Double-stranded RNA-binding protein E3 controls translation of viral intermediate RNA, marking an essential step in the life cycle of modified vaccinia virus Ankara

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Infection of human cells with modified vaccinia virus Ankara (MVA) activates the typical cascade-like pattern of viral early-, intermediate- and late-gene expression. In contrast, infection of human HeLa cells with MVA deleted of the E3L gene (MVA-ΔE3L) results in high-level synthesis of intermediate RNA, but lacks viral late transcription. The viral E3 protein is thought to bind double-stranded RNA (dsRNA) and to act as an inhibitor of dsRNA-activated 2′-5′-oligoadenylate synthetase (2′-5′OA synthetase)/RNase L and protein kinase (PKR). Here, it is demonstrated that viral intermediate RNA can form RNase A/T1-resistant dsRNA, suggestive of activating both the 2′-5′OA synthetase/RNase L pathway and PKR in various human cell lines. Western blot analysis revealed that failure of late transcription in the absence of E3L function resulted from the deficiency to produce essential viral intermediate proteins, as demonstrated for vaccinia late transcription factor 2 (VLTF 2). Substantial host cell-specific differences were found in the level of activation of either RNase L or PKR. However, both rRNA degradation and phosphorylation of eukaryotic translation initiation factor-2α (eIF2α) inhibited the synthesis of VLTF 2 in human cells. Moreover, intermediate VLTF 2 and late-protein production were restored in MVA-ΔE3L-infected mouse embryonic fibroblasts from *Pkr*$^{0/0}$ mice. Thus, both host-response pathways may be involved, but activity of PKR is sufficient to block the MVA molecular life cycle. These data imply that an essential function of vaccinia virus E3L is to secure translation of intermediate RNA and, thereby, expression of other viral genes.

INTRODUCTION

Modified vaccinia virus Ankara (MVA) was attenuated by over 500 serial passages in chicken embryo fibroblast (CEF) cells and is being developed as safe viral vector and third-generation smallpox vaccine (Mayr *et al.*, 1975; Sutter & Staib, 2003; Drexler *et al.*, 2004). The virus was found to be replication-deficient in most cells of mammalian origin (Sutter & Moss, 1992; Carroll & Moss, 1997; Blanchard *et al.*, 1998; Drexler *et al.*, 1998), probably because MVA lacks many of the viral gene products exploited by other orthopoxviruses to regulate virus–host interactions (Antoine *et al.*, 1998). However, several important regulatory-gene sequences of *Vaccinia virus* are still conserved within the MVA genome, including vaccinia virus genes K3L and E3L (Antoine *et al.*, 1998; Moss & Shisler, 2001; Staib *et al.*, 2005).

The vaccinia virus E3L gene encodes the 25 kDa polypeptide E3, which is synthesized early during the viral-infection cycle (Chang *et al.*, 1992) and harbours an amino-terminal Z-DNA-binding domain (Patterson & Samuel, 1995; Herbert *et al.*, 1997; Kim *et al.*, 2003, 2004; Kahmann *et al.*, 2004), as well as a carboxyl-terminal domain with a typical double-stranded RNA (dsRNA)-binding motif (Chang & Jacobs, 1993; Chang *et al.*, 1995; Ho & Shuman, 1996; Shors *et al.*, 1997). The amino-terminal domain of E3 is dispensable for infection of cells in culture, but both amino- and carboxy-terminal domains of E3 are required for pathogenesis in mice (Shors *et al.*, 1998; Brandt & Jacobs, 2001; Brandt *et al.*, 2005). A vaccinia virus E3L-deletion mutant has been shown to be highly sensitive to the antiviral activity of alpha/beta interferon (IFN-α/β) and replication-deficient in Vero and HeLa cells, but retained full replicative capacity in CEF, hamster BHK and rabbit RK13 cells (Beattie *et al.*, 1991, 1995, 1996; Chang *et al.*, 1995; Langland & Jacobs, 2002). By binding dsRNA, the E3 protein is thought to inhibit stimulation of protein kinase (PKR) and activation of 2′-5′-oligoadenylate synthetase (2′-5′OA synthetase), two enzymes that are activated by dsRNA (Chang *et al.*,

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1992; Chang & Jacobs, 1993; Rivas *et al.*, 1998; Langland & Jacobs, 2004). Upon stimulation with dsRNA, 2′-5′OA synthetase polymerizes oligoadenylates with 2′–5′ linkages (Kerr & Brown, 1978). These synthesized 2′-5′-oligoadenylates activate an endoribonuclease (RNase L), which is suggested to affect virus replication by cleaving cellular and viral RNA, thereby leading to a general inhibition of protein synthesis (Floyd-Smith *et al.*, 1981; Wreschner *et al.*, 1981; Silverman *et al.*, 1982, 1983; Kumar *et al.*, 1988; Díaz-Guerra *et al.*, 1997; Shors *et al.*, 1997; Stark *et al.*, 1998). The IFN-inducible PKR can bind to dsRNA, resulting in enzyme activation and phosphorylation of the α subunit of the eukaryotic translation initiation factor eIF2 (eIF2α), with consequent global inhibition of translation (Thomis & Samuel, 1993; Clemens & Elia, 1997; Goodbourn *et al.*, 2000).

An MVA E3L-deletion mutant (MVA-ΔE3L) was unable to replicate in CEFs; however, it possesses full replicative capacity in mammalian BHK-21 cells (Hornemann *et al.*, 2003). This vaccinia virus host-range phenotype in CEFs was associated with induction of apoptosis, enhanced production of chicken IFN-α/β and reduced viral DNA replication and protein biosynthesis (Hornemann *et al.*, 2003).

For regulation of vaccinia virus gene expression, a cascade-like model was suggested (Vos & Stunnenberg, 1988; Keck *et al.*, 1990). Early transcription starts immediately upon entry of the virus, because all necessary components are present in the infectious particle. The early proteins that are newly synthesized are enzymes for DNA replication, RNA polymerase subunits, intermediate transcription factors (VITF-1 and 3 and capping enzyme) and viral host-range or immunomodulatory proteins. Activation of intermediate and late transcription requires the onset of viral DNA replication. Intermediate genes encode, for example, the vaccinia late transcription factors VLTF 1, VLTF 2 and VLTF 3, encoded by G8R, A1L and A2L, respectively (Keck *et al.*, 1990). Late-gene products include RNA polymerase, early-gene transcription factors, poly(A) polymerase and proteins required for virion morphogenesis (reviewed by Moss, 1990; Condit & Niles, 2002; Broyles, 2003).

Previously, we have demonstrated unimpaired late-protein synthesis and complete cascade-like transcription of early, intermediate and late temporal-gene classes following MVA infection of human HeLa cells (Sutter & Moss, 1992; Ludwig *et al.*, 2005). In contrast, we found that only early and intermediate transcripts were made abundantly in MVA-ΔE3L-infected HeLa cells, suggesting an essential role of E3L for completion of the viral molecular life cycle in human cells. Consistent with the proposed function of the vaccinia virus E3L-encoded protein to bind and sequester dsRNA, the dsRNA-activated antiviral 2′-5′OA synthetase/RNase L system was found to be induced in MVA-ΔE3L mutant virus-infected human HeLa cells (Ludwig *et al.*, 2005). The cellular factor(s) responsible for the dramatic block of viral late-gene expression and the interrupted MVA-ΔE3L molecular life cycle has not been investigated.

In this work, we addressed the question of whether, in the context of an MVA-ΔE3L infection, enough dsRNA is formed to induce the 2′-5′OA synthetase/RNase L pathway and/or PKR. Additionally, we determined that the interruption of the MVA-ΔE3L molecular life cycle in human cells is associated with a host cell-dependent differential activation of 2′-5′OA synthetase/RNase L and/or PKR activity. Consequently, we identified a failure in intermediate-protein synthesis as a major impediment to MVA-ΔE3L late-gene transcription. Finally, in mouse embryonic fibroblasts (MEFs), we demonstrate that activation of the PKR pathway is sufficient for the arrest of viral infection at the level of intermediate-gene expression.

METHODS

Viruses and cells. Baby hamster kidney BHK-21 (ATCC CCL-10), HeLa, HaCaT (human adult skin keratinocytes) (Boukamp *et al.*, 1988) and human embryonic kidney 293T (ATCC CRL-11268) cells were grown in RPMI 1640 medium supplemented with 10 % fetal calf serum (FCS). MEFs were prepared from C57BL/6 mice ($Pkr^{+/+}$) or mice devoid of functional PKR ($Pkr^{0/0}$) (Yang *et al.*, 1995) and cultured in Dulbecco's modified Eagle's medium with 10 % FCS. Vaccinia virus MVA (cloned isolate F6 at 582nd CEF passage) (Meyer *et al.*, 1991), MVA-ΔE3L and MVA-ΔE3Lrev (Hornemann *et al.*, 2003) were routinely propagated and titrated on BHK-21 cells.

Northern blot analysis. Cells were mock-infected or infected with MVA or MVA-ΔE3L at an m.o.i. of 5. Total RNA was isolated with TRIzol reagent (Invitrogen), following the manufacturer's instructions. Total RNA was separated by electrophoresis in 1 % agarose formaldehyde gels. Subsequently, RNA was transferred onto positively charged nylon membranes (Roche Diagnostics). Riboprobes for detection of MVA-encoded mRNAs 005R (C11R), 078R (G8R) and 047R (F17R) were synthesized by *in vitro* transcription using PCR products generated from viral DNA templates via primer pairs HLG5 (5′-TTATCTGATGTTGTTGTTGTTCGC-3′)/HLG6 (5′-CTAATACGACTCACTATAGGGAGAGTTTAGTTCGTCGAGTGAAC-3′), HLG3 (5′-CGATAAACTGCGCCAAATTG-3′)/HLG4 (5′-CTAATACGACTCACTATAGGGAGACATAATAGCCAAATGCTGATG-3′) and HLG1 (5′-ATTCTCATTTTGCATCTGCTC-3′)/HLG2 (5′-CTAATACGACTCACTATAGGGAGACTAGAAGCTACATTATCGCG-3′), respectively. A riboprobe specific for human 18S rRNA was synthesized by *in vitro* transcription using a PCR product amplified from HeLa cell DNA via primer pair HLPEI92 (5′-GCGAATGGCTCATTAAATCAG-3′)/HLPEI91 (5′-CTAATACGACTCACTATAGGGAGACGCTGAGCCAGTCAGTGTAG-3′). Reverse primers contained a T7 RNA polymerase promoter-recognition sequence (underlined). Digoxigenin (DIG)-labelled riboprobes were obtained by *in vitro* transcription with T7 RNA polymerase (Roche Diagnostics), using PCR-generated DNA fragments as templates. *In vitro* RNA labelling, hybridization and signal detection were carried out according to the manufacturer's instructions (DIG RNA labelling kit and Anti-DIG detection chemicals; Roche Diagnostics), applying 68 °C for hybridization and high-stringency wash in 0·1 × SSC containing 0·1 % SDS buffer.

Western blot analysis. Cell monolayers were washed with PBS, scraped off and incubated with lysis buffer [50 mM Tris (pH 7·0), 150 mM NaCl, 0·5 % NP-40, 1 mM PMSF, 1 mM sodium vanadate, 20 mM sodium fluoride and 1 mM sodium molybdate] for 10 min on ice. The cell debris was removed by centrifugation. Lysates were separated by SDS-PAGE and transferred to a PVDF membrane. After blocking, membranes were incubated with antibodies specific for eIF2α (Cell Signaling Technology) or eIF2α phosphorylated at

Ser51 (eIF2α-P) (Sigma-Aldrich) at a 1 : 1000 dilution in 5 % skimmed milk powder including 50 μM sodium vanadate overnight at 4 °C. Polyclonal antisera from rabbits, specific for vaccinia virus E3 and A1 proteins and ectromelia virus interleukin 18-binding protein (IL-18bp), were applied at 1 : 1000, 1 : 500 and 1 : 2000 dilutions, respectively. mAbs directed against the A27 envelope protein (Czerny *et al.*, 1994) and β-actin (Sigma-Aldrich) were used at 1 : 2000 and 1 : 10 000 dilutions, respectively.

Detection of dsRNA. HeLa cells were mock-infected or infected with MVA or MVA-ΔE3L at an m.o.i. of 5 in the absence or presence of 40 μg cytosine arabinoside (AraC) ml^{-1}. Total RNA was isolated at indicated hours post-infection (h p.i.) with TRIzol reagent (Invitrogen), following the manufacturer's instructions. For denaturation (90 °C), reannealing (56 °C) and RNase A/T1 treatment (30 °C), 5 μg total RNA was applied per reaction, using an RPA kit (BD RiboQuant) according to the manufacturer's instructions. Following phenol/chloroform extraction and ammonium acetate precipitation, samples were separated electrophoretically and blotted as described for Northern blot analysis. To detect RNase-resistant RNA species, an MVA-specific DIG-labelled probe was synthesized by random-primed labelling with 2 μg *Eco*RV-digested MVA DNA as template, using random hexameric primers and a DIG-High Prime kit (Roche Diagnostics). Prehybridization and hybridization were performed at 50 °C by using EasyHyb (Roche Diagnostics). For low- and high-stringency washes, 2 × SSC containing 0·1 % SDS (at room temperature) and 0·1 × SSC containing 0·1 % SDS (at 50 °C) were used, respectively. Signal detection was carried out as described for Northern blot analysis.

RESULTS

E3L is required for viral late transcription and inhibits degradation of rRNA and phosphorylation of eIF2α following MVA infection of HeLa cells

Recently, we have characterized the essential function of the E3L gene product for completion of the MVA molecular life cycle during infection of human HeLa cells. Striking consequences of E3L inactivation were the lack of viral late-gene transcription and the degradation of cellular rRNA, probably due to activation of the 2′-5′OA synthetase/RNase L pathway (Ludwig *et al.*, 2005). Here, we used a previously generated revertant MVA-ΔE3L virus containing a re-inserted E3L gene copy under transcriptional control of its authentic promoter (MVA-ΔE3rev) (Hornemann *et al.*, 2003) to ascertain that the above-described phenotypes are solely due to the lack of E3L gene function. Upon infection of HeLa cells, we analysed the integrity of rRNA species and monitored by Northern blot for transcription of the MVA 047R late gene (encoding the vaccinia virus 11 kDa DNA-binding protein F17). In MVA-ΔE3rev-infected cells, we were able to detect abundant late viral transcripts and fully preserved rRNA, very similar to infections with wild-type MVA, yet in sharp contrast to MVA-ΔE3L infection (see Supplementary Fig. S1, available in JGV Online). Therefore, we concluded that the E3L gene product is sufficient to allow for late transcription and to inhibit the 2′-5′OA synthetase/RNase L pathway. In addition, we monitored for dsRNA-activated PKR, which is responsible for phosphorylation of eIF2α and represents another prime-candidate host protein described to be regulated by E3 activity (García *et al.*, 2002; Langland & Jacobs, 2002). We determined PKR activation in association with E3L gene function in cell lysates prepared from MVA- and MVA-ΔE3L-infected HeLa cells by Western blot using an antibody directed against the phosphorylated form of the PKR substrate eIF2α. Indeed, we detected phosphorylation of eIF2α exclusively in MVA-ΔE3L-infected HeLa cells, starting at 3 h p.i. (Fig. 1). These data show clearly that both dsRNA-stimulated host responses, i.e. the 2′-5′OA synthetase/RNase L system and PKR-dependent eIF2α phosphorylation, are activated in MVA-ΔE3L-infected HeLa cells.

Intermediate transcripts are the source of dsRNA during MVA-ΔE3L infection

Despite the lack of late transcription and dramatically reduced viral DNA replication, MVA-ΔE3L infection of HeLa cells allows for unimpaired initiation and prolonged activity of viral intermediate transcription (Ludwig *et al.*, 2005). Thus, it was desirable to address the question of whether viral intermediate transcripts would be necessary or sufficient to form enough dsRNA for activation of the 2′-5′OA synthetase/RNase L and PKR pathways in the absence of the dsRNA-binding E3 protein. We therefore analysed intermediate transcripts synthesized during MVA-ΔE3L infection for their capacity to form dsRNA and to stimulate rRNA degradation. HeLa cells were infected with MVA or MVA-ΔE3L in the absence or presence of AraC to block viral DNA replication and, thereby, intermediate and late transcription. In the presence of AraC, DNA synthesis of MVA and residual genome replication of MVA-ΔE3L were reduced to background levels (see Supplementary Fig. S2, available in JGV Online). As expected, transcription of the early gene 005R (encoding MVA VGF/C11R homologue) was not affected by the AraC block of DNA replication, but was rather prolonged and enhanced, as has been shown

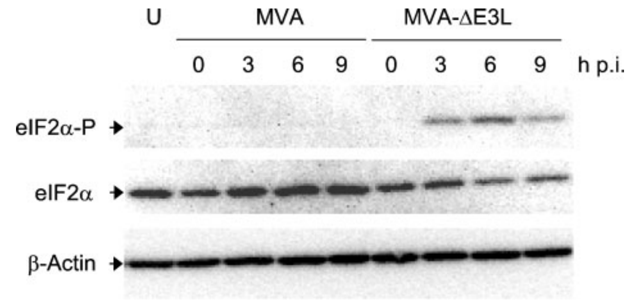

Fig. 1. Deletion of the E3L gene from the MVA genome causes phosphorylation of eIF2α. HeLa cells were mock-infected (U) or infected with MVA or MVA-ΔE3L. HeLa cell lysates were immunoblotted and monitored for phosphorylation of eIF2α by using an antibody specific for the phosphorylated form of eIF2α on serine 51 (eIF2α-P). Total amounts of eIF2α and β-actin were analysed by applying the respective specific antibodies for detection.

previously (Sanz & Moss, 1999) (Fig. 2a). Transcription of the intermediate gene 078R (MVA G8R homologue encoding VLTF 1) was blocked following infection with both viruses in the presence of AraC, as shown by Northern blot (Fig. 2a). Remarkably, upon abrogation of DNA synthesis and, consequently, intermediate transcription, rRNA also remained intact during MVA-ΔE3L infection of HeLa cells. Additionally, phosphorylation of eIF2α was also inhibited in the presence of AraC (Fig. 2a). These results also suggest that intermediate transcripts can form dsRNA and have the capacity to activate the 2′-5′OA synthetase/RNase L and PKR pathways in the absence of E3L expression.

To compare the impact that dsRNA made on MVA-ΔE3L infection with the transfection of synthetic dsRNA poly(I : C) as an approach for 2′-5′OA synthetase/RNase L activation, we also tested rRNA degradation in a highly sensitive Northern blot, using a probe specific for human 18S rRNA. This assay markedly demonstrated the need for E3L gene function to prevent massive RNA degradation during MVA infection of HeLa cells. Again, we detected a substantial repression of rRNA degradation in MVA-ΔE3L-infected cells in the presence of AraC (Fig. 2b, lanes 3 and 9). However, the degradation of rRNA induced by transfection of synthetic dsRNA poly(I : C) was not affected by the addition of AraC to the medium, excluding a direct inhibitory effect of the AraC nucleoside on the 2′-5′OA synthetase/RNase L pathway (Fig. 2b, lanes 5 and 11).

Next, we wished to ascertain the capacity of MVA intermediate transcripts to form dsRNA species. To address this question, we quantified RNase A/T1-resistant RNA species as an indicator for intracellular dsRNA synthesized during vaccinia virus infection (Colby & Duesberg, 1969; Langland & Jacobs, 2002). In MVA-infected HeLa cells, strong signals corresponding to MVA-specific RNase-resistant RNAs were detectable at late times of infection (Fig. 3a, b). This finding is in agreement with the suggestion that dsRNA species originate predominantly from abundant late viral transcripts produced after DNA replication. The latter was confirmed by the lack of dsRNA-specific signals in samples prepared from infections in the presence of AraC as an inhibitor of DNA replication (Fig. 3a, b). Total RNA isolated from MVA-ΔE3L-infected cells also included RNase-resistant RNA, although to a much lower amount than for MVA infection (Fig. 3a, b). These dsRNAs should be formed by intermediate transcripts, as no late transcription is initiated in the absence of E3L expression (Ludwig *et al.*, 2005). These results suggest that intermediate transcripts, synthesized during infection of HeLa cells with MVA-ΔE3L, form dsRNA with the potential to stimulate rRNA degradation by RNase L and phosphorylation of eIF2α by PKR.

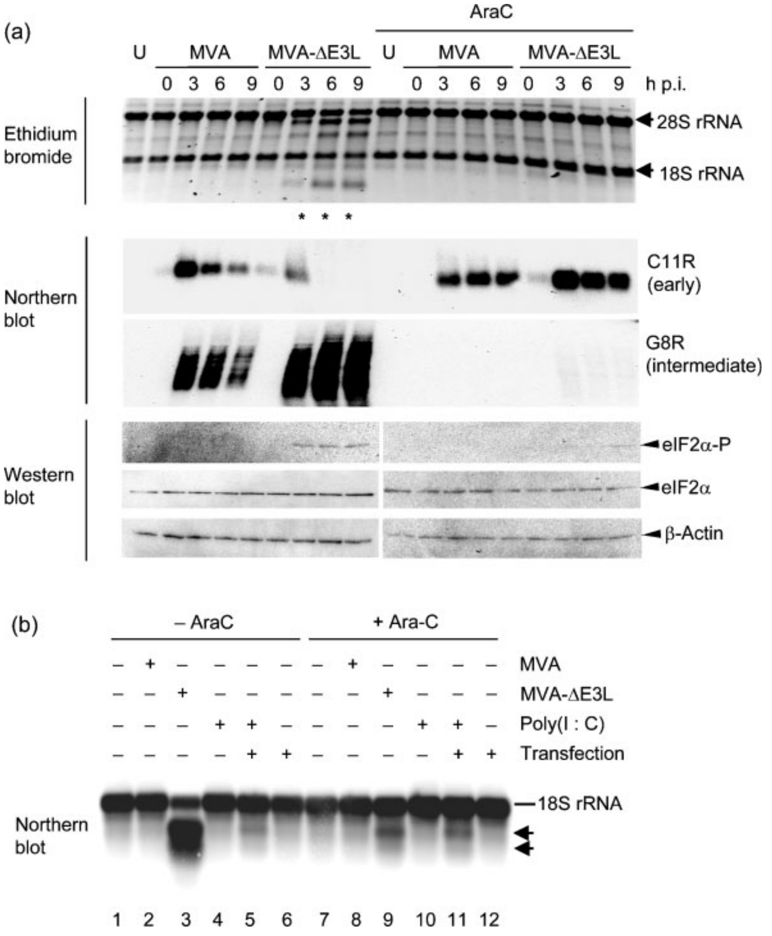

Fig. 2. Inhibition of viral DNA synthesis blocks viral intermediate transcription and degradation of rRNA following MVA-ΔE3L infection. (a) Total RNA was isolated from uninfected cells (U) and at indicated hours post-infection (h p.i.), early 005R (C11R) and intermediate 078R (G8R) transcripts were detected by Northern blot using specific riboprobes. Degradation of rRNA is indicated by asterisks. Phosphorylation of eIF2α was detected as described for Fig. 1. (b) Addition of AraC does not interfere with activity of the 2′-5′OA synthetase/RNase L pathway. HeLa cells were pre-incubated with human IFN-α and IFN-β (R&D Systems) (100 IU ml^{-1} each) for 12 h. Subsequently, cells were infected, treated with 2 μg poly(I : C) ml^{-1} added to the culture medium [poly(I : C)] or transfected with 2 μg poly(I : C) by using FUGENE reagent (Roche Diagnostics) (transfection) in the absence or presence of AraC, respectively. After 12 h incubation, total RNA was prepared and analysed by Northern blotting, using a riboprobe specific for human 18S rRNA. Degradation products of 18S rRNA are indicated by arrows.

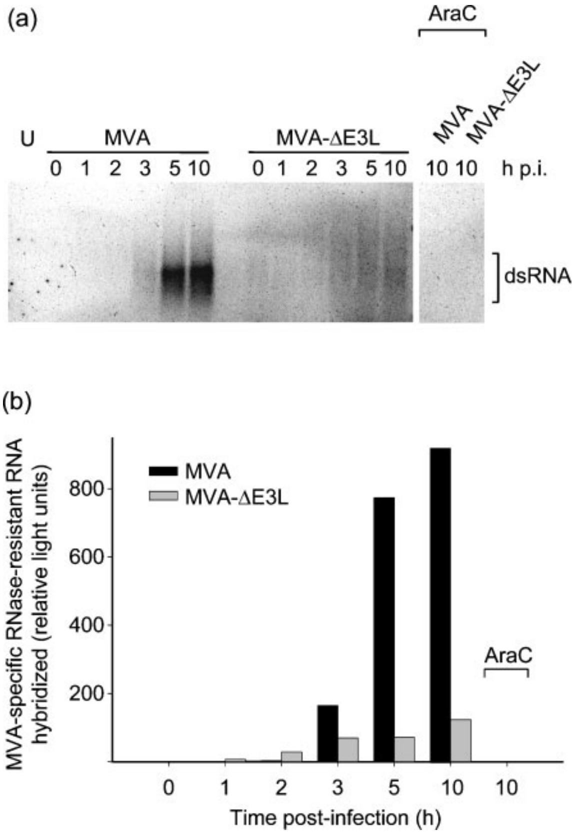

Fig. 3. Detection of RNase A/T1-resistant RNA species in MVA- and MVA-ΔE3L-infected cells. (a) HeLa cells were mock-infected (U) or infected with MVA or MVA-ΔE3L in the absence or presence of AraC. Total RNA was isolated at indicated hours post-infection (h p.i.). For RNase A/T1 degradation, 5 μg total RNA per reaction was applied and RNase-resistant RNA was subsequently analysed by Northern blotting by applying an MVA-specific DIG-labelled probe for detection. (b) Quantification of MVA-specific RNase-resistant RNAs [indicated as dsRNA in (a)].

Differential induction of RNase L and PKR activity in human cells

To ascertain the above-described host responses for MVA-ΔE3L-infected HeLa cells in other human cells, we infected HaCaT and 293T cells with MVA or MVA-ΔE3L and monitored for degradation of rRNA. Starting at 6 h p.i. of HaCaT cells with MVA-ΔE3L, almost-complete degradation of 28S and 18S rRNA was detectable (Fig. 4). In contrast, after infection of 293T cells with MVA-ΔE3L, we noticed only weak RNase L activity (Fig. 4). Thus, compared with the infections of HaCaT or 293T cells, we observed an intermediate level of rRNA degradation in MVA-ΔE3L-infected HeLa cells (Figs 2a and 4).

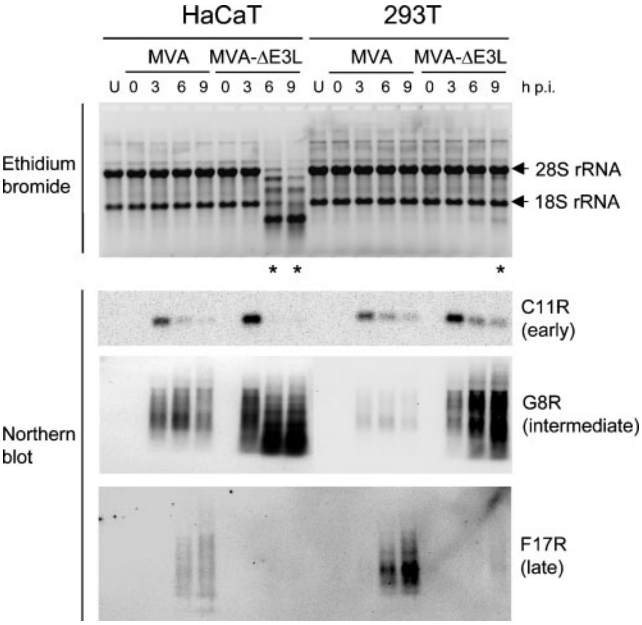

Fig. 4. Analysis of rRNA stability and viral mRNA synthesis following infection of HaCaT and 293T cells with MVA and MVA-ΔE3L. Total RNA was isolated and viral early, intermediate and late mRNA was monitored by Northern blotting, using riboprobes specific for MVA genes 005R, 078R and 047R, respectively. Degradation of rRNA is marked with asterisks.

Analysis of vaccinia viral transcription revealed the typical transient activity of early transcription following infection of HaCaT and 293T cells with MVA and MVA-ΔE3L, respectively. However, in both cell lines, viral intermediate transcription was prolonged and no late transcripts were detectable in the absence of E3L, confirming the pattern of viral transcription observed for MVA-ΔE3L-infected HeLa cells [Fig. 4; compare with Fig. 2a and Ludwig *et al.* (2005)].

Thus, in the context of obvious differences in the levels of MVA-ΔE3L-induced RNase L activities in human HeLa, HaCaT and 293T cells, we observed a clear-cut block of the mutant virus life cycle, being arrested precisely at the level of intermediate transcription in all of these cells. Therefore, we speculated that another antiviral pathway might be involved in triggering this phenotype of infection, at least in 293T cells. As we had noticed strong phosphorylation of eIF2α in infected HeLa cells (Fig. 1), we also determined PKR activity in HaCaT and 293T cells. Indeed, following infection of HaCaT and 293T cells with MVA-ΔE3L, we observed either moderate (HaCaT) or stronger (293T) phosphorylation of eIF2α (Fig. 5). These data suggested that infection of different human cells with MVA-ΔE3L can cause differential activation of the 2′-5′OA synthetase/RNase L pathway or PKR, resulting in different levels of either rRNA degradation or eIF2α phosphorylation.

Block of protein biosynthesis limits late transcription of MVA-ΔE3L virus

Next, we characterized the factor(s) responsible for the block in viral late transcription in more detail. As transcription of

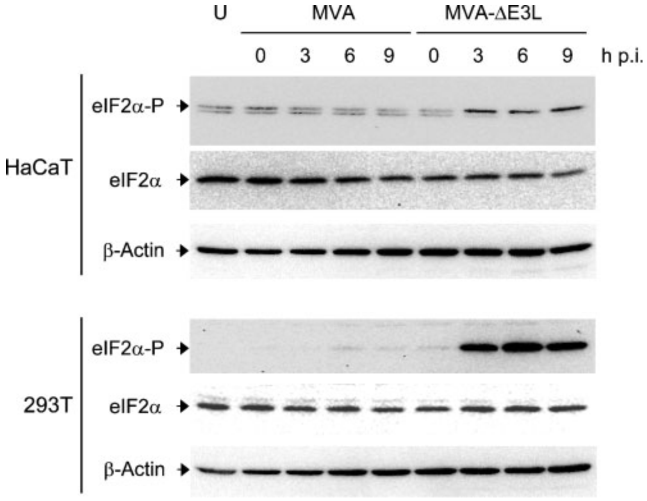

Fig. 5. Analysis of eIF2α phosphorylation in MVA-ΔE3L-infected human cells. Total cell lysates from mock-infected (U) or infected HaCaT and 293T cells were isolated 0, 3, 6 and 9 h p.i. and immunoblotted. For phosphorylation of eIF2α, total amounts of eIF2α and β-actin were monitored as described for Fig. 1.

viral early genes proceeds as normal in MVA-ΔE3L-infected human cells (Figs 2a and 4), first we wished to analyse early-protein synthesis and monitored lysates prepared from MVA- and MVA-ΔE3L-infected HeLa cells by Western blot analysis. When analysing the synthesis of the early vaccinia virus E3 protein in an initial control experiment, we detected two previously described immunoreactive species, p25 and p20 (Watson *et al.*, 1991; Chang *et al.*, 1992), being present at peak levels in MVA-infected cells at 6 and 9 h p.i. (Fig. 6a). As expected, no E3 protein was visualized in lysates from MVA-ΔE3L-infected cells (Fig. 6a). To compare synthesis of a viral early protein in the presence or absence of E3L gene expression, we monitored protein levels of the MVA-encoded IL-18bp (Born *et al.*, 2000; Smith *et al.*, 2000). At 3 h p.i. with MVA or MVA-ΔE3L, similar levels of IL-18bp were detectable, showing clearly that translation of viral early transcripts is independent of E3 protein synthesis. However, at later time points of infection, we noticed reduced levels of IL-18bp in MVA-ΔE3L-infected HeLa cells (Fig. 6a).

Vaccinia virus intermediate transcripts encode vaccinia late transcription factors G8 (VLTF 1), A1 (VLTF 2) and A2 (VLTF 3). These viral intermediate-gene products are essential for late viral mRNA synthesis (Keck *et al.*, 1990). MVA-ΔE3L is still capable of transcribing intermediate genes, as shown for the mRNA encoding the MVA 078R gene (G8R homologue) (Fig. 2a), but no late mRNA is made and no late-protein biosynthesis was detectable (Ludwig *et al.*, 2005). Therefore, it was tempting to look for the presence of late viral transcription factors in MVA-ΔE3L-infected HeLa cells. Western blot analysis using an antiserum against the A1 protein (VLTF 2) (Keck *et al.*, 1993) verified its production in MVA-infected HeLa cells at later times of infection (Fig. 6b). In clear contrast, the late transcription factor A1 was not detectable in the absence of E3L gene expression. Similarly, as expected for intermediate gene products, A1 synthesis was inhibited by the addition of

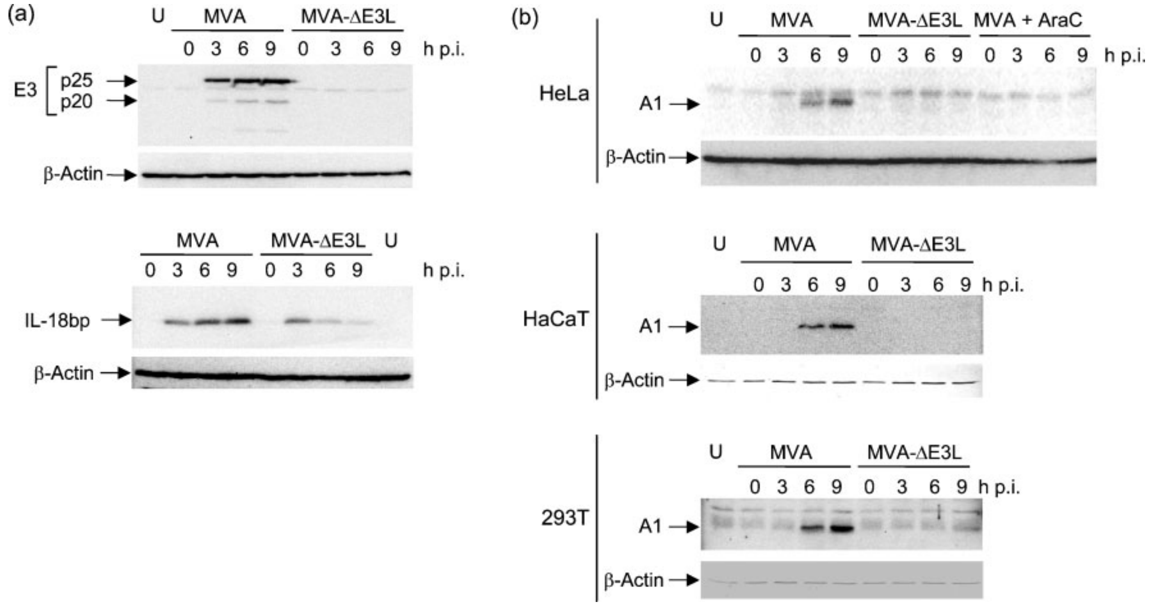

Fig. 6. Synthesis of viral early proteins E3 and IL-18bp (a) and late transcription factor A1 (b) in MVA-ΔE3L-infected human cells. Cell lysates were immunoblotted and analysed for E3 (p25, p20), IL-18bp and A1 proteins by using specific polyclonal rabbit antibodies. AraC was added to the culture medium as indicated.

AraC (Fig. 6b). Moreover, comparable to the infection of HeLa cells, A1 protein synthesis was also absent upon MVA-ΔE3L infection of human HaCaT and 293T cells (Fig. 6b). These results show clearly that a block of translation of intermediate mRNA causes the failure of viral late-gene transcription in MVA-ΔE3L-infected human cells. Interestingly, this inhibition of intermediate protein synthesis in the absence of the E3 protein was observed in all human cell lines tested, irrespective of either the RNase L or the PKR antiviral pathway being dominant (see Figs 1, 5 and 6b).

PKR activity is sufficient to block intermediate- and late-protein expression in MVA-ΔE3L-infected MEFs

To further explore the role of PKR-mediated phosphorylation of eIF2α for interruption of the vaccinia viral life cycle, we analysed late-gene expression upon MVA-ΔE3L infection in the presence and absence of a functional PKR. For this purpose, we infected MEFs derived from wild-type mice (*Pkr*$^{+/+}$) or from mice devoid of functional PKR (*Pkr*$^{0/0}$) (Yang *et al.*, 1995) with MVA or MVA-ΔE3L. Interestingly, we observed no signs of RNase L-mediated rRNA degradation in MVA-ΔE3L-infected *Pkr*$^{+/+}$ or *Pkr*$^{0/0}$ MEFs (data not shown). To analyse the impact of PKR on protein synthesis of intermediate genes, we monitored for A1 (VLTF 2) expression by Western blot analysis. In *Pkr*$^{+/+}$ MEFs, the late transcription factor A1 was detectable following MVA, but not MVA-ΔE3L, infection (Fig. 7a), a finding comparable to the results obtained with different human cells (see also Fig. 6b). Interestingly, in *Pkr*$^{0/0}$ MEFs, both MVA and MVA-ΔE3L were able to synthesize the late transcription factor A1 (Fig. 7a). This result further supported the hypothesis that activated PKR in MVA-ΔE3L-infected cells blocks translation of viral intermediate genes, thus limiting late-gene expression. Finally, we tested the capacity of MVA and MVA-ΔE3L to synthesize the late vaccinia virus 14 kDa envelope protein A27 (Rodriguez *et al.*, 1987) in the presence or absence of a functional PKR. As expected, A27 protein was found easily by Western blot analysis of lysates from MVA-infected cells, but it was not detectable in those from MVA-ΔE3L-infected *Pkr*$^{+/+}$ MEFs (Fig. 7b). We revealed that A27 expression levels were fully restored in MVA-ΔE3L-infected MEFs derived from mice deficient for functional PKR (Fig. 7b). These data clearly demonstrate prevention of PKR activation as a crucial role of the MVA-encoded E3 protein to ensure expression of intermediate and, consequently, late viral proteins.

DISCUSSION

The previously constructed deletion mutant MVA-ΔE3L was shown to initiate intermediate transcription, but completely failed to activate late transcription and late-protein biosynthesis in infected HeLa cells (Ludwig *et al.*, 2005). Such a distinct interruption of the virus life cycle seemed surprising for a mutation targeting a viral immune-evasion factor. In this work, we first confirmed that this particular infection phenotype is linked to the inactivated E3L gene locus, as an MVA-E3L-revertant virus regained full capacity to activate late transcription in human cells (see Supplementary Fig. S1, available in JGV Online). We assumed that the failure of MVA-ΔE3L to initiate late transcription in human cells should be a consequence of activated cellular antiviral activities, rather than being due to a direct function of the E3 protein in the MVA molecular life cycle. Indeed, upon MVA-ΔE3L infection of HeLa cells, we had observed RNase L-mediated degradation of rRNA (Ludwig *et al.*, 2005), and here we show clear evidence for activation of PKR, the other dsRNA-stimulated pathway, following infection of human cells (Figs 1 and 5).

Post-translational activation of the IFN-response effectors 2′-5′OA synthetase/RNase L and PKR requires the presence of dsRNA. Yet, the source of dsRNA during vaccinia virus MVA infection still remained to be investigated. In

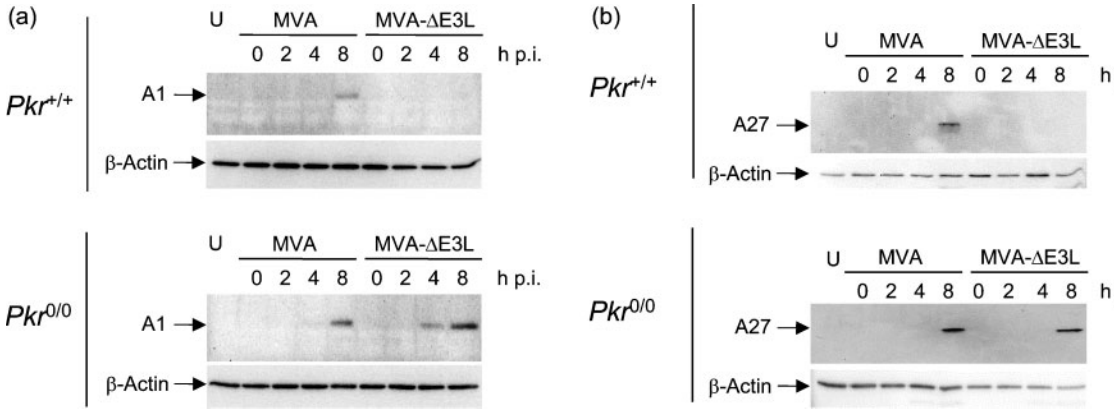

Fig. 7. PKR activity inhibits expression of intermediate protein A1 (VLTF 2) (a) and late vaccinia envelope protein A27 (b) in MVA-ΔE3L-infected MEFs. Cell lysates from wild-type (PKR$^{+/+}$) and PKR-deficient (PKR$^{0/0}$) MEFs were analysed by Western blot using A1- and A27-specific antibodies.

particular, viral late mRNA species are believed to provoke formation of dsRNA molecules, due to read-through transcription by the viral RNA polymerase in the late phase of infection generating heterogeneous 3′ termini (Colby *et al.*, 1971; Boone *et al.*, 1979; Varich *et al.*, 1979; Cooper *et al.*, 1981; Mahr & Roberts, 1984). Interestingly, RNA 3′-end heterogeneity was also demonstrated for intermediate transcripts encoding the vaccinia virus G8 protein (Baldick & Moss, 1993). In MVA-ΔE3L-infected human cells, we exclusively detected early and intermediate transcripts, including RNase A/T1-resistant RNA species. These latter transcripts were recognized as being derived from viral intermediate-gene products because their identification strictly required the unimpaired onset of viral DNA replication (Fig. 3). Thus, synthesis of intermediate transcripts during MVA-ΔE3L infection seemed sufficient for dsRNA formation and critical activation of RNase L and/or PKR.

In addition, the absence of protective E3L function offered an excellent opportunity to define more precisely the step at which activation of RNase L and/or PKR can block the virus life cycle. Both host-response pathways associated with the non-permissive MVA-ΔE3L infection trigger consequences – degradation of 28S and 18S rRNA and the phosphorylation of eIF2α – that are linked to inhibition of the cellular translation machinery. Whilst one might expect that cessation of translation could result in a more insidious inhibition of the virus life cycle, we hypothesized that a failure of viral protein synthesis could explain the peculiarity of MVA-ΔE3L to transcribe intermediate, but not late, viral genes. Activation of viral intermediate transcription requires viral DNA replication and can be prevented by addition of the inhibitor AraC, whilst post-replicative *de novo* protein biosynthesis, in contrast to the requirements for late transcription, is dispensable (Vos & Stunnenberg, 1988) (Fig. 2a). Indeed, upon MVA-ΔE3L infection of different human cells, we found robust levels of intermediate transcription, but failed to detect intermediate proteins, as shown for the A1 protein (VLTF 2) (Figs 2a, 4 and 6b). These results suggest that a block of translation of intermediate mRNA can abruptly prevent the production of essential viral late transcription factors and, thus, cause the complete failure of viral late-gene expression in MVA-ΔE3L-infected human cells.

Interestingly, the inhibition of intermediate protein synthesis in the absence of E3 protein was observed in all human cell lines tested, irrespective of either the RNase L or the PKR antiviral pathway being activated dominantly (Figs 1, 4 and 5). Despite barely detectable degradation of rRNA in MVA-ΔE3L-infected 293T cells, we found no evidence for synthesis of proteins encoded by intermediate genes or late transcription. The strong phosphorylation of eIF2α in 293T cells implies that, in these host cells, PKR activity prevents translation of intermediate messages, rather than stimulation of the 2′-5′OA synthetase/RNase L pathway. In HaCaT cells, however, we observed nearly complete degradation of rRNA, indicating high levels of 2′-5′-oligoadenylates, but only weak eIF2α phosphorylation upon MVA-ΔE3L infection (Fig. 4). Interestingly, activated RNase L can possibly degrade the intermediate viral mRNA encoding the vaccinia virus G8 protein (VLTF 1) directly, as suggested by our detection of faster-migrating G8R-specific transcripts (Fig. 4). This degradation of viral mRNA would be a first example of specific cleavage of a vaccinia virus mRNA by activated RNase L. Until now, specific cleavage of viral mRNA has rarely been demonstrated, e.g. during infection with *Encephalomyocarditis virus* in intact cells (Li *et al.*, 1998) and for reovirus mRNA in a cell-free system (Baglioni *et al.*, 1984). It seems most likely that this mRNA destruction effectively stops G8 protein synthesis, in addition to rRNA degradation. Interestingly, in MEFs derived from mice deficient for functional PKR, we found E3 to be dispensable for unimpaired expression of intermediate A1 (VLTF 2) and late envelope protein A27 (Fig. 7). These data demonstrate a pivotal role of activated PKR to inhibit MVA intermediate-, and thereby late-, protein synthesis in the absence of the dsRNA-binding protein E3. In contrast to the infection of human cells, we detected no signs of rRNA degradation in MEFs infected with MVA-ΔE3L. Thus, inhibition of PKR alone is sufficient to restore viral protein synthesis completely, at least in MEFs. Interestingly, MEFs deficient for PKR, RNase L and Mx1 were shown to not allow for complete rescue of the replicative capacity of a vaccinia virus strain Copenhagen E3L-deletion mutant, possibly indicating the involvement of other cellular activities and/or additional viral regulatory factors (Xiang *et al.*, 2002). In addition to E3, MVA encodes another PKR inhibitor, the viral regulatory protein K3, being suggested to act as a viral eIF2α decoy to competitively block phosphorylation of eIF2α (Beattie *et al.*, 1991; Davies *et al.*, 1992; Carroll *et al.*, 1993; Jagus & Gray, 1994). Despite conservation of functional K3 protein-encoding sequences in the MVA-ΔE3L genome, we observed eIF2α phosphorylation upon infection of different human cells. These data imply that, at least in human cells, the MVA-ΔE3L-induced phosphorylation of eIF2α by PKR cannot be prevented fully by the virus-encoded K3 protein. The cell line-dependent impact of this viral decoy has been shown by the specific need for K3 function for permissive vaccinia virus infection of hamster BHK-21 cells (Langland & Jacobs, 2002).

Given the described function of a recently described RNA helicase (retinoic acid-inducible gene-I, RIG-I) (Yoneyama *et al.*, 2004; Kato *et al.*, 2005), it is tempting to speculate about activation of its downstream signalling modulators depending on the presence of the dsRNA-binding protein E3 during vaccinia virus infection, as was suggested for IRF-3 activity (Smith *et al.*, 2001; Xiang *et al.*, 2002). Moreover, stimulation of such innate responses has also been shown to enhance antigen-specific immune responses (Leitner *et al.*, 2003; Schulz *et al.*, 2005). Thus, in future experiments, the mutant virus MVA-ΔE3L should represent a promising tool to investigate the potential impact of E3L on adaptive immune responses induced after MVA vaccination.

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