were cloned into p Δ K1L to direct insertion of the K1L selectable marker by homologous recombination, resulting in the deletion of the E3L ORF from the MVA genome. K1L gene expression allows selective growth of the unstable intermediate virus MVA- Δ E3L+K1L in RK-13 cells. The final mutant virus MVA- Δ E3L resulted after the deletion of the K1L marker gene during a second homologous recombination involving additional repetitive sequences (rep). (B) PCR analysis of viral DNA. Genomic template DNA was prepared from MVA- Δ E3L without K1L (lane 1) or with K1L marker gene sequences (lane 2) or from MVA (lane 3). Oligonucleotides E3A and E3B from gene sequences adjacent to the E3L gene locus were used for amplification of specific DNA fragments. PCR products were separated by agarose gel electrophoresis. The 1-kb ladder (Roche Diagnostics) served as molecular weight marker (M). (C) Western blot analysis of lysates from CEF infected with MVA- Δ E3L or MVA in the presence (+AraC) or absence (-AraC) of AraC. The E3L protein bands detected by the E3L-specific mouse monoclonal antibody TW9 are marked by arrowheads.

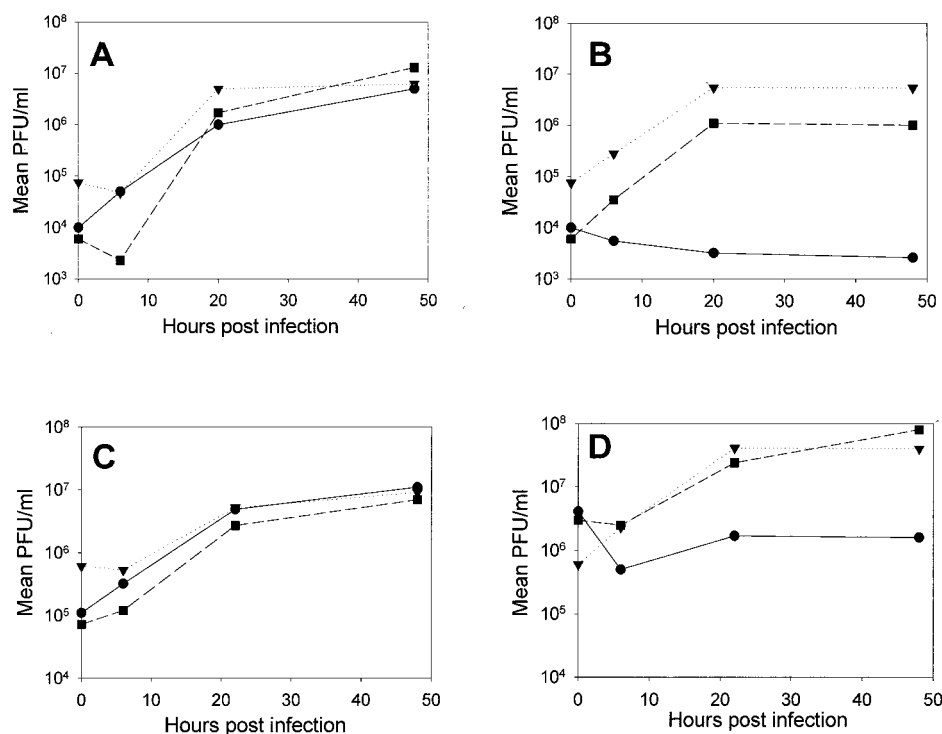


FIG. 2. Analysis of virus growth in BHK (A and C) and CEF (B and D) cells after infection with a low (A and B) or high (C and D) dose of MVA-ΔE3L (●), MVA (■), or MVA-E3rev (▼).

virus growth (Fig. 2B). In addition, using an MOI of 10 for infection, we analyzed one-step virus growth and again we found that MVA and MVA-ΔE3L were equally capable of multiplying in BHK-21 cells. Paralleling the data from multiple-step-growth analysis, infectivity titers of MVA-ΔE3L detected in CEF after 8, 22, or 48 h of infection did not reach the level of infectivity found after virus adsorption (Fig. 2C and D). We concluded from these experiments that MVA-ΔE3L has a specific replication defect in CEF.

To verify that this host range phenotype is solely due to the deletion of the E3L gene sequences, we reinserted the E3L gene under transcriptional control of its authentic promoter sequence into the genome of MVA-ΔE3L to generate the revertant virus MVA-E3rev. Briefly, we used the plasmid vector pE3rev, containing a DNA fragment comprising the complete E3L gene expression cassette together with its genomic flanking sequences, for transfection of MVA-ΔE3L-infected BHK cells. Revertant virus MVA-E3rev could be readily isolated by plaque purification in CEF monolayers. Stable reinsertion of E3L was confirmed by PCR with genomic MVA-E3rev DNA, and Western blot analysis of cell lysates revealed synthesis of E3L protein at levels equal to that of wild-type MVA (data not shown). Already the fact that MVA-E3rev could be isolated without need for additional screening or selection suggested a successful reversion of the growth defect of MVA-ΔE3L in CEF. Correspondingly, upon multiple-step and one-step-growth analysis of MVA-E3rev in both BHK-21 and CEF cells, we found levels of virus replication similar to that of wild-type MVA (Fig. 2). From this data we concluded

that E3L function is necessary for maintenance of MVA replication in CEF.

Reduced viral protein and DNA synthesis in CEF infected with MVA-ΔE3L. To better characterize the nonpermissive infection of CEF with MVA-ΔE3L, we sought to determine whether the failure to produce infectious viral progeny resulted from reduced viral protein synthesis. CEF and BHK cells were metabolically labeled with [³⁵S]methionine at various times after infection with MVA, MVA-ΔE3L, or MVA-E3rev. After each labeling period, lysates were prepared and analyzed by SDS-PAGE and autoradiography. In BHK cells infected with MVA, late viral protein synthesis occurred at 5 h after infection and became prominent, with profound shutoff of cell protein synthesis, at 10 h after infection (Fig. 3A). Similar patterns of viral proteins were found in BHK cells infected with MVA-ΔE3L or MVA-E3rev (Fig. 3A). In CEF infected with MVA or MVA-E3rev, abundant late viral protein synthesis was found at 5 and 10 h after infection (Fig. 3B). In CEF cells infected with MVA-ΔE3L, by contrast, we could hardly detect polypeptide bands specific for viral protein production (Fig. 3B). Some weak bands of polypeptides comigrating with typical late viral proteins became visible with the prominent shutoff of host cell protein synthesis at times after 5 h of infection.

Because synthesis of the abundant viral late proteins is dependent on the replication of viral DNA, we further examined this step in the life cycle of MVA-ΔE3L, comparing infection of BHK-21 and CEF cells. Viral DNA synthesis was monitored by isolating total DNA from infected cells at different times

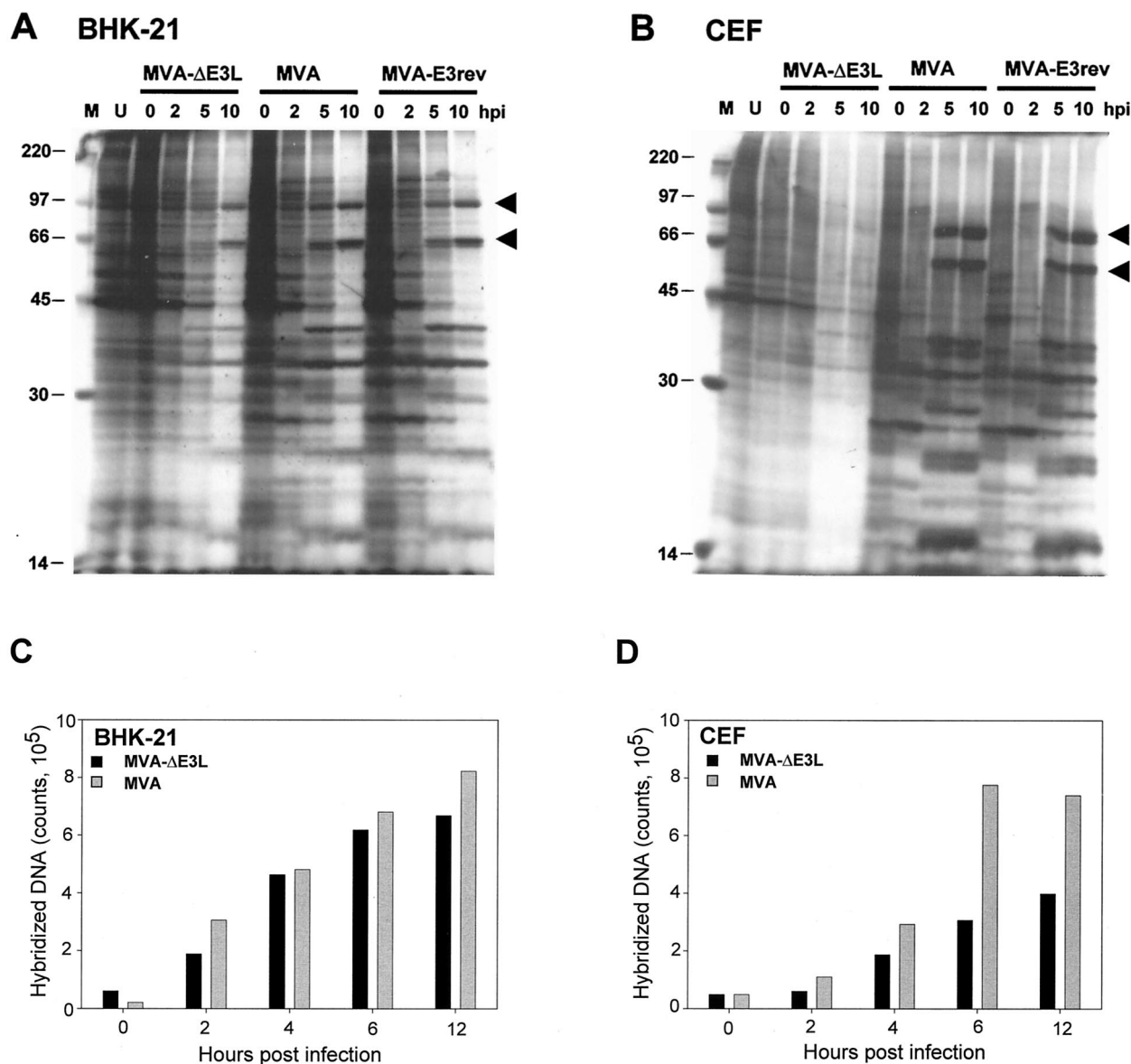


FIG. 3. Viral polypeptide synthesis. (A and B) BHK (A) and CEF (B) cells were infected with MVA-ΔE3L, MVA, or MVA-E3rev and labeled with [³⁵S]methionine for 30 min at the indicated hour p.i. (hpi). Cell lysates were analyzed by gel electrophoresis on a 10% polyacrylamide gel and visualized by autoradiography. Protein standards (lanes M) are indicated by their molecular masses (in kilodaltons). Uninfected cells (lanes U) served as controls. Representative late virus-induced proteins are marked by arrowheads. (C and D) Viral DNA synthesis. DNA isolated from BHK-21 cells (C) or CEF (D) at 0, 2, 4, 6, and 12 h after infection by MVA-ΔE3L or MVA was immobilized on a Hybond N+ membrane and analyzed by hybridization of a ³²P-labeled MVA DNA probe. Radioactivity was quantitated with a phosphorimager analyzer.

during a one-step infection and transferring this DNA onto a membrane which was hybridized with a radioactively labeled MVA-DNA probe (Fig. 3C and D). In BHK-21 cells infected with MVA-ΔE3L or MVA, accumulations of viral DNA occurred with very similar kinetics and quantities, as shown in Fig. 3C. We also found viral DNA in CEF cells being increasingly made after infection with both viruses (Fig. 3D). In the case of the nonpermissive CEF infection with MVA-ΔE3L, however, we detected lesser amounts of DNA (compared to permissive conditions) at time points 6 and 12 h after infection. Thus, the drastically diminished protein synthesis found in MVA-ΔE3L-infected CEF was not correlated with a gross

block of virus-specific DNA replication. Nevertheless, production of viral DNA appeared to be arrested at later times during nonpermissive infection.

Induction of apoptosis in CEF infected with MVA-ΔE3L. This infection phenotype, being characterized by prominent shutdown of viral protein synthesis together with maintained capacity for viral DNA replication, was reminiscent of the nonpermissive vaccinia virus infection of Chinese hamster ovary (CHO) cells (43, 54). The abortive vaccinia virus-CHO infection is associated with induction of apoptosis that can be overcome by coexpression of the cowpox virus gene *CHOhr* (27, 44). Furthermore, the vaccinia virus E3L gene product was

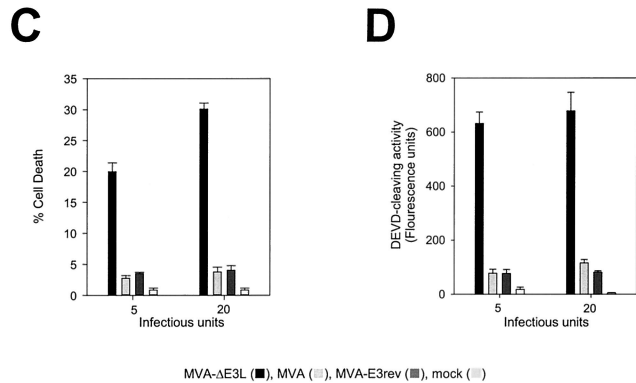
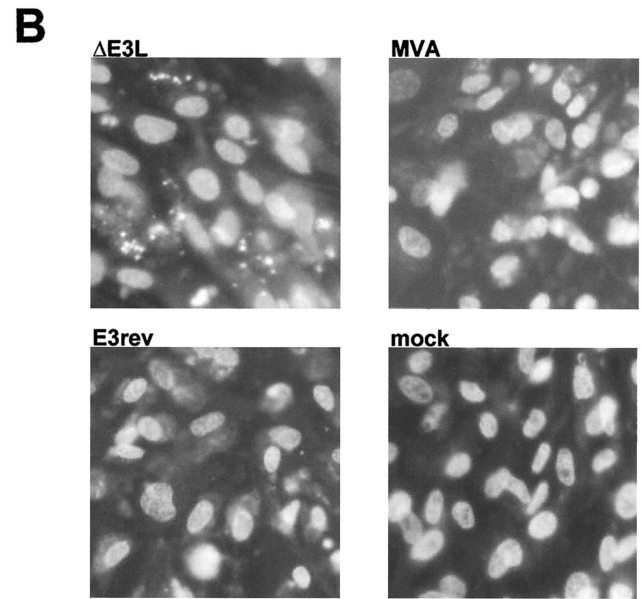
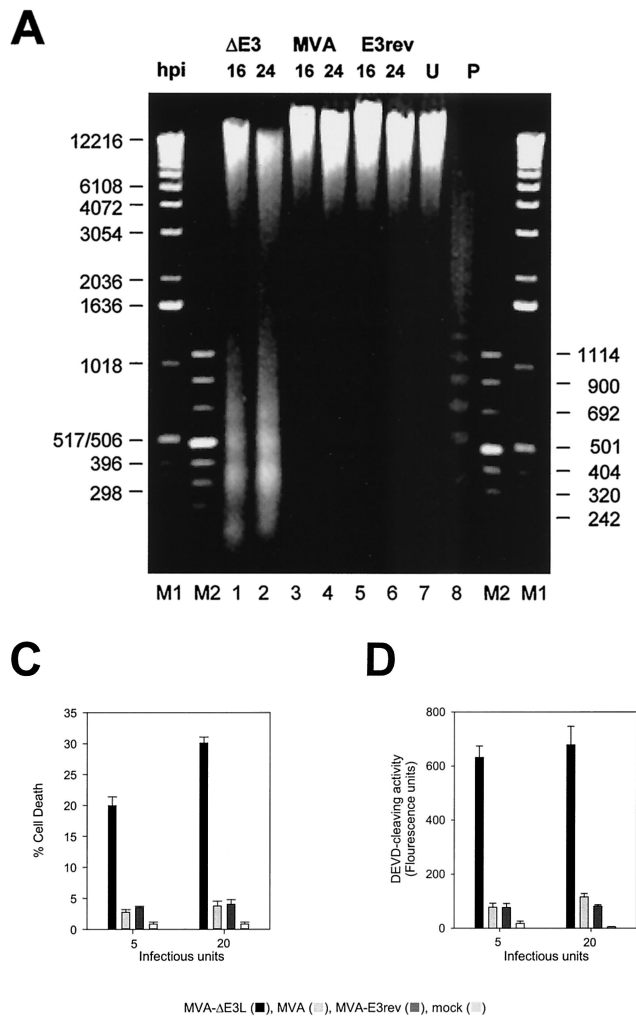


FIG. 4. Induction of apoptosis by MVA-ΔE3L in CEF cells. (A) For DNA fragmentation analysis, semiconfluent monolayers of CEF cells were either left uninfected (lane 7) or infected with MVA-ΔE3L (lanes 1 and 2), MVA (lanes 3 and 4), or MVA-E3rev (lanes 5 and 6) at an MOI of 20 IU/cell. Total DNA extracts were obtained at 16 h p.i. (lanes 1, 3, and 5) and 24 h p.i. (lanes 2, 4, and 6), separated by gel electrophoresis through 1% agarose, and visualized by ethidium bromide staining. As a positive control, a sample from a DNA laddering kit (Roche Diagnostics) was applied (lane 8). Lanes M1 and M2, molecular weight standards. (B) The Hoechst 3343 staining was performed with mock-infected or MVA-infected CEF cells grown on coverslips. After 16 h, cells were stained for 30 min with Hoechst 3343. Micrographs were taken of CEF cells either mock infected (mock) or infected with MVA-ΔE3L (ΔE3L), nonrecombinant MVA (MVA), or MVA-E3rev (E3rev) at an MOI of 5 IU/cell. (C) Apoptotic cells stained with Hoechst 3343 were counted in CEF cells infected with MVA-ΔE3L, MVA, or MVA-E3rev at an MOI of 5 or 20 PFU/cell and in mock-infected cells. Results are given as means plus or minus 3× SEM. (D) Induction of DEVD-cleaving activity was determined in CEF cells infected with MVA-ΔE3L, MVA, and MVA-E3rev at an MOI of 5 or 20 IU/cell or in uninfected CEF cells. DEVD-cleaving activity levels were measured in triplicate with 10 μl from each mixture as described in Materials and Methods. Results are given as means plus or minus 3× SEM.

also described as inhibiting induction of apoptosis in vaccinia virus-infected HeLa cells (29, 32). Because we had observed unusual cell shrinkage when monitoring CEF cultures by light microscopy within 24 h of infection with MVA-ΔE3L, it appeared important to probe whether the growth restriction of MVA-ΔE3L in CEF cells could possibly be linked to apoptosis. In a first standard assay for apoptosis, we monitored the characteristic cleavage of DNA into 180-bp multimers corresponding to a nucleosomal DNA ladder (71). Total cellular DNA was isolated from CEF cultures infected with MVA-ΔE3L, MVA, or MVA-E3rev, separated by agarose gel electrophoresis, and visualized with ethidium bromide staining (Fig. 4A). We observed the typical fragmentations of cellular DNA indicative of apoptosis in samples from CEF cells infected with MVA-ΔE3L for 16 and 24 h. In contrast, no such DNA laddering was detectable in samples from CEF cells infected with MVA or the revertant virus MVA-E3rev (Fig. 4A). Nuclei of infected cells were further stained with Hoechst reagent and analyzed by fluorescence microscopy. As shown in Fig. 4B, numerous CEF nuclei displayed an apoptotic morphology (chromatin condensation and disintegration) at 16 h after infection with MVA-ΔE3L. Using an MOI of 5 IU/cell, apoptosis

was seen by this criterion in about 20% of all cells. In experiments done at an MOI of 20 IU/cell, the percentage of apoptotic cells increased to about 30%. In contrast, 4% (at most) of cells infected with wild-type MVA or revertant virus MVA-E3rev and only 1% of mock-infected cells were counted positive for extranuclear staining.

The activation of proteases of the caspase family is central to apoptosis (65). To confirm the activation of the apoptotic pathway by infection of CEF cells with MVA-ΔE3L, we measured effector caspase activation as cleavage of the artificial substrate DEVD-AMC in cell lysates. This enzymatic activity correlates with the activation of caspase-3 during apoptosis (48). Again, after infection with MVA-ΔE3L we found strongly enhanced DEVD cleavage activities, while extracts from MVA- or MVA-E3rev-infected cells contained little if any detectable activity (Fig. 4D). These results clearly demonstrated that the growth

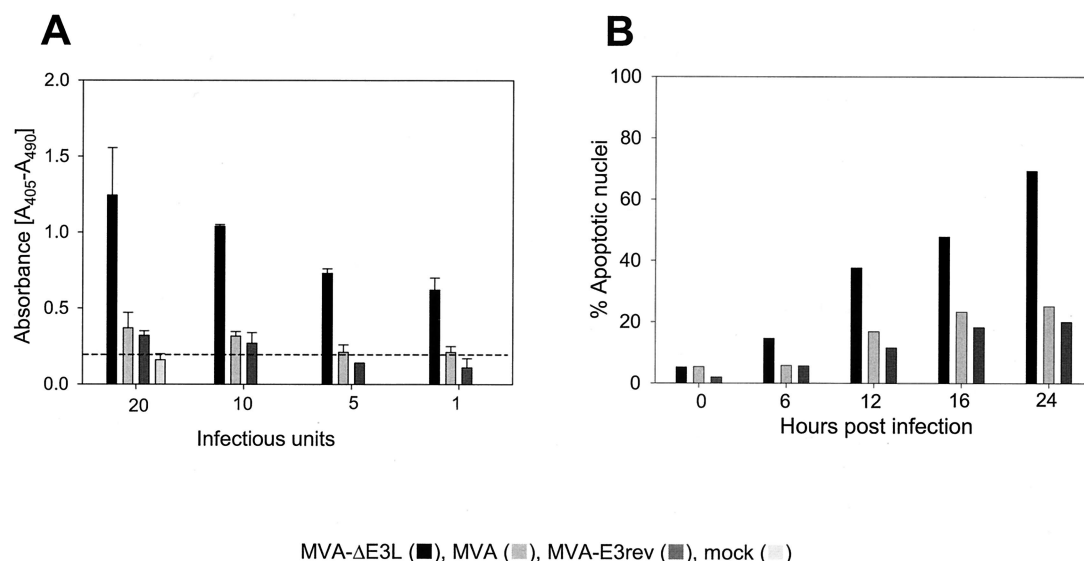


FIG. 5. (A) Detection of apoptosis after infection with different doses of virus. The extent of apoptosis was measured by ELISA either in mock-infected CEF or in cells infected with MVA-ΔE3L, MVA, or MVA-E3rev. Cell death detection ELISAs were performed according to the manufacturer's instructions at 16 h p.i. Absorbance at 405 nm (reference, 490 nm) was measured. Results are given as means plus or minus 2× SEM. (B) Kinetics and extent of apoptosis were analyzed in CEF cells that were mock infected or infected with MVA-ΔE3L, MVA, or MVA-E3rev at an MOI of 20 IU/cell. Cells were harvested at indicated time points, fixed overnight, stained with propidium iodide, and analyzed by flow cytometry.

defect of MVA-ΔE3L in CEF was associated with an induction of apoptosis.

Furthermore, we wished to assess whether the onset of apoptosis in MVA-ΔE3L-infected CEF could be more obviously linked to the host range restriction phenotype. Although our previous experiments were done at an MOI of 20 IU/cell, which should guarantee an infection of all the cells, only approximately 30% of the cells were scored apoptotic upon Hoechst staining. Therefore, we were first interested in more carefully titrating MVA-ΔE3L with regard to the extent of apoptosis. We infected CEF cells with 1, 5, 10, or 20 IU of MVA-ΔE3L, MVA, or MVA-E3rev per cell, and using an ELISA based on specific detection of apoptotic nucleosomes with mouse monoclonal antibodies at 16 h after infection, we quantified apoptosis (Fig. 5A). We found clear evidence for apoptosis in CEF inoculated with the lowest dose of 1 IU of MVA-ΔE3L per cell. Levels of apoptosis steadily augmented with increasing amounts of MVA-ΔE3L used for infection, while even higher-dose infections with MVA or MVA-E3rev resulted in amounts of cell death that barely exceeded background levels. The accuracy of enumeration of apoptotic cells following Hoechst staining might have been limited by the fact that a fraction of apoptotic cells intended for analysis was likely to have been lost due to detachment during handling of infected-cell monolayers. To use an alternative protocol for analysis of the percentage of dead cells present after infection at an MOI of 20, we stained fixed cells with propidium iodide and quantitated apoptotic cells as "sub-G₁ nuclei" by flow cytometry (39) at various time points (Fig. 5B). As early as 6 h after infection with MVA-ΔE3L, we detected an increased number of apoptotic nuclei in comparison to those seen with CEF infected with MVA or MVA-E3rev. At later time points, the percentage of dead cells in MVA-ΔE3L-infected CEF rose

continuously, accounting for about 70% of all the cells at 24 h p.i. These data suggested that the extent and kinetics of apoptosis found in MVA-ΔE3L-infected CEF correlated with the growth restriction phenotype characterized by reduced protein and DNA synthesis.

Induction of IFN-α/β in CEF infected with MVA-ΔE3L.

Induction of apoptosis in HeLa cells by vaccinia virus WR or Copenhagen from which E3L had been deleted has been linked to the function of E3L as a potent dsRNA-binding protein which interferes with the activation of the principal IFN-regulated antiviral enzymes PKR and RNase L (29, 32). Another characteristic feature of this vaccinia virus mutant is its increased sensitivity to IFN treatment (4, 6). Interestingly, infection with an E3L deletion mutant of vaccinia virus Copenhagen also results in the induction of IFN-β synthesis through activation of IFN regulatory factor 3 (72). Therefore, we wished to comparatively monitor the presence of IFN after infection of CEF with MVA-ΔE3L. We inoculated CEF monolayers grown in 6-well tissue culture plates with 10 IU/cell of MVA-ΔE3L, MVA, or MVA-E3rev or the wild-type vaccinia virus CVA or WR. Cell-free supernatants were collected 24 h after infection and tested for antiviral activities in comparison to those of recIFN (250 U/ml) (Fig. 6). Supernatants from cells infected with wild-type vaccinia virus CVA contained no detectable IFN activity (Fig. 6A). The same result was obtained after infection with vaccinia virus WR (data not shown). In contrast, IFN activity was clearly present in medium from MVA-infected CEF (Fig. 6B). Surprisingly, the highest levels of IFN were found after infection with MVA-ΔE3L, reaching activity levels similar to those obtained with recIFN used as a control (Fig. 6C). After infection with MVA-E3rev, we also detected IFN activity, albeit at lower levels comparable to the activity level found after infection with nonmutated MVA (Fig.

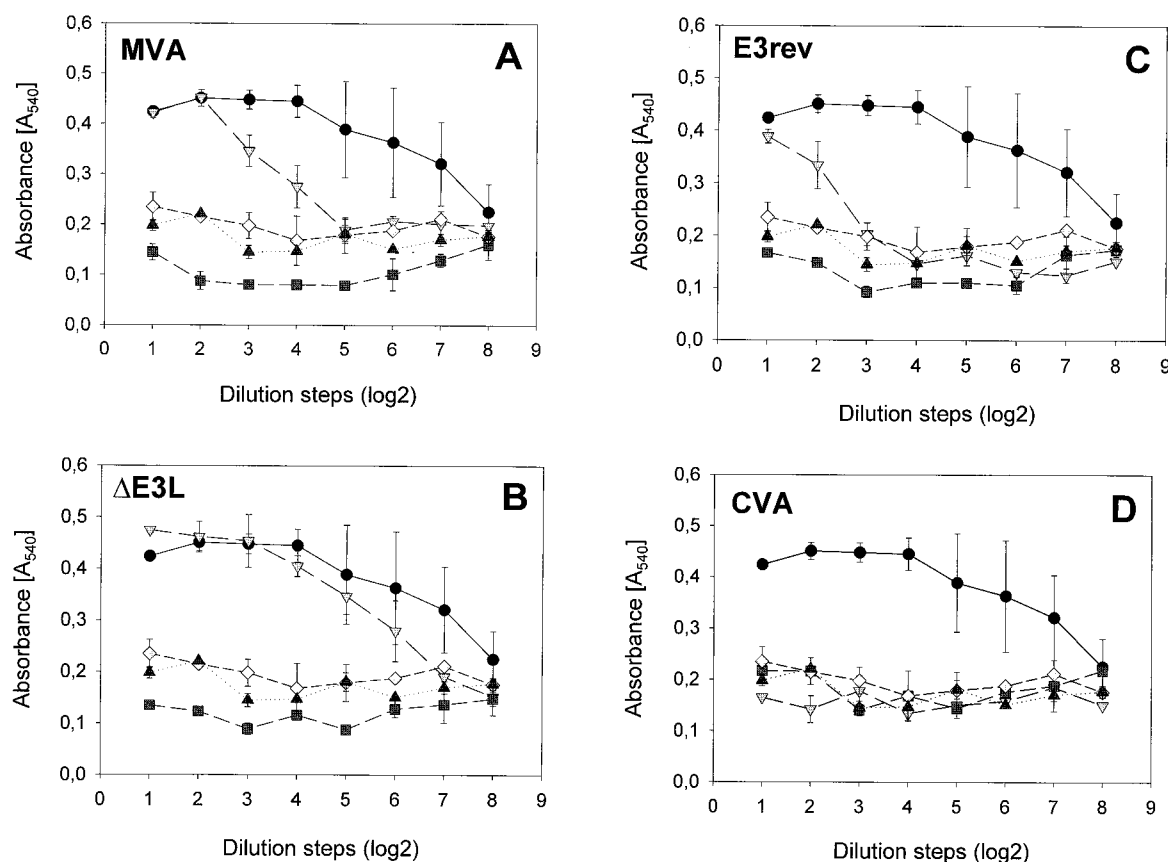


FIG. 6. Synthesis of chicken IFN- α/β after infection of CEF with MVA- Δ E3L. CEF monolayers grown in 6-well tissue culture plates were inoculated with 10 IU/cell of MVA (A), MVA- Δ E3L (B), MVA-E3rev (C), or wild-type vaccinia virus CVA (D). Cell-free supernatants were collected at 0 h (\blacktriangle , mock infected; \blacksquare , virus infected) or 24 h (\diamond , mock infected; ∇ , virus infected) after infection and tested for antiviral activities of chicken IFN- α/β in comparison to those of recIFN (\bullet ; 250 U/ml).

6D). This data demonstrated that the permissive infection of CEF with MVA already induced an accumulation of biologically active IFN- α/β and that the nonpermissive MVA- Δ E3L infection produced even more IFN.

Effect of IFN treatment on vaccinia virus infection in CEF.

To determine the possible inhibitory effect of chicken IFN- α/β on MVA replication in CEF, we pretreated cell monolayers with increasing amounts of recIFN for 24 h before infecting the cultures at a low MOI with MVA, MVA- Δ E3L, or MVA-E3rev or (for comparison) with vaccinia virus CVA or WR. At 48 h after infection, cell monolayers were fixed and foci of virus infected cells were visualized using vaccinia virus-specific immunostaining (Fig. 7). After vaccinia virus WR or CVA was used for infection, we found multiple virus plaques that had formed in monolayers that had been mock treated or treated with low amounts of IFN (1 or 10 U of IFN/ml of medium). Yet the presence of 10 U of IFN/ml of medium resulted in fewer virus plaques that were usually of a smaller size. No more plaque formation was detectable in monolayers preincubated with 100 U of IFN/ml of medium. Immunostaining of MVA-infected CEF revealed foci of virus-positive cells which remained associated with the cell monolayer, demonstrating the typical lower cytopathogenicity of MVA infection. Despite this difference in plaque phenotypes, formation of MVA-in-

fect cell foci upon IFN treatment was very comparable to the plaque formation seen with vaccinia virus WR or CVA. We found MVA-infected cells in monolayers treated with up to 10 U of IFN/ml of medium. This latter amount clearly affected the number and size of the foci, while higher IFN concentrations resulted in complete growth inhibition. In sharp contrast, there were no detectable virus-infected cells in monolayers that were inoculated with MVA- Δ E3L irrespective of IFN treatment, confirming the inability of this mutant to productively replicate in CEF, whereas upon infection with MVA-E3rev we again found a formation of foci that was identical to the pattern established with wild-type MVA. These data suggested that the IFN-mediated inhibition of CEF-adapted MVA is very comparable to the IFN effect found upon infection with vaccinia virus strains WR and CVA.

MVA- Δ E3L growth in fibroblast cultures from young chicken embryos. Previous work on the programmed development of the chicken IFN system suggests that young (5-day-old) chicken embryos are still immature with regard to IFN expression (51). To possibly obtain further evidence of a connection between IFN induction and the abortive MVA- Δ E3L infection in CEF, we comparatively assessed virus growth in primary fibroblast cultures prepared from small-size 5-day-old or standard 10-day-old chicken embryos. We determined virus

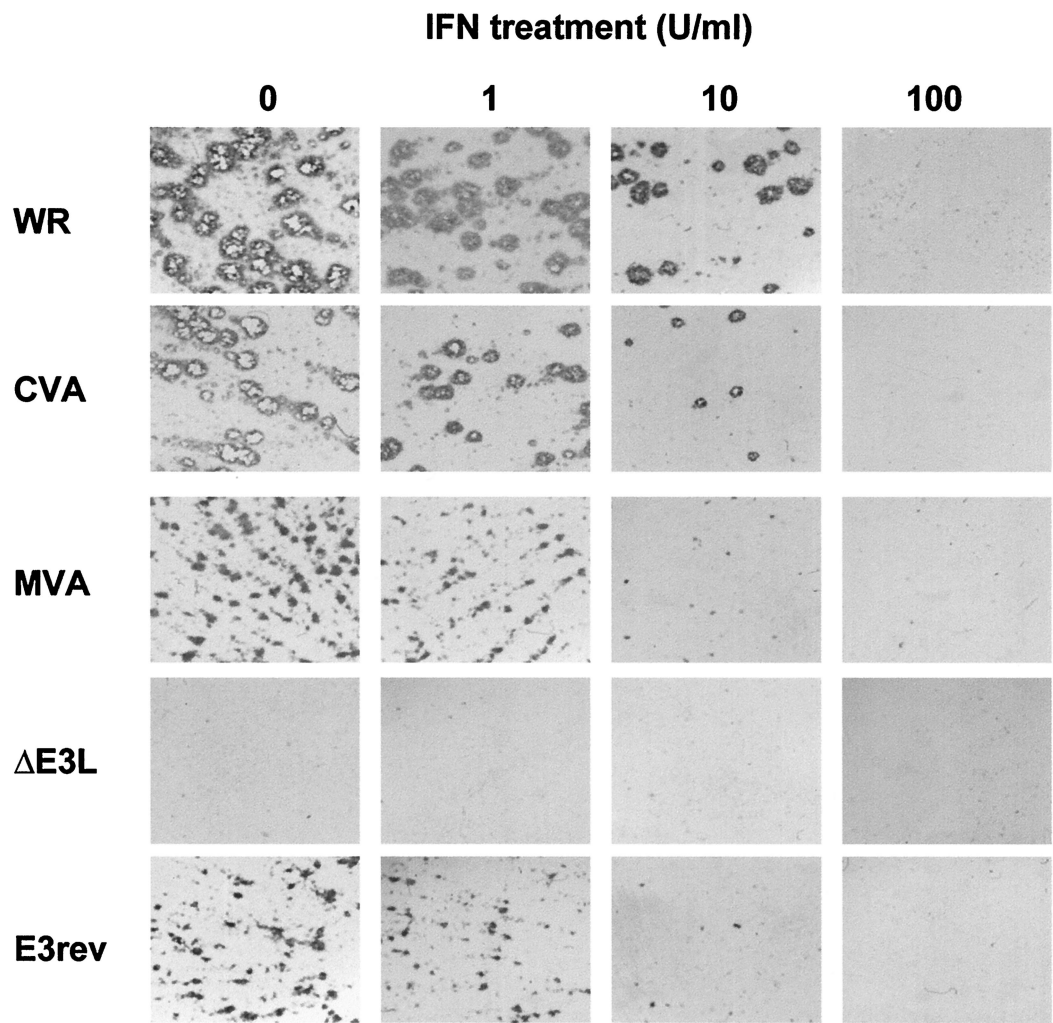


FIG. 7. Effect of IFN treatment on MVA infection. CEF monolayers were incubated with increasing amounts of recIFN- α/β for 24 h before the cultures were infected at low MOIs with MVA, MVA- Δ E3L, or MVA-E3rev or with vaccinia virus CVA or WR. At 48 h after infection, cell monolayers were fixed and foci of virus infected cells were visualized using vaccinia virus-specific immunostaining.

yields in low-multiplicity growth experiments, infecting the two CEF cultures with MVA or MVA- Δ E3L at 0.05 IU per cell (Fig. 8). MVA efficiently replicated in fibroblasts from both 5- and 10-day-old embryos, increasing to very similar infectivity titers at 24, 48, and 72 h after infection. In contrast to its growth failure in CEF prepared from 10-day-old embryos, MVA- Δ E3L was able to productively replicate in fibroblast cultures made from 5-day-old embryos, as clearly shown by the more than 500-fold increase of infectivity found within 24 h of infection. Yet we did not find further multiplication of MVA- Δ E3L at later times after infection. These data suggest that E3L gene expression is not essential for MVA replication in CEF at a stage of embryonic development in which the IFN system is not sufficiently matured. Interestingly, the IFN system has the capacity to continue its development in in vitro CEF cultures (51), which might be the reason for the merely transient productive growth of MVA- Δ E3L.

Generation of recombinant MVA by restoration of E3L function. Single-gene-dependent host range phenotypes in vaccinia virus infection can be elegantly used for efficient selection

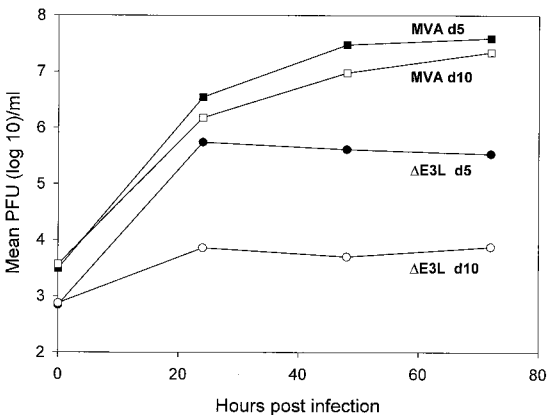


FIG. 8. Analysis of virus growth in primary fibroblast cultures prepared from 5-day-old (d5) chicken embryos (closed symbols) or 10-day-old (d10) chicken embryos (open symbols) after infection with a low-level dose of MVA- Δ E3L (Δ E3L) or MVA.

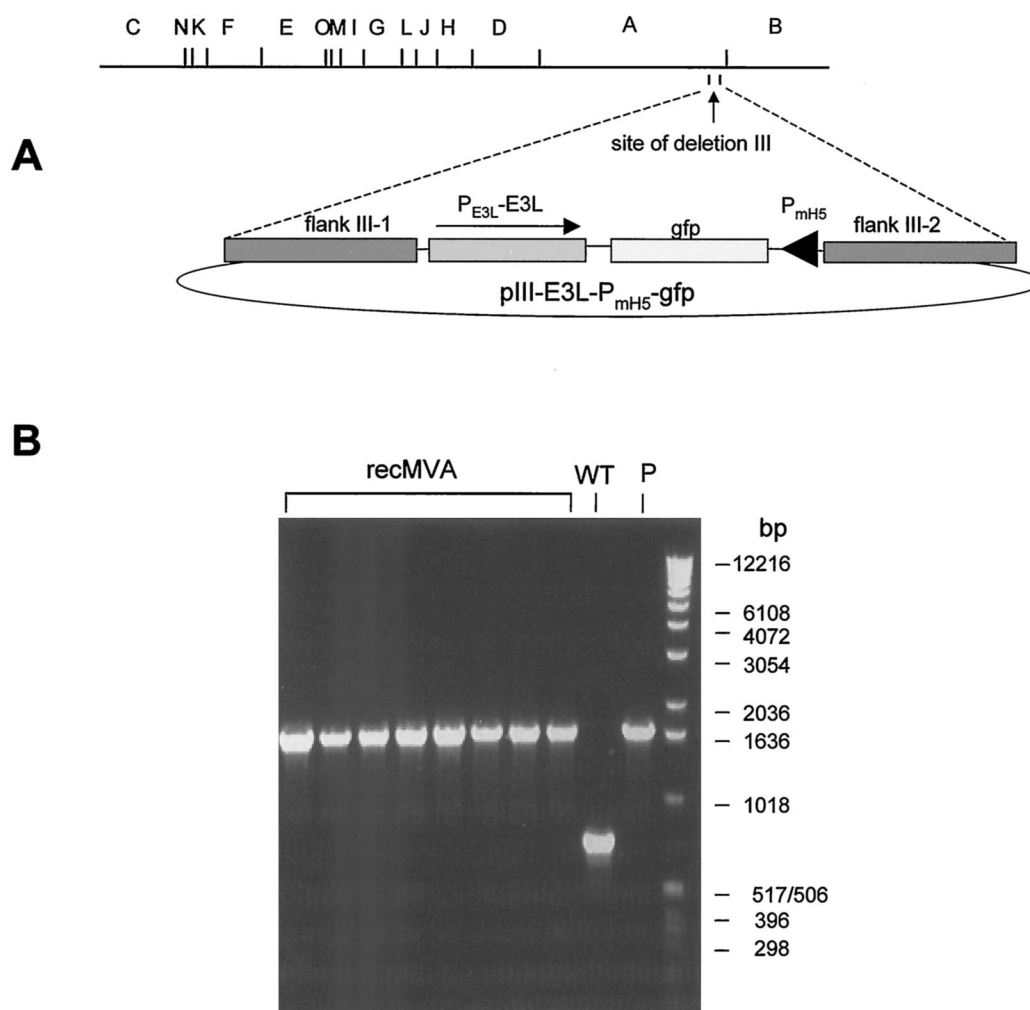


FIG. 9. Generation of recombinant MVA by E3L rescue and growth selection in CEF cells. (A) Schematic maps of the MVA genome (*Hind*III restriction map) and the vector plasmid pIII-E3L-P_{mH5}-gfp are shown. Flank III-1 and flank III-2 correspond to DNA sequences which target foreign genes as well as the selectable marker E3L in the site of deletion III within the MVA genome. The foreign gene is controlled by the modified vaccinia virus early-late promoter P_{mH5}. (B) PCR analysis of viral DNA. Genomic DNA isolated from eight different clones of recombinant MVA-P_{mH5}-gfp (recMVA) or from nonrecombinant MVA (WT) and plasmid pIII-E3L-P_{mH5}-gfp DNAs (P) served as template DNAs for the amplification of characteristic DNA fragments. A 1-kb DNA ladder was used as a standard.

of recombinant viruses through reinsertion of the host range gene into the mutant virus genome (26, 41, 55). To verify whether the growth restriction of MVA-ΔE3L in CEF would allow for such host range selection, we constructed an MVA insertion plasmid to target reintroduction of the E3L coding sequences under transcriptional control of its authentic promoter into the site of deletion III in the MVA genome. The addition of the *gfp* gene (which served as a model recombinant gene expressed with the vaccinia virus-specific promoter P_{mH5}) resulted in the MVA vector plasmid pIII-E3L-P_{mH5}-gfp (Fig. 9A). After transfection of this plasmid into MVA-ΔE3L-infected BHK-21 cells, we observed a few *gfp* gene-expressing cell clusters among the many cells showing virus-

specific cytopathic effects, which suggested that recombinant MVA had formed. To test for a selective restoration of virus growth on CEF, we inoculated 10-fold dilutions of the virus material obtained following transfection on CEF monolayers grown in 6-well tissue culture plates. After 3 days we observed the formation of virus plaques, virtually all of which contained green fluorescent protein-producing cells. From 10 well-separated plaques processed for further amplification on CEF, we recovered eight different isolates of recombinant MVA-P_{mH5}-gfp after one additional passage. PCR analysis of viral DNA demonstrated that all of the virus isolates represented bona fide recombinants carrying stable insertions at the site of deletion III within their genomes (Fig. 9B). The ease with which

these virus isolates were obtained reflects the essential requirement of E3L function for MVA growth on CEF and suggests that the E3L-specific rescue of MVA- Δ E3L can be proposed as an efficient host range selection protocol for the generation of recombinant MVA.

DISCUSSION

For the present study, we constructed the mutant virus MVA- Δ E3L by deleting the sequences of the vaccinia virus IFN resistance gene E3L. The latter belongs to the wide spectrum of vaccinia virus genes that encode factors known or predicted to evade host immune defenses (for a review, see reference 37). During attenuation of MVA, large numbers of genomic deletions occurred which were mostly located nearer to the termini of the genome and affected multiple genes that were initially considered nonessential because they are not required for virus replication in tissue culture (35). However, E3L (among other regulatory viral genes) had been conserved in the MVA genome upon over 570 serial propagations in CEF, which made the ORF an attractive target for mutagenesis (3). The ability to use transient K1L-based host range selection to isolate mutant viruses (55) to obtain mutant MVA- Δ E3L demonstrated that E3L is dispensable for MVA growth in hamster BHK-21 and rabbit RK-13 cells. This finding is in good agreement with previous work demonstrating that a vaccinia virus Copenhagen from which E3L was deleted is replication deficient in human HeLa and monkey Vero cells yet can productively grow in rabbit RK-13 and BHK cells (6, 7, 29, 30). In addition, this previous work showed that the vaccinia virus Copenhagen E3L deletion mutant replicated with wild-type efficiency in CEF. More surprising was our finding that MVA- Δ E3L failed to propagate in CEF. Reinsertion of E3L gene sequences yielded the revertant virus MVA-E3rev that again replicated efficiently in CEF and confirmed that the loss of E3L had indeed caused the growth restriction of MVA- Δ E3L.

The ease with which the revertant virus MVA-E3rev was obtained upon passage in CEF reflected an essential requirement of E3L function and spurred our further characterization of the host restriction in MVA- Δ E3L infection. Previous host range phenotypes found with MVA include a characteristic late block upon nonproductive infection of many mammalian cells, allowing for unimpaired viral DNA replication and late gene expression (11, 18, 60), and the inhibition of the MVA life cycle during nonpermissive infection of RK-13 cells, with only viral early mRNAs being made and a block in both DNA replication and viral intermediate transcription (62). The defect of MVA- Δ E3L in CEF showed a somewhat intermediate manifestation, with normal onset of viral DNA replication but complete cessation thereafter of host and viral protein synthesis. Similar poxvirus host range phenotypes have been described for the nonpermissive infection of CHO cells with wild-type vaccinia virus (43, 54) and the defective infection with the myxoma virus deletion mutant M-T5 of a rabbit CD4⁺ T-cell line (38).

The fact that these host cell restrictions were found associated with the induction of programmed cell death (27, 38, 44), together with previous evidence showing that vaccinia virus E3L can inhibit induction of apoptosis in human HeLa cells (29, 32), prompted us to search for signs of apoptosis in MVA-

Δ E3L-infected CEF. Indeed, in assaying for DNA fragmentation, caspase activity, and the presence of mono- and oligonucleosomes in the cytoplasmic fractions of infected cells, we were able to clearly demonstrate that the host range defect of MVA- Δ E3L in CEF was also linked to apoptosis. This onset of apoptosis was blocked upon infection with revertant virus MVA-E3rev, illustrating that E3L specifically functions as an efficient inhibitor of apoptosis in CEF. In similarity to findings for vaccinia virus-infected mammalian cells, the presence of dsRNA or the thereby-activated PKR might be responsible for a suicide response in vaccinia virus-infected chicken cells. In contrast to MVA- Δ E3L, the replicative capacity of vaccinia virus Copenhagen from which E3L was deleted is not impaired in CEF, which implies the contribution of another vaccinia virus-encoded protein(s) that rescues growth and blocks apoptosis in CEF and suggests that a corresponding gene sequence(s) was deleted or inactivated within the MVA genome. The hypothesized existence of such additional regulatory poxvirus gene function may be supported by the fact that fowlpox virus and canarypox virus (avipoxviruses that are routinely propagated in CEF) do not contain genes homologous to E3L (1, 23).

Another issue to explore is why productive MVA infection of CEF is obviously more dependent on E3L function than infection of BHK-21 or RK-13 cells. Interestingly, recent data suggest that mammalian cell lines can differ in regard to their levels of PKR and the quantities of dsRNA made during vaccinia virus infection (29, 30). Both RK-13 and BHK cells appear to endogenously produce relatively low amounts of PKR (30), which is a likely explanation for E3L's being nonessential during MVA infection as long as sufficient E3L gene product is available to function as competitive inhibitor of PKR. In MVA-infected primary CEF, on the other hand, there could be high-level induction of IFN response gene products such as PKR and an essential need for E3L-dependent sequestration of dsRNA to prevent their activation. The relevance of IFN response gene functions has been well established by the use of IFN treatment to modulate the requirement for E3L function upon vaccinia virus infection (6, 15, 17). Interestingly, when monitoring the presence of IFN after vaccinia virus infection of CEF, we found active IFN in supernatants of MVA-infected cultures but not in those from infections with vaccinia viruses CVA and WR. Similarly, only vaccinia virus MVA induced IFN- α/β after infection of human, ovine, or porcine peripheral blood mononuclear leukocytes (10).

A likely explanation for the increased IFN levels seen after MVA infection of primary cell cultures is the failure of MVA to produce functional vaccinia virus IFN- α/β receptor due to fragmentation of the corresponding gene in the MVA genome (3, 8). This virus-encoded IFN receptor (ORF B18R in vaccinia virus WR) has been shown to bind IFN- α/β with broad species specificity (16, 64) and might also have the ability to block chicken IFN. Moreover, we found increased amounts of IFN upon infection with MVA- Δ E3L, which suggests that the presence of E3L can prevent activation of IFN regulatory factor 3 and also a subsequent induction of IFN- β synthesis in chicken cells (72). The importance of the IFN system for the mediation of antipoxvirus activity in avian cells is well established. While vaccinia virus replication has been found to be relatively resistant to IFN activity in mammalian cells (40, 56,

68), pretreatment of CEF with chicken IFN appears to more efficiently inhibit the vaccinia virus life cycle (22, 28). In our experiments monitoring vaccinia virus infection after IFN treatment, we found that CEF-adapted MVA was as susceptible to IFN as the wild-type vaccinia virus strains CVA and WR. The data point to the possibility that the IFN made upon MVA or MVA- Δ E3L infection of CEF is sufficient to inhibit virus replication in the absence of E3L. This notion is further supported by our observation that MVA- Δ E3L can at least transiently grow in primary fibroblasts prepared from five-day-old chicken embryos which are still lacking a fully developed IFN system (51). In contrast, MVA- Δ E3L shows such a distinct growth restriction in standard CEF prepared from 10-day-old embryonated eggs that rescue of E3L gene function can be used as an efficient selection protocol to generate recombinant MVA.

Taken together, the data described here define the importance of the E3L gene product for vaccinia virus replication in IFN-competent primary avian cells. We demonstrated that E3L is essential for the propagation of the highly attenuated strain MVA in CEF, the cell culture it has been adapted to during more than a decade of serial passage. Our data reveal the induction of apoptosis and enhanced IFN synthesis as characteristics of the growth failure in the absence of E3L gene function and imply that the failure to block different IFN response pathways is responsible for the inability of MVA- Δ E3L to replicate in CEF. The identification of vaccinia virus E3L as an essential regulatory protein in avian cells may contribute to the identification of the corresponding IFN response proteins and help to elucidate their functional activity.

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