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Stable Expression of the Vaccinia Virus K1L Gene in Rabbit Cells Complements the Host Range Defect of a Vaccinia Virus Mutant

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Modified vaccinia virus Ankara (MVA), having acquired genomic deletions during passage in chicken embryo fibroblasts, is highly attenuated and unable to productively infect most mammalian cell lines. Multiplication in rabbit kidney-derived RK13 cells, but not other nonpermissive cells, can be restored by insertion of the vaccinia virus K1L gene into the MVA genome. During nonproductive infection of RK13 cells by MVA, transcription of representative viral early genes was revealed by Northern (RNA) blotting, whereas synthesis of an intermediate mRNA and replication of viral DNA could not be detected. Despite the persistence of viral early mRNA for at least several hours, synthesis of virus-induced polypeptides occurred only during the first hour and was followed by abrupt inhibition of all protein synthesis. Transfection of RK13 cells with a eukaryotic expression plasmid that contained the K1L gene allowed MVA infection to proceed to late stages of viral protein synthesis. Moreover, RK13 cell lines that stably expressed the K1L gene were permissive for MVA as well as a K1L deletion mutant of the WR strain of vaccinia virus. This is the first description of the complementation of a poxvirus mutant by cells that stably express a viral gene.

Vaccinia virus, the prototype *Orthopoxvirus*, can replicate in a wide range of cells. At least three viral genes, the vaccinia virus K1L and C7L and the cowpox 77-kDa genes, affect the host range of orthopoxviruses in tissue culture (9, 21, 25). The desire to develop attenuated poxvirus expression vectors has stimulated efforts to determine the roles of host range genes in virulence (5, 28). Both the K1L and 77-kDa genes encode proteins that contain ankyrin-like repeats, common to such diverse eukaryotic proteins as transcription factors, membrane receptors, and cell cycle regulators (3, 14). Neither the functions of the viral host range genes nor the precise stages at which replication is blocked by their loss or inactivation have been determined. Transformed cell lines that express poxvirus genes might provide new approaches to study virus-host interactions. Indeed, cell lines expressing adenovirus and herpesvirus genes have proven to be powerful tools for studying the replication of those viruses (7, 12, 24). Complementing cell lines that express poxvirus genes have not been described, perhaps because of the incompatibilities between the poxvirus and cellular transcription systems and the rapid inhibition of host gene expression following infection (18, 19).

We decided to develop a complementing cell line to investigate host range defects in the highly attenuated modified vaccinia virus Ankara (MVA) strain. During serial passage in primary chicken embryo fibroblasts (CEF), the virus became highly attenuated and restricted for most mammalian cells (15). MVA has six major genomic deletions affecting at least two host range genes (1, 16, 26). Marker rescue of the partially deleted K1L host range gene with wild-type DNA enabled recombinant MVA to grow in the rabbit kidney cell line RK13 but not in other mammalian cells tested (16). In the present study, we further characterized the block in MVA replication in RK13 cells and demonstrated that it could be overcome by transient transfection of a eukaryotic expression plasmid containing the K1L gene. Moreover, RK13 cells stably expressing the recombinant K1L gene were permissive for MVA and a K1L deletion mutant of the WR strain of vaccinia virus.

MATERIALS AND METHODS

Cells and viruses. Vaccinia virus strains Ankara (wild type [WT]) and MVA were kindly provided by A. Mayr, Veterinary Faculty, University of Munich, Munich, Germany. MVA-K1L is an MVA recombinant virus in which the partially deleted K1L gene and flanking DNA were restored with the corresponding WT DNA (16). The viruses were routinely propagated and titered in CEF grown in minimal essential medium (MEM) supplemented with 10% fetal calf serum (FCS). The rabbit kidney cell line RK13 was also grown in MEM supplemented with 10% FCS. Virus multiplication was determined by infecting cell monolayers with 10 PFU of virus per cell. After adsorption for 45 min at 37°C, the residual virus inoculum was removed, and the cell monolayer was washed twice with MEM and incubated with fresh medium (MEM, 2% FCS) at 37°C in a 5% CO_2 atmosphere. At indicated times after the absorption period, virus was harvested by freeze-thawing and brief sonication of the infected cells. The titer of the resulting lysate was determined in CEF.

Recombinant strain WR vaccinia viruses vREβCAT(K1L$^+$) and vREK1L$^-$ both contain the *Escherichia coli lacZ* gene regulated by the vaccinia virus 11K late promoter and will be described elsewhere.

Antibodies. Rabbit antipeptide antibody against the carboxy-

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terminal end of the vaccinia virus K1L protein (10) was kindly provided by R. Drillien, University of Strasbourg, Strasbourg, France.

Analysis of viral DNA. Cytoplasmic DNA from infected RK13 cells was transferred with a dot blot apparatus to a Hybond N+ membrane (Amersham International, Amersham, England) and hybridized to a ^{32}P-labeled MVA DNA probe. Radioactivity was quantitated with a Betascope 603 blot analyzer (Betagen, Waltham, Mass.).

Analysis of [^{35}S]methionine-labeled polypeptides. Cell monolayers, in 12-well plates, were infected with virus at a multiplicity of 15 PFU per cell. Following a 45-min adsorption period at 4°C, MEM supplemented with 2% FCS was added and the cell cultures were incubated at 37°C in a 5% CO_2 atmosphere. At indicated times after virus adsorption, the medium was removed and each culture was washed once with 1 ml of methionine-free MEM. To each well, 0.2 ml of methionine-free MEM supplemented with 50 μCi of [^{35}S]methionine was added, and the mixture was incubated for 30 min at 37°C. Cytoplasmic extracts of infected cells, prepared by incubating each well with 0.2 ml of 0.5% Nonidet P-40 (Sigma, St. Louis, Mo.) lysis buffer for 10 min at 37°C, were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE).

Isolation and Northern (RNA) blot analysis. Total cellular RNA was extracted from infected cells by the guanidinium isothiocyanate-CsCl procedure (6). RNA was electrophoresed on 1% agarose–1.1 M formaldehyde gels and transferred to nitrocellulose filters as described by Sambrook et al. (23). The DNA probes were prepared by random-primed ^{32}P labeling of the total DNA sequences of the vaccinia virus genes C11R, D9L, and G8R. Prehybridization (3 h) and hybridization (18 h) were performed at 42°C in a solution containing 50% formamide, 5× SSPE (0.75 M NaCl, 50 mM sodium phosphate, 5 mM EDTA [pH 7.4]), 0.5% (wt/vol) SDS, 5× Denhardt's mixture, and 100 μg of sheared and denatured salmon sperm DNA per ml. The filters were washed twice in 2× SSPE plus 0.1% SDS at room temperature for intervals of 15 min and then twice for 15 min each time in 1× SSPE containing 0.1% SDS at 50°C. A final stringent wash was carried out in 0.1× SSPE containing 0.1% SDS at 55°C. All washing solutions contained 0.03% (wt/vol) sodium pyrophosphate.

Plasmid construction. The K1L open reading frame (ORF), containing an *Eco*RI site at the start and a *Bam*HI site at the end, was amplified by PCR using WT DNA as a template and cloned into unique restriction sites of eukaryotic expression vector pSG5 (Stratagene, La Jolla, Calif.). The primers were 5′-GGG GGG GAA TTC GCG TCT CGT TTC AGA CAT GGA-3′ (*Eco*RI site underlined, K1L start codon in boldface) and 5′-GGG GGG GGA TCC TGT GGG AGA ATC TAA TTA GTT-3′ (*Bam*HI site underlined, K1L termination codon in boldface).

Transient expression assays. Monolayers of nearly confluent RK13 cells in 12-well Costar tissue culture plates were transfected with 10 μg of pSG-K1L DNA per well, using the Lipofectin reagent (GIBCO/BRL, Gaithersburg, Md.) as recommended by the manufacturer. At 24 h after transfection, cells were infected with 10 PFU of MVA per cell. At 6 h after infection, cultures were pulse-labeled in methionine-free medium to which [^{35}S]methionine had been added, and cytoplasmic extracts were analyzed by SDS-PAGE.

Isolation of stably transfected RK13 cell lines. Monolayers of 90% confluent RK13 cells in six-well Costar tissue culture plates were cotransfected with 20 μg of pSG-K1L and 2 μg of pRc/CMV (Invitrogen, San Diego, Calif.) per well, using the Lipofectin reagent. At 24 h after transfection, cells were trypsinized, resuspended in medium (MEM, 10% FCS) containing 800 μg of G418 (Sigma) per ml, transferred to 10-cm^2 Falcon tissue culture dishes, and incubated for 12 days at 37°C in a 5% CO_2 atmosphere. At days 4 and 8, the spent medium was replaced with fresh selection medium. G418-resistant cell colonies were picked, transferred into 24-well Costar tissue culture plates, and propagated as separate cell clones. Monolayers, in 12-well tissue culture plates, derived from the cell clones were infected with 0.05 PFU of MVA per cell and monitored for development of a cytopathic effect. Positive cell clones were amplified in Costar T75 tissue culture flasks, trypsinized, resuspended in medium (MEM, 20% FCS) containing 10% dimethyl sulfoxide (Sigma), and stored in liquid nitrogen.

A second, independent isolation of stably transfected RK13 cell lines was performed. Monolayers of 50% confluent RK13 cells were transfected with 2 μg of pRc/CMV or cotransfected with 18 μg of pSG-K1L and 2 μg of pRc/CMV per 10-cm^2 dish. G418-resistant colonies were selected as described above except that the medium contained 500 μg of G418 per ml and was replaced every third day. After 10 days, individual colonies were transferred to 24-well culture plates. Each clone was expanded to six-well and then to 75-cm^2 dishes, at which time glycerol stocks were made in freezing medium (GIBCO/BRL). Screening was accomplished by seeding duplicate wells of 12-well plates with each cloned cell line and then infecting them at 30 PFU per cell with either vREβCAT(K1L$^+$) or vREK1L(K1L$^-$) vaccinia virus. After 12 h, the cells were harvested and β-galactosidase activity was measured as described below.

Analysis of cellular DNA. Total cellular DNA was isolated by standard procedures (23) and PCR amplified by using 35 cycles of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C. The primers used for amplification were GS49 (5′-GAC GAT ATC GAG ATC TTT TGT ATA GGA GTC AGA-3′) and GS54 (5′-CCA TTA CAT CAG GCA GCC ACA TTA GAA GAT ACC-3′). PCR products were analyzed by agarose gel electrophoresis. The same preparation of DNA was also digested with *Eco*RI and analyzed by electrophoresis on a 1% agarose gel. The DNA was transferred to a Hybond N+ membrane and hybridized with a ^{32}P-labeled probe of 600 bp of vaccinia virus WT DNA from within the K1L ORF.

Reverse transcription-PCR analysis of RNA. Total RNA (3 μg) from infected cells was isolated as described by Chirgwin et al. (6) and transcribed with 25 U of avian myeloma virus reverse transcriptase (Boehringer Mannheim, Indianapolis, Ind.), using 0.5 μg of oligo(dT)$_{12-18}$ in a total volume of 50 μl at 42°C for 90 min in 50 mM Tris-HCl (pH 8.0)–70 mM KCl–10 mM $MgCl_2$–1 mM each of the four deoxyribonucleoside triphosphates–4 mM dithiothreitol. Subsequently, the RNA was purified by phenol-chloroform extraction, precipitated with ethanol, and resuspended in 50 μl of H_2O. A 1-μl portion was PCR amplified by using 40 cycles (as described above) with a DNA thermal cycler. PCR products were analyzed by agarose gel electrophoresis. The oligonucleotide primers used for the reaction were GS49 and GS54 (sequences are shown above).

Western immunoblotting. Proteins were resolved by electrophoresis on an SDS–11% polyacrylamide gel and electroblotted onto nitrocellulose for 2 h at 4°C in a buffer containing 25 mM Tris, 192 mM glycine, and 20% methanol (pH 8.6). The following procedures were done at room temperature. The blots were blocked in a phosphate-buffered saline (PBS) buffer containing 1% nonfat milk and 0.1% Nonidet P-40 (Sigma) for 1 h and then incubated for 3 h with antisera diluted 100-fold in the same PBS buffer. After being washed with 0.1% Nonidet

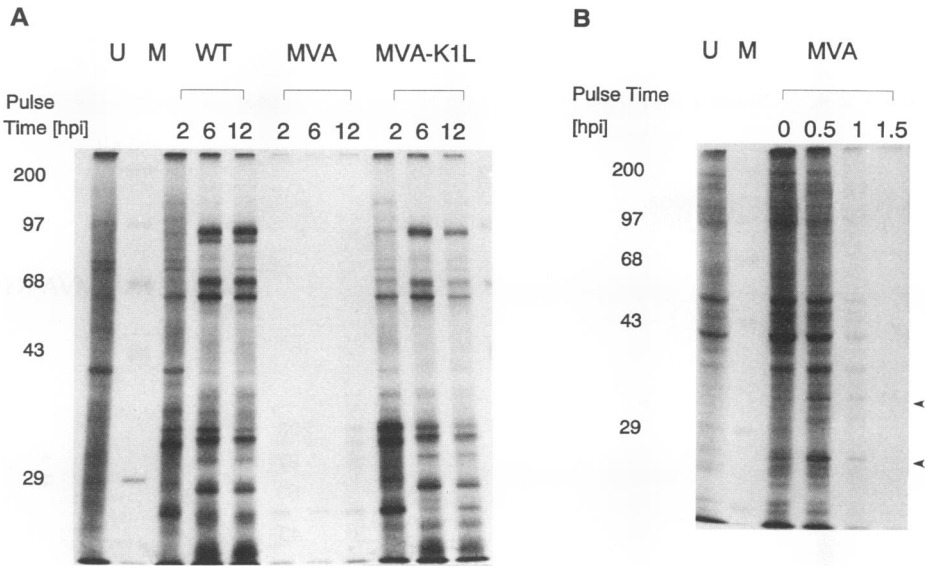

FIG. 1. Polypeptide synthesis in virus-infected RK13 cells. (A) Uninfected RK13 cells (U) or cells at the indicated hour after infection (hpi) with WT, MVA, or MVA-K1L were labeled with [^{35}S]methionine for 30 min. Cell lysates were heated in 2% SDS–1% dithiothreitol and analyzed by electrophoresis on a 10% polyacrylamide gel. An autoradiogram is shown. Protein standards (lane M) are indicated by their molecular masses (in kilodaltons) on the left. (B) RK13 cells were infected with MVA, labeled with [^{35}S]methionine at the indicated hours after infection, and analyzed as in panel A. Representative early virus-induced proteins are marked by arrowheads.

P-40 in PBS, the blots were incubated with ^{125}I-labeled protein A (Amersham, Arlington Heights, Ill.) for 1 h, washed again, and exposed to X-ray film overnight.

β-Galactosidase assay. Confluent cell monolayers were infected with recombinant MVA LZ (15 PFU per cell), which expresses the *E. coli lacZ* gene under the control of the vaccinia virus late promoter P11 (27). After overnight incubation at 37°C, cytoplasmic extracts were prepared and the protein concentration (4) and β-galactosidase specific activity (17) were determined.

RESULTS

Macromolecular synthesis in MVA-infected RK13 cells. We had previously shown that viral early and late proteins were synthesized in human cells infected with MVA and that the block to replication occurred during virion assembly (27). Similar analyses were performed by metabolically labeling RK13 cells with [^{35}S]methionine for 30-min intervals at early and late times after infection. Autoradiograms of SDS-polyacrylamide gels containing proteins labeled at 2 h after infection with either WT or MVA-K1L, a recombinant MVA containing an intact K1L gene, revealed several prominent virus-induced early proteins of less than 40 kDa (Fig. 1A). By 6 h, host protein synthesis was inhibited and prominent viral late proteins, including some greater than 60 kDa, were detected. By contrast, no protein synthesis was detected between 2 and 12 h after infection of RK13 cells with MVA (Fig. 1A). To better understand what was happening, we pulse-labeled RK13 cells immediately after adsorption of MVA and at short intervals thereafter. During the first hour, host as well as virus-induced early polypeptides were made, but protein synthesis was abruptly terminated after that time (Fig. 1B). This effect seemed to be specific for loss of the K1L gene, since the pattern of protein synthesis in cells infected with MVA-K1L was similar to that of WT-infected cells. These data suggested that the MVA host range defect in RK13 cells, unlike that in human cells, was manifested shortly after the induction of viral early protein synthesis, preventing the expression of intermediate and late viral genes.

To further investigate the host range defect, the synthesis of representative viral early and intermediate RNAs in RK13 cells infected with MVA-K1L and MVA was analyzed. Total cellular RNA obtained at 0, 0.5, 1, 2, and 4 h after infection was fractionated by agarose gel electrophoresis and transferred to membranes. Probes for two early RNAs, encoded by the C11R and D9L ORFs, were used. With both viruses, the C11R RNAs were most abundant at 2 h after infection (Fig. 2A), although in the case of MVA-K1L, there was more of this RNA species at 1 h than at 4 h, and the reverse was true for MVA. The trend to delayed and prolonged early mRNA accumulation in cells infected with MVA, compared with MVA-K1L, was more obvious in the case of D9L (Fig. 2B). The RNA bands of high molecular weight detected at 4 h after infection with MVA-K1L are probably read-through products of the D10L gene, which is expressed after DNA replication (Fig. 2B). We note that both early mRNAs examined were present in MVA-infected cells at times well beyond the shutoff of protein synthesis (Fig. 1).

When the blots were probed with the viral intermediate G8R gene, transcripts were detected in RK13 cells at 2 and 4 h after infection with MVA-K1L but not after infection with MVA (Fig. 2C). Since viral intermediate gene expression is dependent on DNA replication (2, 13, 29), we analyzed the viral DNA present in infected RK13 cells (Fig. 3). Viral DNA replication was not detected in MVA-infected RK13 cells, providing one reason for the absence of intermediate and late gene expression. By contrast, DNA replication occurred in MVA-K1L-infected RK13 cells.

Transient expression of the K1L gene regulated by the SV40 promoter. Experiments with the MVA-K1L recombinant virus suggested that intermediate and late viral gene expression in MVA-infected RK13 cells depends on the product of the viral K1L gene. To prove this, however, it is necessary to express the

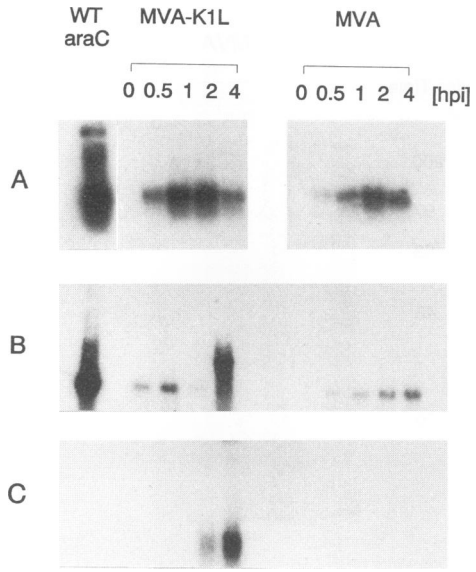

FIG. 2. Northern blot analysis of viral RNA extracted from virus infected RK13 cells. Total cellular RNA was extracted from cells at the indicated hours after infection (hpi) with MVA-K1L or MVA, electrophoresed on an agarose-formaldehyde gel, blotted onto a nitrocellulose filter, and autoradiographed. Total cellular RNA isolated from HeLa cells infected with WT in the presence of cytosine arabinoside served as control and was loaded on the gel in the lanes marked WT araC. Hybridization with ^{32}P-labeled DNA probes derived from the early viral gene C11R (A), early viral gene D9L (B), and intermediate viral gene G8R (C) is shown.

gene in *trans*. We therefore constructed plasmid pSG-K1L, in which the K1L gene is regulated by the simian virus 40 (SV40) early promoter (Fig. 4A). The plasmid was transfected into RK13 cells, which were then infected with MVA and labeled with [^{35}S]methionine 6 h later. SDS-PAGE analysis of these extracts demonstrated that synthesis of viral late polypeptides

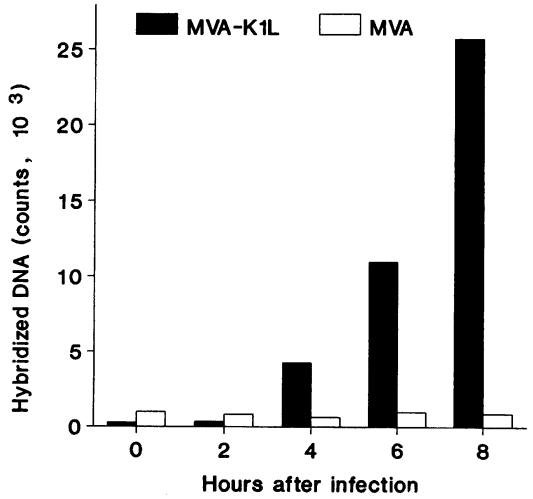

FIG. 3. Viral DNA synthesis in infected RK13 cells. DNA from RK13 cells at 0, 2, 4, 6, or 8 h after infection with MVA or MVA-K1L was immobilized on a Hybond N+ membrane. Viral DNA was detected by hybridization of a ^{32}P-labeled viral DNA probe. Radioactivity was quantitated with a Betascope 603 blot analyzer.

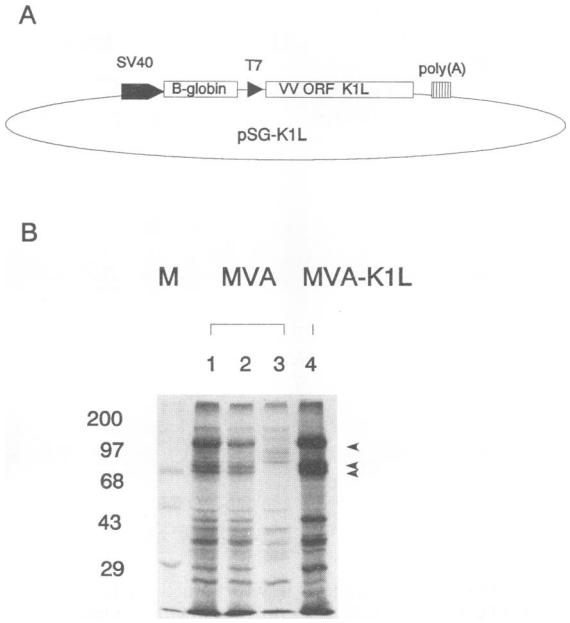

FIG. 4. Effects of transient expression of the vaccinia virus K1L gene on MVA-induced protein synthesis. (A) Schematic of pSG-K1L derived by insertion of the K1L ORF into the expression vector pSG5. The positions of the SV40 early promoter, the β-globin intron, the T7 promoter, the vaccinia virus (VV) K1L ORF, and the SV40 poly(A) signal sequence are shown. (B) Synthesis of viral late polypeptides in transfected RK13 cells infected with MVA. RK13 cells were transfected with pSG-K1L (lanes 1 and 2) or pSG5 (lanes 3 and 4) and infected with MVA (lanes 1 to 3) or MVA-K1L (lane 4) at 24 h after transfection. At 6 h after infection, cells were labeled with [^{35}S]methionine for 30 min. Cell lysates were prepared and analyzed by electrophoresis on an 11% polyacrylamide gel. The numbers on the left indicate the positions and molecular masses (in kilodaltons) of protein standards (lane M). Representative late viral proteins are marked by arrowheads.

was dependent on transfection with the K1L expression plasmid (Fig. 4B). In fact, the viral protein patterns observed in pSG-K1L-transfected and MVA-infected cells (lanes 1 and 2) were similar to those of MVA-K1L-infected RK13 cells (lane 4). In contrast, MVA failed to induce the synthesis of viral late proteins in RK13 cells transfected with the vector plasmid pSG5 (lane 3).

Isolation of RK13 cell lines that stably express the viral K1L gene. Since transient expression of the viral K1L gene permitted late gene expression to occur in MVA-infected RK13 cells, we attempted to make stably transfected cell lines. RK13 cells were cotransfected with pSG-K1L and pRc/CMV, a plasmid containing the aminoglycoside phosphotransferase resistance (*neo*) gene under the control of the SV40 early promoter. Since no cytopathic effects were observed when RK13 cells were infected with low multiplicities of MVA, this procedure was used to screen G418-resistant cell lines. Of 66 such cell lines, 8 exhibited moderate to strong cytopathic effects, suggesting that they were permissive for MVA. In a second transfection experiment, 26 G418-resistant cell lines (24 monoclonal and 2 polyclonal) that had been transfected with the selection plasmid pRc/CMV alone, as well as 30 G418-resistant cell lines (29 monoclonal and 1 polyclonal) that had been cotransfected with pRc/CMV and pSG-K1L, were isolated. In addition, two nonrecombinant RK13 cell lines were obtained by limiting dilution. An alternative screening procedure, in which dupli-

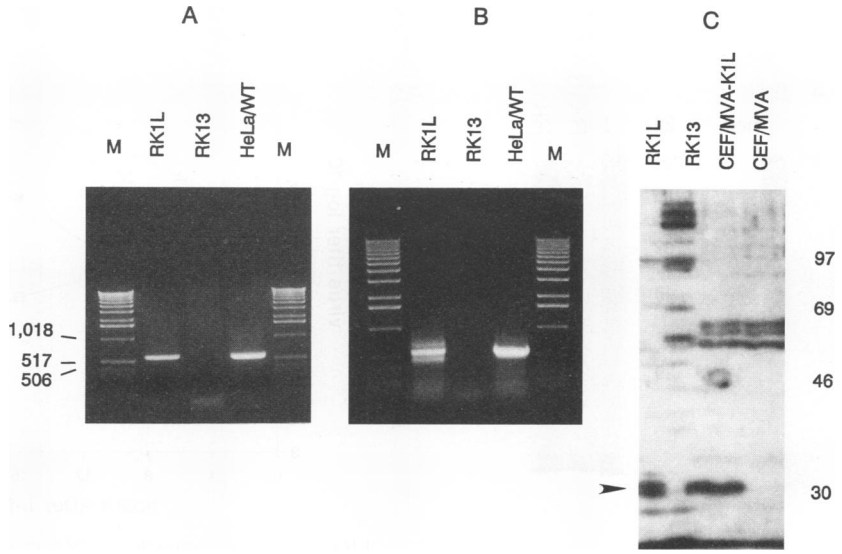

FIG. 5. Analysis of RK13 cells stably transfected with pSG-K1L. (A) PCR analysis of DNA from RK13 cells, an RK13-K1L cell line (abbreviated RK1L) stably transfected with pSG-K1L, and HeLa cells infected with WT virus (designated HeLa/WT). M, double-stranded DNA markers indicated in base pairs. (B) Reverse transcription-PCR analysis. Purified cellular RNA was reverse transcribed into first-strand cDNA, and the latter was used as a PCR template. Lanes are designated as in panel A. (C) Western blot analysis. Whole-cell extracts prepared from RK13-K1L cells (RK1L), RK13 cells (RK13), CEF infected with MVA-K1L (CEF/MVA-K1L), and CEF infected with MVA (CEF/MVA) were dissociated with SDS and dithiothreitol, electrophoresed on a 10% polyacrylamide gel, electroblotted onto nitrocellulose, probed with an antipeptide antiserum, incubated with ^{125}I-protein A, and autoradiographed. Numbers on the right indicate the positions and molecular masses (in kilodaltons) of protein standards. The band representing the K1L gene product is marked by an arrowhead.

cate wells were seeded with each cell line and then infected with a high multiplicity of K1L$^-$ or a K1L$^+$ recombinant vaccinia virus (strain WR) that contained *lacZ* regulated by the late 11K promoter, was devised. Although this screen had some background, β-galactosidase activity induced by the K1L$^-$ virus did not approach that of the K1L$^+$ virus in either the untransfected cell lines or the cell lines that had been transfected with pRc/CMV alone. By contrast, one of the cotransfected cell lines had as much β-galactosidase after infection with the K1L$^-$ virus as after infection with the K1L$^+$ virus, and a few had intermediate levels.

Slot blot DNA-RNA hybridizations were carried out to determine whether there was a correlation between complementation of K1L$^-$ virus in the screening procedure and expression of the K1L gene. RNA was isolated from a total of 24 representative cell lines, including 3 nonrecombinant RK13 cell lines, 9 from the control pRc/CMV transfection group, 9 from the second doubly transfected group, and 2 that permitted strong MVA cytopathic effects in the first screen. Reactivity with the K1L probe was detected only with RNA samples from three cell lines: the two noted above from the first screen and the strongly positive one from the second screen. No attempts were made to further analyze cell lines that appeared weakly permissive during screening.

Further characterization of a stably transfected RK13 cell line. One of the permissive cell lines was chosen for further study. Integration of the viral K1L gene was demonstrated by PCR. Primers were designed to amplify a DNA fragment of 587 bp from within the vaccinia virus K1L ORF. Using DNA from RK13-K1L, we amplified a DNA fragment of the same size (Fig. 5A). This fragment comigrated with a PCR product that was amplified by using DNA derived from WT-infected HeLa cells. In contrast, PCR of DNA extracted from RK13 cells yielded no amplification product. When DNA isolated from RK13-K1L cells was digested with the restriction enzyme *Eco*RI, a single DNA fragment (2.8 kbp) that hybridized with a ^{32}P-labeled K1L probe was detected (data not shown). This finding suggested that integration of the viral K1L gene occurred at a single site in the cell genome.

Reverse transcription-PCR analysis was used to confirm the presence of K1L RNA in the RK13-K1L cell line (Fig. 5B). First-strand products derived from purified RNA of WT-infected HeLa cells and uninfected RK13-K1L cells were amplified with the primers GS49 and GS54 to give PCR products of the expected sizes. The specificity of the PCR was confirmed with RNA from untransformed RK13 cells.

Synthesis of the K1L polypeptide by RK13-K1L cells was established by Western blotting using the anti-K1L peptide antiserum (10) (Fig. 5C). The antipeptide serum reacted specifically with a 32-kDa protein from MVA-K1L-infected CEF and uninfected RK13-K1L cells (marked by an arrowhead) corresponding in size to the K1L gene product.

Late gene expression in MVA-infected RK13-K1L cells. Two stably transfected RK13 cell lines were infected with MVA and incubated with [^{35}S]methionine to determine whether the cells would support late gene expression. As negative controls, an untransfected RK13 cell line and one stably transfected with the *neo* gene alone were infected with MVA. As a positive control, RK13 cells were infected with the recombinant MVA-K1L. In each case, the labeled polypeptides were separated by SDS-PAGE and detected by autoradiography. Late viral proteins were made in MVA-infected RK13-K1L cells (Fig. 6A, lanes 1 and 2) but not in the control RK13 cells (Fig. 6A, lanes 3 and 4).

To provide further evidence for viral late gene expression, we infected RK13 and RK13-K1L cells with MVA LZ, an MVA recombinant virus that expresses the *E. coli lacZ* gene under the control of the well-characterized vaccinia virus late promoter P11. As expected, MVA LZ-infected RK13-K1L cells synthesized a protein of the size, 116,000 Da, expected for

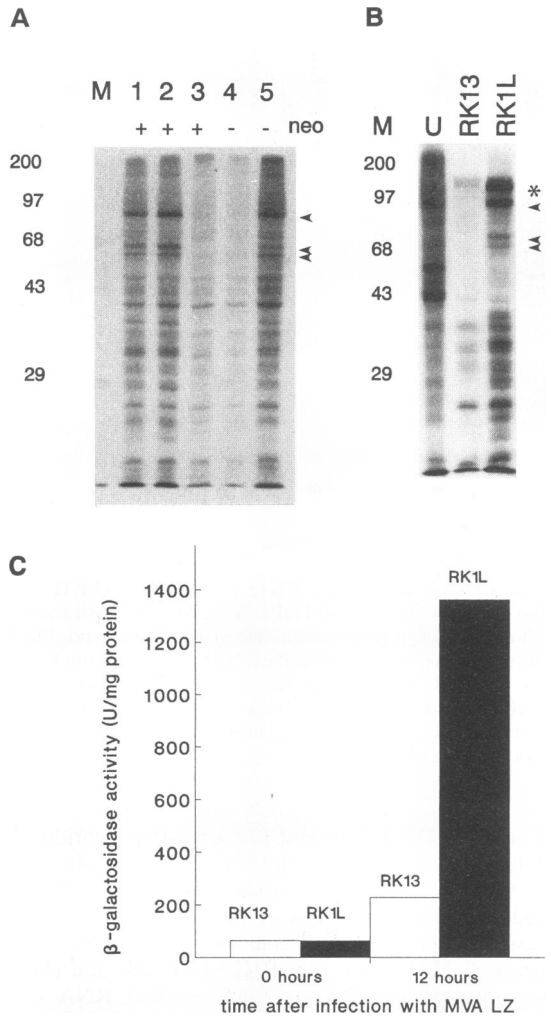

FIG. 6. MVA late gene expression in RK13-K1L cells. (A) Synthesis of viral late polypeptides during MVA infection. RK13-K1L cells infected with MVA (lanes 1 and 2) and RK13 cells infected with MVA (lanes 3 and 4) or MVA-K1L (lane 5) were labeled with [^{35}S]methionine at 6 h postinfection. Cell lysates were analyzed by electrophoresis on a 10% polyacrylamide gel. The ability (+) or inability (−) of the cells to express the *neo* gene is shown. The positions of protein standards (lane M) and their molecular masses (in kilodaltons) are indicated on the left. The positions of representative viral late polypeptides are marked by arrowheads. (B) SDS-PAGE of RK13 and RK13-K1L (RK1L) cells infected with MVA LZ. The infected cultures were labeled with [^{35}S]methionine at 6 h postinfection. Cell lysates were analyzed by electrophoresis on a 10% polyacrylamide gel. Lane U contained uninfected RK13 cell extract. Numbers on the left indicate positions and molecular masses (in kilodaltons) of protein standards. The positions of some representative viral late proteins are marked by arrowheads. The protein band representing the enzyme β-galactosidase is indicated by an asterisk. (C) β-Galactosidase activity. RK13 and RK13-K1L (RK1L) cells were infected with MVA LZ. After 12 h, cytoplasmic extracts were prepared and β-galactosidase activities were determined.

β-galactosidase (27). The recombinant protein, as well as late viral proteins, were absent in RK13 cells infected with MVA LZ (Fig. 6B). These results were confirmed in a more quantitative manner by β-galactosidase assays of extracts of MVA LZ-infected RK13-K1L cells (Fig. 6C). In contrast, the activity in extracts of infected RK13 cells only slightly exceeded background levels.

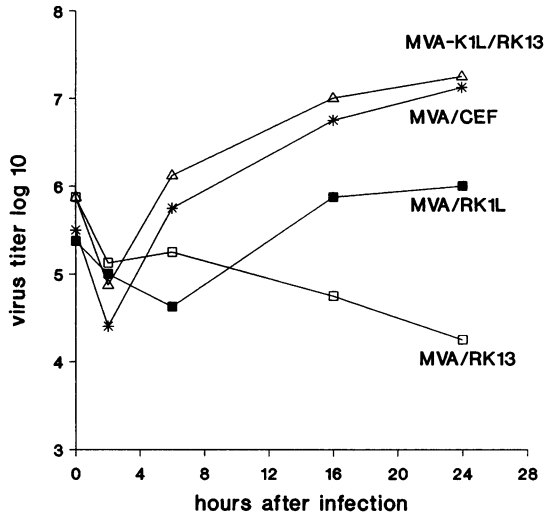

FIG. 7. One-step growth of MVA in RK13-K1L cells, RK13 cells, and CEF and of MVA-K1L in RK13 cells. Titers were determined in CEF.

Multiplication of MVA in R13-K1L cells. One-step virus growth analyses were made to determine whether cellular expression of the viral K1L gene could fully complement the host range defect of MVA in RK13 cells (Fig. 7). In MVA-infected RK13 cells, the residual infectivity of the inoculum dropped progressively during 24 h of infection. By contrast, between 6 and 24 h after infection of RK13-K1L cells with MVA, more than a 10-fold increase in viral titer occurred. The titer was still about 1/10 below that achieved with MVA in CEF or the MVA-K1L recombinant virus in RK13 cells.

DISCUSSION

Complementing cell lines that stably express one or more poxvirus genes could provide useful research tools. For the selection of a suitable viral gene to develop this approach, we considered the following criteria. First, loss of the gene function should entirely abrogate virus replication. Second, the product of the viral gene should be required early in infection and in relatively low amounts because of the rapid inhibition of host gene expression. Third, the gene product should be stable and not toxic to cells. We felt that the vaccinia virus K1L gene probably met at least the first two criteria. The K1L gene is expressed early in infection (10) and is required for replication of vaccinia virus in rabbit kidney cells and in human cells in the absence of the C7L gene (9, 21). Although the function of the K1L gene is not known, homology searches did not suggest a similarity to any genes with known toxic effects. We decided to use the MVA strain of vaccinia virus, which has a truncated K1L gene and cannot grow in most mammalian cell lines (1, 16). Previous marker rescue studies suggested that an MVA recombinant containing the K1L gene allowed MVA to replicate in RK13 cells but not in human cells (16). Although this interpretation was a likely one, the DNA used for marker rescue contained flanking sequences that could have a role in host range. Therefore, we considered that the success of our experiments could resolve this question.

Although the determination of the role of the K1L gene in virus replication was not the main object of these studies, we

did carry out experiments to further characterize the host range defect of MVA in RK13 cells. Transient synthesis of virus-induced proteins was followed by an abrupt inhibition of all protein synthesis, similar to that described by Drillien et al. (8) for human cells infected with the Copenhagen strain of vaccinia virus containing a deletion that included both the K1L and C7L host range genes (21). We extended the results of Drillien et al. (8) by using Northern blotting to show the accumulation of two specific early mRNAs in MVA-infected RK13 cells well beyond the time of almost complete cessation of protein synthesis. This result argues against a general effect on mRNA stability and suggests a block in translation of early mRNA. The absence of DNA replication, demonstrated by DNA hybridization instead of [^{3}H]thymidine incorporation, provided one explanation for our failure to detect the synthesis of intermediate mRNAs. It is likely that MVA has a functional C7L gene, since the host range defect occurs at a late stage of virus assembly in human cells (27) and C7L sequences can be detected by DNA hybridization (26).

Since the MVA-K1L recombinant virus synthesized viral RNA and proteins normally, we attempted to determine whether the K1L gene expressed in *trans* would allow expression of viral late genes in RK13 cells infected with MVA. We inserted the K1L ORF into the plasmid vector pSG5 (11), which contains the SV40 early promoter for expression and intron II of the rabbit β-globin gene and a polyadenylation signal for splicing and 3′ modification of transcripts, respectively. Initial evidence for functional expression of the viral K1L gene was obtained by transient assays. The expression vector was transfected into RK13 cells that were subsequently infected with MVA. Metabolic labeling experiments demonstrated that late viral proteins were made. Encouraged by this result, we isolated stable transfectants by G418 selection. An initial screening was performed by assaying for either cytopathic effects or late reporter gene expression by K1L$^-$ virus. Importantly, only permissive cell lines had detectable K1L RNA when screened by DNA hybridization. One cell line was further analyzed and shown to synthesize immunoreactive protein of the correct size, to support late viral gene expression, and to support the production of infectious MVA K1L$^-$ virus. Although the yield of MVA was not as high as that obtained with recombinant virus, more efficient eukaryotic promoters are available for increasing the levels of the K1L protein, should that be limiting.

The RK13-K1L cell lines should be useful for determining the nature of the host range block. Recent studies indicate the presence of an active factor required for in vitro transcription of intermediate genes in the stably transfected RK13 cells that is undetectable in untransfected RK13 cells (22). It will be interesting to test whether the K1L gene product has activated cellular functions required for viral protein, RNA, and DNA synthesis. It seems likely that a similar approach could be used to make permissive human cell lines that contain either the K1L or C7L gene and Chinese hamster ovary cells that contain the cowpox 77-kDa gene. However, the usefulness of this method should not be limited to host range studies. Cell lines that express viral proteins required for DNA replication and transcription would be useful for isolating and propagating viral deletion mutants. Such mutants would be useful for genetic studies and might make exceptionally safe live recombinant vaccine vectors (20).

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