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The Role of the PKR-Inhibitory Genes, E3L and K3L, in Determining Vaccinia Virus Host Range

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Vaccinia virus encodes two regulators of the cellular antiviral response. The E3L gene is thought to act primarily by sequestering double-stranded RNA, whereas the K3L gene is thought to act as a competitive inhibitor of the double-stranded RNA-dependent protein kinase, PKR. The broad host range associated with vaccinia virus replication appears to be related to the presence of these genes. The E3L gene is required for replication in HeLa cells, but is not required for replication in BHK cells. On the contrary, the K3L gene is required for replication in BHK cells, but is dispensable for replication in HeLa cells. Our results suggest that these cell lines varied in the expression of endogenous activatable PKR and that replication of vaccinia virus in different cell lines led to altered levels of double-stranded RNA synthesis from the virus. Vaccinia virus was able to overcome these cellular variations by regulating PKR activity through the synthesis of either E3L or K3L. The results suggest that vaccinia virus has evolved a broad host range by maintaining both the E3L and the K3L genes.

Key Words: PKR; K3L; E3L; interferon; dsRNA; vaccinia virus; host range.

INTRODUCTION

Vaccinia virus (VV), a member of the *Orthopox* group, has previously been characterized as a virus with a broad host range and significant resistance to the antiviral effects of interferon (IFN) (Beattie *et al.,* 1995; Davies *et al.,* 1993). The antiviral state present in interferon-treated cells involves the induction of at least two interferon-regulated pathways (Samuel, 1991). The double-stranded RNA (dsRNA)-dependent protein kinase, PKR, pathway involves the up-regulation of this enzyme in an inactive form and activation requires binding to dsRNA or other polyanions (Meurs *et al.,* 1990; Gunnery and Mathews, 1998). PKR bound to dsRNA undergoes an autophosphorylation event and subsequently phosphorylates various substrates including the α subunit of protein synthesis initiation factor eIF2, NF-90, and HIV tat protein (Brand *et al.,* 1997; Langland *et al.,* 1999). The phosphorylation of eIF2α leads to the interference of the eIF2B-mediated exchange of GTP for GDP necessary for the catalytic function of eIF2. This ultimately leads to the inhibition of protein synthesis at the level of initiation. Similar to the PKR pathway, the 2′-5′ oligoadenylate synthetase (OAS) pathway involves the interferon-induction of an inactive form of the enzyme and activation requires interaction with dsRNA (Kerr and Brown, 1978; Carroll *et al.,* 1997). Activated OAS synthesizes 2′-5′-linked oligoadenylates, which activate RNase L. Activated RNase L cleaves mRNA and rRNA leading to the inhibition of protein synthesis at the level of RNA stability. Both PKR and OAS are present in most cell lines at a basal level even in the absence of interferon treatment, but activation requires the presence of dsRNA, or possibly endogenous cellular protein activators such as PACT/RAX (Patel *et al.,* 2000; Ito *et al.,* 1999).

Vaccinia virus, similar to many other viruses, has evolved mechanisms to evade the antiviral activities associated with interferon. The VV E3L gene encodes the dsRNA-binding proteins p20 and p25. These proteins contain a highly conserved dsRNA-binding motif found in many dsRNA-binding proteins, including PKR. The E3L gene products inhibit activation of both PKR and OAS by binding to and sequestering potential dsRNA activator molecules. Various assays have demonstrated that dsRNA binding is necessary for p20/p25 inhibition of PKR activity and that excess dsRNA can overcome the inhibitory effects of p20/p25 (Chang and Jacobs, 1993; Shors *et al.,* 1997). Recently, though less well characterized, the E3L gene products have been suggested to inhibit PKR by direct interaction with PKR leading to heterodimer formation (Romano *et al.,* 1998).

The VV K3L gene has limited homology to the amino-terminal region of eIF2α. The K3L gene product inhibits autophosphorylation of both PKR and eIF2α substrate phosphorylation (Carroll *et al.,* 1993). Both K3L and eIF2α bind to PKR at a similar or overlapping region in the catalytic domain of the enzyme. It has been suggested that the K3L gene product acts as a pseudosubstrate, binding to PKR in competition with eIF2α, thereby block-

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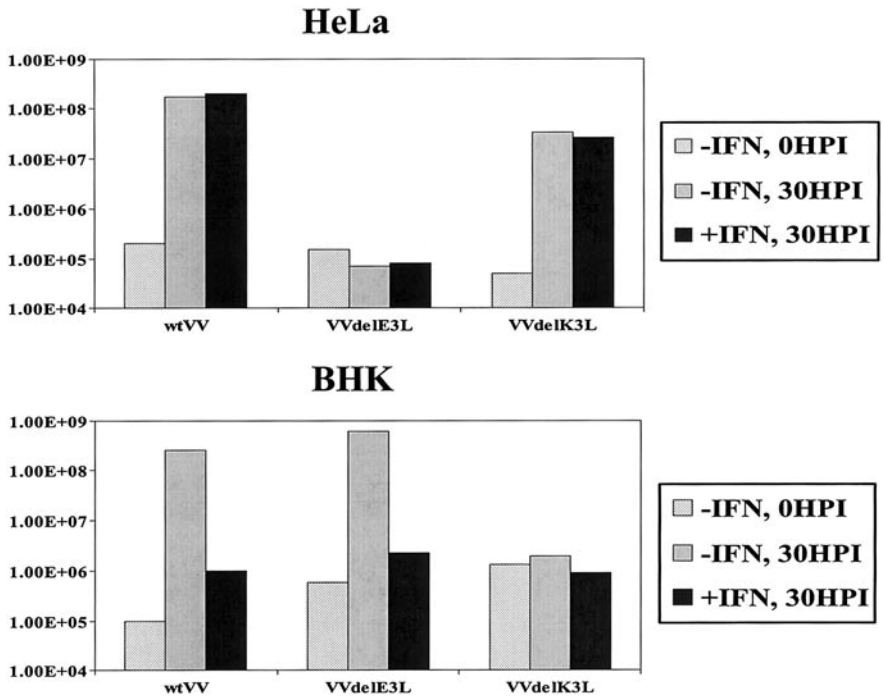

FIG. 1. Dependence of VV host range on the expression of E3L or K3L. The ability of VV to replicate in different cell lines was determined by a single-cycle growth assay. Mock or interferon-treated HeLa or BHK cells were infected with wtVV, VVΔE3L, or VVΔK3L. Infected cells were harvested at 0 and 30 h.p.i. and the amount of virus present in the lysate determined by a standard plaque assay using permissive RK_{13} cells.

ing the inhibition of protein synthesis (Davies *et al.,* 1992; Carroll *et al.,* 1993).

Both the E3L and the K3L gene products likely contribute to the interferon-resistant phenotype associated with VV. VV deleted of the E3L gene (VVΔE3L) has a severely limited host range and is interferon-sensitive in permissive cell lines (Beattie *et al.,* 1995, 1996). VV deleted of K3L (VVΔK3L) has a more subtle phenotype. This virus has a much broader host range and has only been found to be interferon-sensitive in mouse L-929 cells (Beattie *et al.,* 1995). In a heterologous system, the E3L gene products were able to rescue vesicular stomatitis virus (VSV) from the antiviral effects of interferon, but were unable to rescue the interferon sensitivity of encephalomyocarditis virus (EMCV) (Shors *et al.,* 1998). In this same system, the K3L gene product was unable to rescue VSV, but could rescue EMCV. These results suggest that the necessity for either the E3L or the K3L gene products for virus replication may be virus specific and dependent on the cell type.

The work presented in this study further characterizes dependence of VV on both E3L and K3L. WtVV expressing both E3L and K3L was able to replicate in both HeLa cells and BHK cells in the absence of interferon. WtVV was resistant to the effects of interferon in HeLa cells, but was interferon sensitive in BHK cells. VVΔE3L had a limited host range where, even in the absence of interferon treatment, this virus was unable to replicate in HeLa cells, but could replicate in the BHK cell line. An opposite dependence of the virus for K3L expression was observed where VVΔK3L was unable to replicate in BHK cells, but had a wild-type phenotype in HeLa cells.

RESULTS

Host range of VV mutants

WtVV has previously been shown to have a broad host range (Shors *et al.,* 1997; Beattie *et al.,* 1995). To determine the role of the interferon-resistance genes, E3L and K3L, related to host range, viral constructs were obtained which were deleted of these respective genes (Beattie *et al.,* 1995). As shown in Fig. 1, WtVV replicated well in both HeLa cells and BHK cells over a single cycle of replication with over a three-log increase in viral titer. WtVV was completely interferon-resistant in HeLa cells but, surprisingly, was very interferon-sensitive in BHK cells where replication was reduced by approximately two-logs. VVΔE3L had a much more limited host range where no replication of the virus was observed in HeLa cells, but the virus replicated to wild-type levels in BHK cells. This virus, similar to wtVV, was interferon-sensitive in BHK cells. An opposite host range phenotype was observed for VVΔK3L. This virus replicated to nearly wild-type levels in HeLa cells and was interferon-resistant, but in BHK cells, no replication was detected.

To determine whether an inhibition in protein synthesis was responsible for altering the host range of the viruses, proteins being synthesized at 6 h postinfection (h.p.i.) were radiolabeled with [^{35}S]methionine. With wtVV, a switch from cellular protein synthesis to a preference

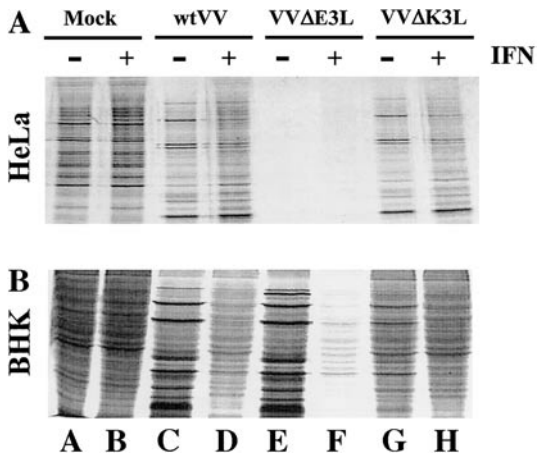

FIG. 2. Inhibition of protein synthesis correlated with the viral host range. To determine level of protein synthesis during viral infection, infected HeLa and BHK cells were radiolabeled at 6 h.p.i. with [^{35}S]methionine. Detergent lysates were then prepared and the radiolabeled proteins separated by SDS–PAGE and visualized by autoradiography. Cells were either mock-infected (lanes A and B), or infected with wtVV (lanes C and D), VVΔE3L (lanes E and F), or VVΔK3L (lanes G and H). Prior to infection, cells were either mock-treated (lanes A, C, E, and G) or treated with interferon (lanes B, D, F, and H).

to viral protein synthesis was observed in both HeLa and BHK cells (Figs. 2A and 2B, lanes A and C). Viral translation was still observed in HeLa cells pretreated with interferon (Fig. 2A, lane D), but a complete block in viral protein synthesis and a decrease in host protein synthesis was observed in interferon-treated BHK cells (Fig. 2B, lane D). These results agree with the lack of wtVV replication observed in interferon-treated BHK cells.

VVΔE3L demonstrated a complete shutoff of both viral and host protein synthesis in mock-treated and interferon-treated HeLa cells (Fig. 2A, lanes E and F). However, in replication-permissive BHK cells, levels of protein synthesis were indistinguishable from that of wtVV (Fig. 2B, lane E). Again, upon interferon treatment of BHK cells, VVΔE3L translation was completely blocked and host protein synthesis was also significantly decreased (Fig. 2B, lane F). As expected, VVΔK3L demonstrated viral protein synthesis in HeLa cells similar to that of wtVV (Fig. 2A, lanes G and H). However, in BHK cells, the presence of viral translation products was significantly reduced (Fig. 2B, compare lanes C and G). Interferon treatment of BHK cells infected by VVΔK3L completely blocked all viral protein synthesis, but only slightly decreased host protein synthesis (Fig. 2B, lane H).

The interferon pathway(s) involved in determining host range

Since a reduction in viral protein synthesis appeared to correlate with the limited host range observed with the VV mutants, activity associated with the two well-characterized interferon response pathways was assayed. Activation of the OAS pathway has previously been characterized by the degradation of the 28S- and 18S-ribosomal RNAs (Floyd-Smith *et al.,* 1981). RNA extracts were prepared from HeLa and BHK cells infected by the various viral constructs. In HeLa cells, significant rRNA degradation was observed only in cells infected by VVΔE3L (Fig. 3A, lane C). This degradation was increased when cells were pretreated with interferon (Fig. 3A, lane G). All other viral infections did not show any significant rRNA degradation (Fig. 3A, lanes B, D, F, and H). In viral-infected BHK cells, rRNA degradation was not observed in any of the samples, including VVΔE3L and VVΔK3L lysates, either in the presence or in the absence of interferon (Fig. 3B, lanes A–H). These results suggest that the OAS pathway may be involved in the inhibition of VVΔE3L replication in HeLa cells and that this pathway is not involved in the inhibition of VV replication in BHK cells.

To determine whether the PKR pathway may be involved in regulating viral replication in these cell lines, various assays were performed. In Fig. 4, extracts from viral-infected HeLa and BHK cells were assayed for the presence of phosphorylated eIF-2α by Western blot. In HeLa cells, infection by VVΔE3L showed high levels of eIF2α phosphorylation either in the presence or in the absence of interferon (Fig. 4, lanes E and F). The percentage of eIF2α phosphorylated is calculated relative to the maximal amount of phosphorylation that could be detected in that cell line. Therefore, 85% of the eIF2α phosphorylated during the infection of interferon-treated HeLa cells by VVΔE3L (Fig. 4, lane F) could be phosphorylated during the infection by this virus in non-interferon-treated HeLa cells (Fig. 4, lane E). In BHK cells, eIF2α phosphorylation could be detected in all VV-infected

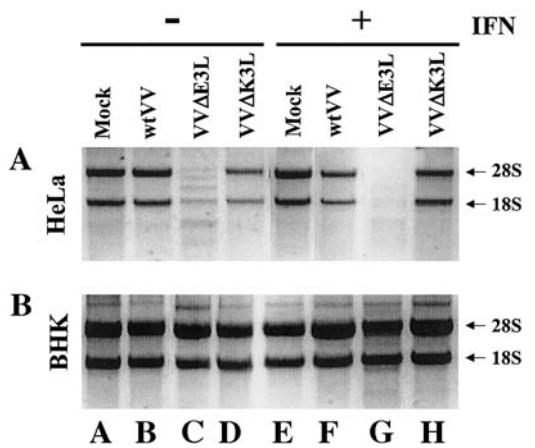

FIG. 3. Activation of the 2′5′ oligoadenylate synthetase pathway in HeLa cells. To monitor activation of the OAS pathway, total RNA was isolated from virally infected HeLa and BHK cells at 12 h.p.i. Isolated RNA was separated on an agarose-formaldehyde gel and visualized by staining with ethidium bromide. The position of the 28S- and 18S-ribosomal RNAs is indicated. Mock (lanes A–D) or interferon-pretreated (lanes F–H) cells were either mock-infected (lanes A and E) or infected with wtVV (lanes B and F), VVΔE3L (lanes C and G), or VVΔK3L (lanes D and H).

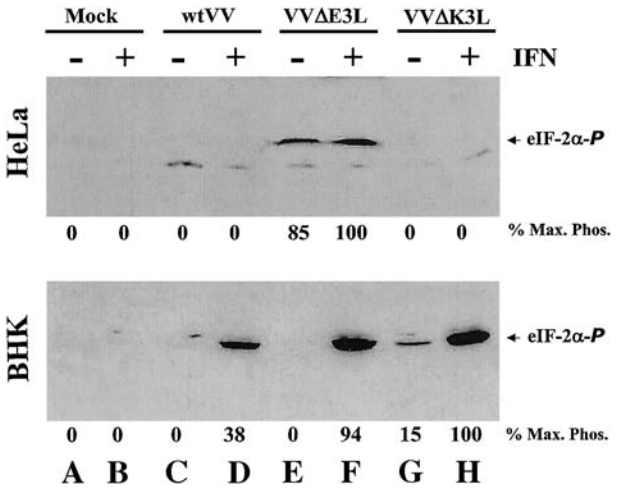

FIG. 4. eIF2α phosphorylation in HeLa and BHK cells. HeLa or BHK cells were untreated (lanes A, C, E, and G) or pretreated with IFN (lanes B, D, F, and H). The monolayers were then mock-infected (lanes A and B) or infected with wtVV (lanes C and D), VVΔE3L (lanes E and F), or VVΔK3L (lanes G and H). At 6 h.p.i., RIPA cell lysates were prepared and assayed by Western blot using antiphosphorylated eIF2α antiserum. Bound antibodies were detected by chemiluminescence and visualized by autoradiography. The position of eIF2α is shown and the relative level of eIF2α phosphorylation indicated.

cells which had been pretreated with interferon (Fig. 4, lanes D, F, and H). In the absence of interferon pretreatment of BHK cells, eIF2α phosphorylation was only observed in cells infected by VVΔK3L (Fig. 4, lane G). These results clearly suggest the need for K3L for replication of VV in BHK cells. Interestingly, infection with VVΔK3L in BHK cells treated with IFN had similar levels of eIF2α phosphorylation as infection with VVΔE3L, but the level of host translational shutoff was minor in the cells infected with VVΔK3L and nearly complete in cells infected with VVΔE3L (Fig. 2, lanes F and H).

To further confirm the activation of PKR leading to eIF2α phosphorylation, Western blot assays were done to detect the activated form of PKR. Two different antiserums were used in this assay, anti-PKR 71/10 and anti-PKR M-550, neither of which were sensitive enough to detect PKR in the absence of interferon treatment (Fig. 5, lane A). Upon activation of PKR, previous investigators have observed an electrophoretic mobility shift of the enzyme relative to the nonactivated state (Langland and Jacobs, 1992). As shown in Fig. 5, when HeLa cell extracts are separated by SDS–PAGE and the PKR protein detected by Western blot using anti-PKR 71/10 serum, a shift in PKR electrophoretic mobility was observed in extracts from VVΔE3L-infected cells compared to mock-infected or cells infected by the other VV constructs (Fig. 5, compare lane F to lanes B, D, and H). When a similar assay is performed using a different PKR antiserum (anti-PKR M-550), the shifted or activated form of the protein could not be detected (Fig. 5, lane F). Since the anti-PKR 71/10 serum was able to detect the activated/shifted form of PKR, the loss of signal using the anti-PKR M-550 was likely due to the loss of an epitope and not protein degradation.

Unfortunately, the anti-PKR 71/10 serum will not detect PKR from BHK cells even in the presence of interferon (data not shown). When a similar Western blot was performed using extracts from VV-infected BHK cells and probing for PKR using the anti-PKR M-550 serum, a reduction in the PKR signal was observed. Infection of interferon-treated BHK cells with WtVV reduced the PKR signal in extracts from virus infected cells (reduced 44%) (Fig. 5, compare lanes B and D). Similar results were also observed upon infection by VVΔE3L (nondetectable PKR level) (Fig. 5, lane F) and VVΔK3L (PKR reduced by 85%) (Fig. 5, lane H). Since with HeLa cells a loss of signal corresponded to activation of PKR, it was likely that a similar activation event occurred in interferon-treated infected BHK cells.

To confirm that the loss of the PKR signal in the BHK Western blot in Fig. 5 was due to the loss of an epitope upon phosphorylation/activation of PKR and not degradation of PKR, extracts from VVΔE3L-infected BHK cells were phosphatase treated. Phosphatase-treated samples were then assayed by Western blot using anti-PKR M-550 serum. Again, as shown in Fig. 5, PKR could not be detected in interferon-treated BHK cells infected with VVΔE3L (Fig. 6, lane E). When the VVΔE3L-infected cell

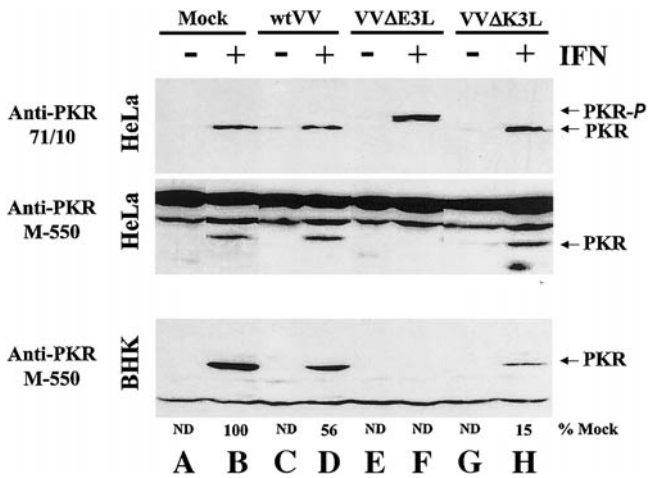

FIG. 5. Activation of PKR in HeLa and BHK Cells. HeLa or BHK cells were untreated (lanes A, C, E, and G) or pretreated with IFN (lanes B, D, F, and H). The monolayers were then mock-infected (lanes A and B) or infected with wtVV (lanes C and D), VVΔE3L (lanes E and F), or VVΔK3L (lanes G and H). At 6 h.p.i., RIPA cell lysates were prepared and assayed by Western blot using two different antisera to PKR. Bound antibodies were detected by chemiluminescence and visualized by autoradiography. The upper two gels represent HeLa cell extracts probed with monoclonal anti-PKR 71/10 from Ribogene (upper gel) or anti-PKR M-550 from Santa Cruz Biochemicals (middle gel). The lower gel represents BHK extracts probed with anti-PKR 71/10. The position of nonphosphorylated PKR (PKR) and phosphorylated PKR (PKR-P) is shown. The relative level of nonphosphorylated PKR (as compared to mock-treated cells) in BHK cells is indicated.

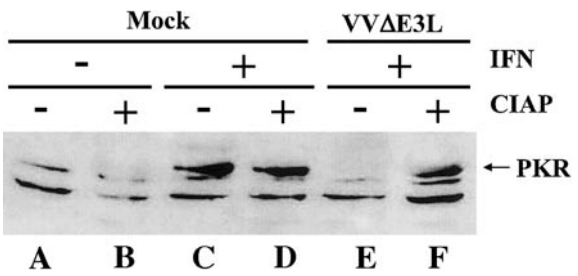

FIG. 6. PKR Antiserum M-550 did not recognize phosphorylated/activated PKR. BHK cells were untreated (lanes A and B) or pretreated with IFN (lanes C–F). The monolayers were then mock-infected (lanes A–D) or infected with VVΔE3L (lanes E and F). At 6 h.p.i., RIPA cell lysates were prepared and left untreated (lanes A, C, and E) or treated with CIAP (lanes B, D, and F). PKR was detected by Western blot using anti-PKR M-550 antiserum (Santa Cruz Biochemicals). Bound antibodies were detected by chemiluminescence and visualized by autoradiography. The position of nonphosphorylated PKR (PKR) is shown.

extract was treated with phosphatase, PKR detection was restored with an electrophoretic mobility corresponding to that of the nonactivated form of PKR (Fig. 6, lane F). The amount of PKR present in this sample was similar to that observed in mock-infected cells (Fig. 6, compare lane F to lanes C and D). These results suggest that PKR was phosphorylated/activated upon infection of interferon-treated BHK cells by all the VV constructs shown in Fig. 5.

Mechanism of PKR activation in BHK cells

The presence of excess free dsRNA is one mechanism by which PKR may become activated in interferon-treated BHK cells. Free dsRNA could be present in these infected cells if lower levels of E3L were synthesized by VV. To test this hypothesis, extracts from both interferon-treated HeLa and BHK cells were assayed for levels of E3L protein at various times postinfection by wtVV. In both cell lines, similar rates of synthesis and levels of accumulation of E3L protein were observed (data not shown). Pretreatment of cells with interferon did not alter these levels (data not shown). These results suggest that the levels and timing of E3L protein synthesis was not cell-line specific.

Free dsRNA may also be observed in BHK cells if VV produced higher levels of dsRNA when replicating in BHK cells as compared to replication in HeLa cells. If this occurred, at some point, the concentration of dsRNA may be high enough to overcome the sequestering effect of E3L protein, thereby allowing activation of PKR. The relative amount of RNase-resistant RNA, equivalent to dsRNA, was determined from BHK and HeLa cells infected by wtVV. The purified total RNA was either rehybridized to assay the total amount of dsRNA possible (Fig. 7B) or tested directly to assay the amount of dsRNA present in the extract (Fig. 7A). With HeLa cells, only a minor detectable increase in the amount of dsRNA was observed between 0 and 12 h.p.i. (Figs. 7A and 7B). With BHK cells, 3–10-fold increase in the amount of dsRNA was observed between 6 and 12 h.p.i., relative to 0 h.p.i. (Figs. 7A and 7B). Both HeLa and BHK cells began at 0 h.p.i. with approximately the same percentage of dsRNA; therefore, the increase in dsRNA levels in BHK cells was likely synthesized during the replication of VV in this cell line.

These results suggest that during the replication of VV in BHK cells, more dsRNA was produced than replication in HeLa cells. If this were the case, the amount of free E3L protein unbound to dsRNA would be expectedly higher in HeLa cells than BHK cells. To assay for this, the E3L protein present in detergent lysates from wtVV-infected HeLa and BHK cells was affinity purified using dsRNA-agarose. The E3L protein binds to dsRNA with a very high affinity, and therefore, any E3L protein bound to dsRNA present in the cell extract would be unable to bind to the dsRNA agarose resin. The amount of free E3L protein present in the extract was then ascertained by comparing the total amount of E3L protein to the amount of E3L protein which could be affinity purified on the dsRNA-agarose resin. As shown in Fig. 8, approximately 5–10% of the E3L protein in BHK cells existed in a free state (i.e., unbound to dsRNA) as compared to approximately 40–60% in HeLa cells. These results support the idea that VV replication in BHK cells led to higher levels

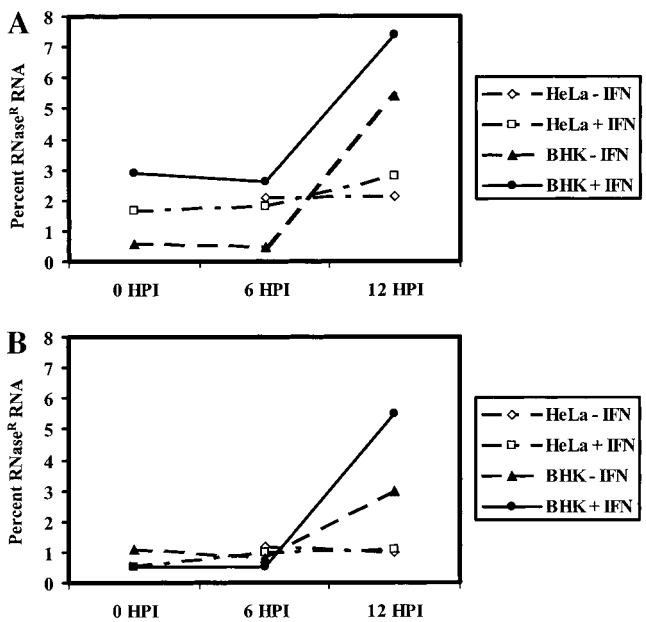

FIG. 7. Vaccinia virus replication in BHK cells synthesized higher levels of dsRNA than replication in HeLa cells. HeLa or BHK cells were untreated or pretreated with IFN. The monolayers were then infected with wtVV. Two hours prior to harvest, cells were radiolabeled with [^{3}H]uridine. At the indicated times, cells were harvested and total RNA isolated and quantified. In A, the total RNA was treated with RNase A and RNase T1 and the percentage of RNase-resistant RNA plotted. In B, the total RNA was denatured and reannealed prior to RNase treatment and quantification of RNase-resistant RNA.

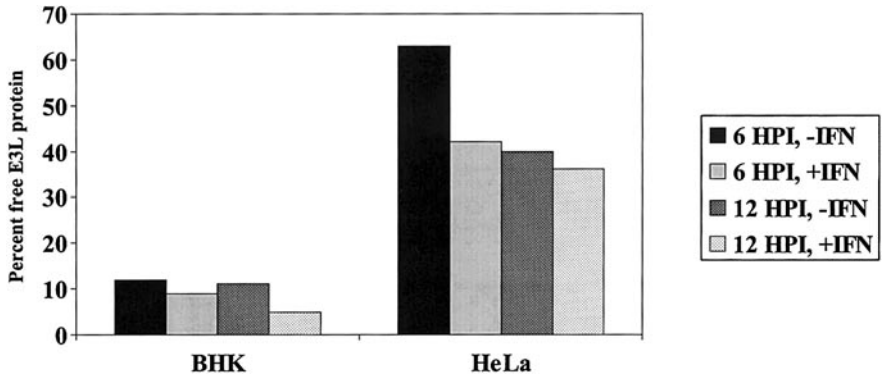

FIG. 8. The level of unbound E3L protein was reduced in BHK cells as compared to HeLa cells. HeLa or BHK cells were untreated or pretreated with IFN. The monolayers were then infected with wtVV. Cells were harvested at 6 and 12 h.p.i. and the total amount of E3L protein determined by Western blot analysis. Extracts were also incubated with poly(rI)•poly(rC)-agarose and the amount of E3L protein bound to the resin determined by Western blot analysis. Given the high affinity of E3L for dsRNA, the amount of E3L bound to the resin represented the level of free E3L present in the extract. By densitometric analysis, the percentage of free E3L was calculated and plotted.

of dsRNA synthesis, and consequently, lower levels of free E3L protein as compared to HeLa cells.

DISCUSSION

The vaccinia E3L and K3L gene products have previously been shown to inhibit the interferon antiviral response, thereby allowing the virus to circumvent the shutoff of protein synthesis in the infected cell (Chang and Jacobs, 1993; Shors *et al.,* 1997; Beattie *et al.,* 1995). One mechanism by which the E3L gene products are known to function is by sequestering dsRNA (Chang and Jacobs, 1993). As demonstrated here, the E3L gene products were both necessary and sufficient to inhibit the antiviral response and permit replication of the virus in HeLa cells. In HeLa cells, the E3L products likely bind to and "mask" virtually all the dsRNA synthesized by the virus at late times postinfection. This sequestering prevents recognition of the dsRNA by either PKR or OAS, thereby inhibiting the activation of these enzymes. Deletion of the K3L gene appeared to have little, if any, effect on the replication of VV in HeLa cells. Furthermore, the presence of the K3L gene was unable to rescue replication of virus deleted of the E3L gene. We have previously observed that HeLa cells endogenously synthesize relatively large amounts of PKR even in the absence of interferon treatment. Since the K3L gene product is thought to function as a competitive inhibitor for both eIF2α phosphorylation and possibly PKR autophosphorylation (Davies *et al.,* 1992; Carroll *et al.,* 1993), excess molar amounts of PKR relative to K3L would be expected to overcome to the inhibitory effects of K3L. The data presented support this concept that the E3L gene products were able to sequester any dsRNA synthesized by the virus, preventing PKR binding to the dsRNA. However, in the absence of the E3L gene, PKR could bind to the dsRNA, but due to the excess levels of PKR, the K3L gene product was unable to fully inhibit PKR activation and subsequently led to eIF2α phosphorylation (Fig. 9).

In the BHK cell line an opposite dependence of K3L vs E3L on virus replication was observed. Here VV deleted of E3L replicated to levels indistinguishable from wtVV, but virus deleted of the K3L gene was debilitated. Furthermore, the presence of E3L, K3L, or both were unable to rescue replication of the virus from the antiviral effects of interferon. This cell line has been demonstrated to have phenotypic differences from HeLa cells in two important ways. First, while immunoreactive PKR could be detected in BHK cells (Fig. 5), no activation of PKR could be detected under *in vitro* phosphorylation conditions. This is due to the presence of a cellular PKR inhibitor (Kibler *et al.,* 1997; data not shown). Second, VV replication in BHK cells appeared to lead to higher levels of dsRNA synthesis than viral replication in HeLa cells. It is our hypothesis that the replication of VV in BHK cells led to high enough concentrations of dsRNA such that the E3L gene products could no longer fully sequester all the dsRNA present. PKR was then able to bind the nonsequestered dsRNA. However, in the absence of interferon treatment, the molar amounts of the K3L product were in excess over the endogenous PKR levels, thereby allowing K3L to function as a competitive inhibitor, thereby preventing PKR activation (Fig. 9). These are the first results to demonstrate K3L inhibiting PKR activation in an *in vivo* viral system. When cells were pretreated with interferon, the E3L products were still unable to mask the dsRNA completely, and due to the interferon treatment, PKR levels were now in excess over the K3L protein thereby allowing PKR activation to occur (Fig. 9). Since BHK cells did not appear to have a functional OAS system, the excess dsRNA synthesized in the absence of interferon did not lead to activation of this pathway. Therefore, we propose that VVΔK3L cannot replicate in untreated BHK cells since the high-level dsRNA formed during virus replication cannot be fully sequestered by E3L, leading to subsequent PKR binding and activation in the absence of K3L. For VVΔE3L, the virus is not able to

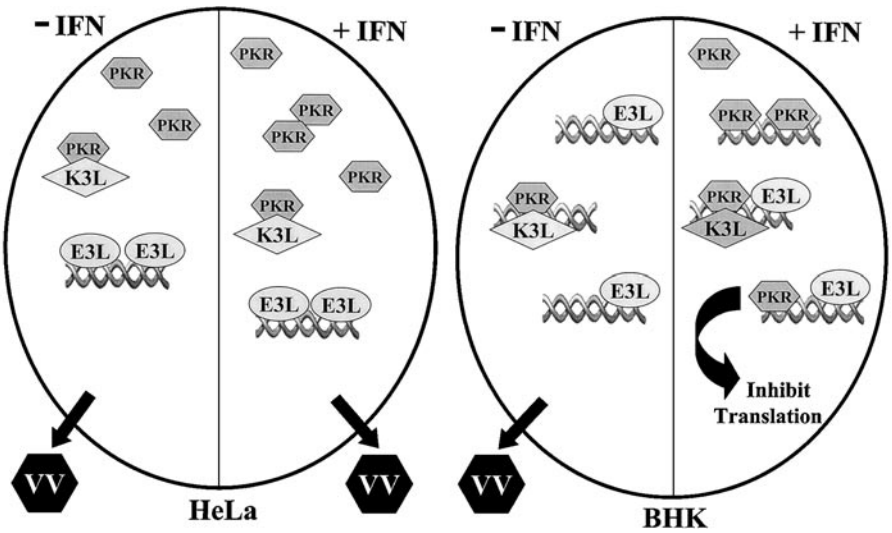

FIG. 9. Model demonstrating the host-range dependence of VV replication on the presence of E3L or K3L. In HeLa cells, VV replication is dependent on the presence of the E3L gene product to sequester the low levels of dsRNA synthesized during viral replication since the K3L gene product cannot competitively inhibit the high levels of PKR present. In BHK cells, the E3L gene product cannot sequester the high levels of dsRNA synthesized, but the K3L gene product, in the absence of interferon treatment, can competitively inhibit the lower levels of PKR present.

sequester the lower levels of dsRNA present in HeLa cells allowing PKR binding, but since PKR levels are much higher in HeLa cells, viral replication was blocked irrespective of K3L synthesis. It should be noted that host and viral translation is blocked in interferon-treated BHK cells infected with VVΔE3L (Fig. 2B, lane F). However, in cells infected with VVΔK3L a more specific block only in viral protein synthesis was observed (Fig. 2B, lanes G and H).

Since VV has a broad host range, these results suggest an importance for the virus to maintain both a functional E3L and a functional K3L gene in relationship to this host range. Our results suggest that cell lines, and likely different cell types in a mammal, differ in regards to endogenous levels of PKR present and in the amount of dsRNA made during a poxvirus infection. Late transcription of VV genes leads to transcripts with extremely heterogeneous 3′ ends (Mahr and Roberts, 1984). Unfortunately, relatively little is known about the mechanism of termination of late transcription. VV transcription is regulated by a complex of proteins including the viral products of the H5R, G2R, and A18R genes and the viral RNA polymerase (Black *et al.,* 1998). Varying the levels of these viral proteins or possibly unidentified host factors, may alter the level of "promiscuous transcription," resulting in the synthesis of longer-than-normal RNAs. These effects could lead to varying concentrations of dsRNA in different cell types.

These results suggest that VV may have evolved to encode both the E3L gene and the K3L gene as a means of expanding the viral host range. Depending on the cell type infected, the necessity of either the E3L or the K3L gene may vary. Cellular and viral factors likely will come into play which will alter components of the cellular antiviral response, such as PKR, or alter viral regulation of transcription which will have a role in levels of dsRNA synthesized.

MATERIALS AND METHODS

Cells and viruses

HeLa cells were maintained as monolayers in Dulbecco's minimum essential media (DMEM) supplemented with 50 μg/ml gentamicin sulfate and 10% fetal bovine serum (Hyclone). BHK cells were maintained in Eagle's minimum essential media (MEM) supplemented with gentamicin and 10% fetal bovine serum. RK_{13} cells were maintained in BHK media containing 5% fetal bovine serum. Vaccinia virus VC-2 (Copenhagen strain), designated in this article as wtVV, was the parent strain for all viruses used in this study. VV deleted for either E3L (VVΔE3L or vP1080) or K3L (VVΔK3L or vP872) were generated as previously described (Chang and Jacobs, 1993; Chang *et al.,* 1995). For interferon treatment, cells were treated with 100 IU/ml recombinant human α A/D interferon (Hoffman-La Roche) for 18 h prior to infection.

Single-cycle virus growth

Indicated cell monolayers were infected at an m.o.i. = 1. At 1 h.p.i., the inoculum was removed, the monolayer washed two times with PBS, and the media replaced. Triplicate monolayers were infected to represent harvests at 0 h.p.i., and 30 h.p.i. with and without interferon treatment. The 0 h.p.i. monolayers were harvested after the 1 h infection process. Monolayers were harvested by scraping the cells off the dish and into the media. Virus was released from cells by three cycles of freeze/thaw-

ing. Virus titers were obtain by plaquing in RK_{13} cells which has previously been shown to be permissive for all viruses used in this study (Shors *et al.,* 1997).

In vivo protein labeling

Cell monolayers, infected at an m.o.i. = 5, were starved for methionine at 5.5 h.p.i. using methionine-free DMEM. At 6 h.p.i. newly synthesized proteins were labeled with 50 μCi of [^{35}S]methionine per ml (800 Ci/mmol) for 30 min. At that time, cytoplasmic extracts were prepared by NP-40 detergent lysis (Chang and Jacobs, 1993)

RNA degradation assay

The amount of ribosomal RNA degradation was determined by isolating total RNA from infected cells at 12 h.p.i. Cells (4×10^6 cells) were scraped into the media and washed one time in PBS. The cell pellet was resuspended in 1 ml of a 1:1 solution containing RNase-free Tris-buffered saline (TBS) pH 7.0 and 25:24:1 phenol:chloroform:isoamyl alcohol. The mixture was immediately vortexed for 1 min and centrifuged at 10,000 *g* for 5 min. An amount of 400 μl of the aqueous phase was transferred to a fresh tube and then 40 μl of 3 M sodium acetate pH 5.2 and 1 ml 100% ethanol were added. The RNA was precipitated at −80°C for 30 min, followed by centrifugation at 10,000 *g* for 30 min. The RNA pellets were resuspended in 20 μl 0.5% SDS and quantified. An amount of 40 μg ethidium bromide/ml was added to the RNA (10 μg) which was then separated on a 1.8% agarose formaldehyde gel.

Level of eIF-2α phosphorylation

Indicated cell monolayers were infected at an m.o.i. = 5. At 6 h.p.i., RIPA cell lysates were prepared. Briefly, cells were scraped into the media and pelleted at 500 *g* for 5 min. The cell pellet was resuspended in RIPA lysis buffer (1× PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM NaF; 100 μl per 4×10^6 cells) and incubated on ice for 10 min. The lysate was centrifuged at 10,000 *g* for 10 min and the supernatant transferred to a new tube. An equal volume of 2× SDS-sample buffer was added to the supernatant. The samples were boiled and 30 μl was analyzed on a 12% polyacrylamide minigel. Separated proteins were transferred to nitrocellulose using a CAPS transfer buffer (20 mM CAPS pH 11.0, 20% methanol). The nitrocellulose was blocked with BLOTTO (20 mM Tris pH 7.5, 120 mM NaCl, 30% nonfat dry milk). The blot was probed with rabbit anti-phosphorylated eIF2α (1:250 dilution; Research Genetics) for 18 h. Secondary goat anti-rabbit IgG conjugated with horseradish peroxidase (1:5000) was added, followed by chemiluminescent development and autoradiography. All extracts were also probed by Western blot with goat anti-eIF2α (Santa Cruz Biochemicals) to confirm equal amounts of the protein (data not shown).

Levels of activated PKR

To determine the levels of activated PKR, Western blot assays were performed. Two different antiserum were used for these assays: monoclonal anti-PKR 71/10 (Ribogene), which recognized human PKR (active and inactive forms), and rabbit anti-PKR M-550 (Santa Cruz Biochemicals; currently listed as M-115), which recognized nonactive forms of human and BHK cell PKR. Extracts were prepared as described for determining "levels of eIF2α phosphorylation." Proteins were separated on a large format (12 cm) 7.5% polyacrylamide gel. Prestained standards were loaded on the gel and the proteins separated until the 50-kDa prestained standard migrated to the bottom of the gel. Western blot analysis was performed as described above using the manufacturer's recommended antiserum dilution.

To dephosphorylate activated PKR in the samples, extracts were prepared as described above; only the RIPA lysis buffer did not contain NaF. Cellular extracts were diluted fourfold (in 20 mm HEPES pH 7.5, 10% glycerol) followed by the addition of 400 U calf intestinal alkaline phosphatase/ml (Boehringer Mannheim) and incubation for 5 min at 37°C (Langland and Jacobs, 1992).

Levels of E3L protein

To determine the levels of E3L protein, Western blot analysis was performed as described above using a 12% polyacrylamide minigel and rabbit anti-E3L antiserum (1:500). To determine the levels of E3L not bound to dsRNA (free E3L), NP-40 detergent lysates were prepared. A portion of the extract was incubated with poly(rI)•poly(rC) agarose (Langland *et al.,* 1995) and the amount of E3L protein bound to the resin determined by Western blot analysis (free E3L). The amount of total E3L protein present in the extract was also determined by Western blot analysis. By densitometric analysis, the percentage of free E3L was then calculated.

Levels of dsRNA

A modified procedure as described by Bayliss and Condit (1993) was used to measure the amount of hybridized RNA (dsRNA) present in an infected cell. Briefly, 100-mm confluent monolayers of cells were infected at an m.o.i. = 10. Two hours prior to harvest, the media was removed and replaced with 1 ml DMEM containing 1 mCi [^{3}H]uridine. The media was rocked over the cells every 15 min to prevent drying. At the indicated harvest times, cells were scraped into the media and washed two times in cold PBS. The cells were lysed and the RNA purified using the SNAP RNA isolation kit (Invitrogen). The RNA was resuspended in 10 mM Tris pH 7.6 (125 μl) and 1/50 of this isolated RNA was spotted directly onto a glass

fiber filter, washed two times in cold 10% TCA and one time in 95% ethanol, and the amount of radioactivity determined by scintillation counting (total RNA). The remaining purified RNA sample was divided equally. One aliquot (50 μl) was heated to 100°C for 2 min and allowed to slow cool to room temperature. The sample was overlaid with 100 μl mineral oil and incubated for 20 h at 68°C. An amount of 20 μg tRNA was added to both of the RNA samples (boiled/hybridized and non-boiled/nonhybridized). The samples were then brought to 0.4 M NaCl and 0.03 M sodium citrate along with the addition of 50 μg RNase A and 2.5 μg RNase T1. The samples were incubated at 37°C for 1 h; an additional 10 μg tRNA was added and the samples spotted on glass fiber filters. The RNA was precipitated and washed two times in cold 10% TCA containing 0.01 M sodium pyrophosphate and one time in 95% ethanol, and the amount of radioactivity determined by scintillation counting. The percentage RNase-resistant RNA (dsRNA) was then calculated by comparison to the total amount of RNA.

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