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# Rescue of Vaccinia Virus Lacking the E3L Gene by Mutants of E3L

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**Vaccinia virus with the E3L gene deleted was able to replicate in RK-13 but not HeLa cells. This host range phenotype could be complemented by an E3L gene expressed transiently from a plasmid. Analysis of mutants of E3L indicates that the ability to complement deletion of E3L correlates with the ability of mutated proteins to bind double-stranded RNA but not with their ability to migrate to the nucleus.**

Vaccinia virus (VV) is resistant to the antiviral effects of interferon (IFN) in most cell types tested (21) and can rescue normally IFN-sensitive viruses, such as vesicular stomatitis virus (19) and encephalomyocarditis virus (20), from the antiviral effects of IFN. The VV E3L (2) and K3L (4) genes are necessary for IFN resistance of VV replication in mouse L929 cells. Both genes can inhibit the IFN-induced, double-stranded RNA (dsRNA)-dependent protein kinase (PKR) (6, 9, 10). The E3L gene encodes the dsRNA-binding proteins p20 and p25 (8, 18, 22), which are thought to function by binding dsRNA and inhibiting both PKR and the IFN-induced, dsRNA-dependent enzyme 2′,5′-oligoadenylate synthetase (2). VV with E3L deleted also has a host range phenotype in that it replicates normally in CEF (3) or RK-13 cells but cannot replicate in Vero (3) or HeLa cells. Vaccinia virus with K3L deleted replicates normally in Vero cells (4). Results of single-step growth experiments with RK-13 and HeLa cells for wild-type (*wt*) VV and VV with E3L deleted (vP1080) are shown in Fig. 1. Confluent monolayers of RK-13 or HeLa cells were treated with the concentrations of rabbit or human IFN-α (Lee Biomolecular), respectively, indicated in Fig. 1. After 24 h, the cells were infected with *wt* VV or VV vP1080 at a multiplicity of infection of 5 PFU per cell. After 24 h, monolayers were harvested, virus was released by repeated freezing and thawing, and released virus was quantified by a plaque assay on monolayers of RK-13 cells. In the absence of IFN treatment, VV vP1080 replicated as efficiently as *wt* VV in RK-13 cells. While replication of *wt* VV was not affected by IFN treatment of RK-13 cells, replication of VV vP1080 was inhibited approximately 20-fold. Even in the absence of treatment with IFN, replication of VV

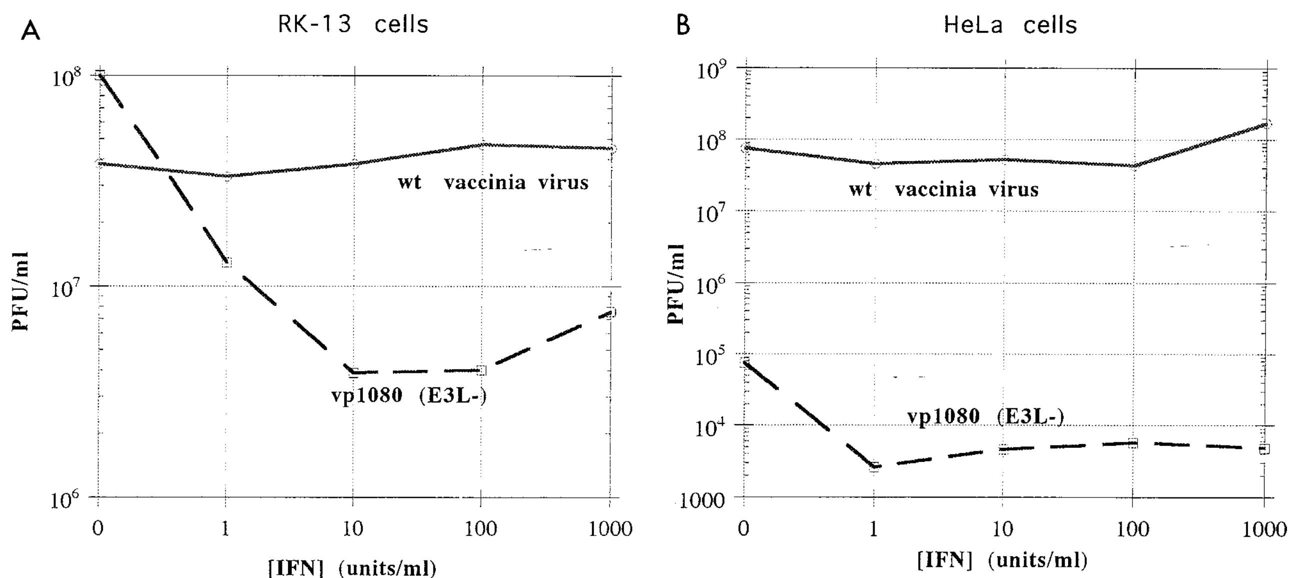


FIG. 1. Replication and interferon sensitivity of VV lacking E3L in RK-13 and HeLa cells. Shown are virus yields from RK-13 (A) and HeLa (B) cells treated with the indicated concentrations of rabbit or human IFN-α, respectively, and subsequently infected with *wt* VV or VV vP1080 at a multiplicity of infection of 5 PFU per cell.

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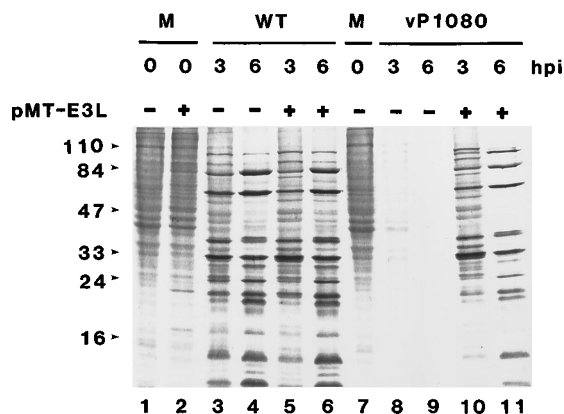


FIG. 2. Rescue of viral protein synthesis by transiently expressed E3L proteins in HeLa cells infected with VV lacking E3L. Subconfluent monolayers of HeLa cells were transfected with pMT-E3L (8) (lanes 2, 5, 6, 10, and 11) by the modified $CaPO_4$ precipitation technique, as previously described (1), or were left untransfected (lanes 1, 3, 4, 7, 8, and 9). At 48 h posttransfection, cells were infected with either *wt* VV (lanes 3 to 6) or VV vP1080 (lanes 8 to 11), or were mock infected (M) (lanes 1, 2, and 7). At 3 h postinfection (hpi) (lanes 3, 5, 8, and 10) or 6 hpi (lanes 4, 6, 9, and 11), cultures were labeled with [$^{35}$S]methionine and newly synthesized proteins were identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography. Molecular masses (in kilodaltons) are indicated on the left.

vP1080 in HeLa cells was inhibited 1,000-fold compared with *wt* VV replication. IFN treatment of HeLa cells had little effect on replication of *wt* VV but inhibited replication of VV vP1080 approximately 30-fold further.

In order to analyze the mechanism by which E3L promotes replication of VV in HeLa cells, we have developed a transient-transfection assay for rescue of VV vP1080 replication in HeLa cells. HeLa cells were transfected with E3L constructs (indicated in Fig. 2 and 3) derived from the eukaryotic expression vector pMT-2Va− (pMT) (7, 13). Transcription of the E3L gene in these constructs is driven by an adenovirus major late promoter. At 48 h posttransfection, cells were infected with either *wt* VV or VV vP1080 at a multiplicity of infection of 5 PFU per cell. Synthesis of proteins was analyzed after the cells were labelled with [$^{35}$S]methionine at 3 or 6 h postinfection, and virus replication was analyzed by assay of virus yield.

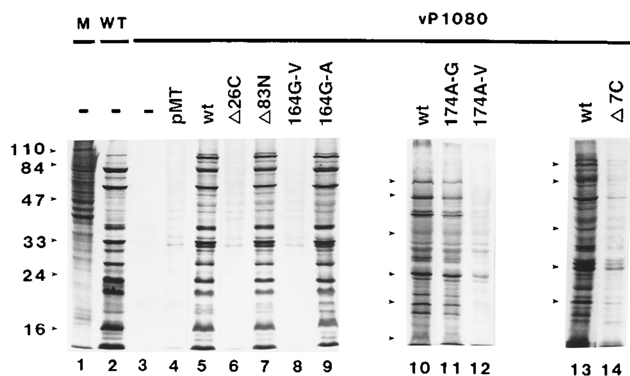


FIG. 3. Rescue of viral protein synthesis by transiently expressed mutants of E3L in HeLa cells infected with VV lacking E3L. Cultures of HeLa cells were treated as described in the legend to Fig. 2, except that protein synthesis was analyzed only at 6 h postinfection. The cells were either untransfected (lanes 1 to 3) or transfected with the parental plasmid pMT (lane 4), with pMT-E3L (wt) (lanes 5, 10, and 13), or with the indicated mutant E3L gene in pMT. M, mock infection; WT, infection with *wt* VV; vP1080, infection with VV vP1080. Molecular masses (in kilodaltons) are indicated on the left.

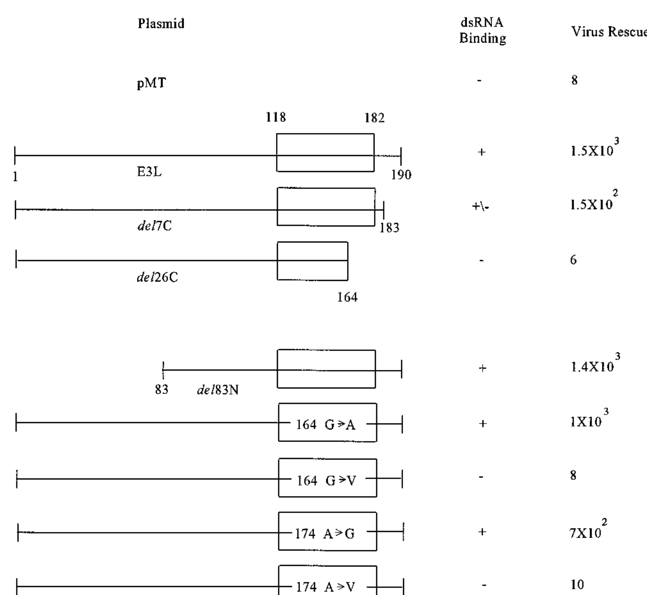


FIG. 4. Rescue of replication of VV with E3L deleted by transiently expressed mutants of E3L. Cultures of HeLa cells were treated as described in the legend to Fig. 2, except that infected cells were harvested after 24 h and assayed for infectious virus on monolayers of RK-13 cells by a plaque assay. Boxes indicate the dsRNA-binding domain (amino acids 118 to 182) conserved among known dsRNA-binding proteins. dsRNA-binding data are from reference 7. Virus rescue is given as the fold increase in PFU relative to the level in mock-transfected cells. *del*, Δ.

The results for protein synthesis are presented in Fig. 2 and 3, and the results of the virus yield experiments are shown in Fig. 4; all results are summarized in Table 1. Synthesis of both viral and cellular proteins was severely inhibited by 3 h postinfection in HeLa cells infected with VV vP1080 (Fig. 2, lane 8). Synthesis of viral proteins in VV vP1080-infected cells was restored to nearly the level in cells infected with *wt* VV after expression of E3L from the transfected plasmid, pMT-E3L (Fig. 2; compare lanes 3 and 4 with lanes 10 and 11). The parental plasmid (pMT) had only a marginal effect on protein synthesis in VV vP1080-infected cells in comparison with untransfected cells (compare lanes 3 and 4 of Fig. 3). Expression of E3L from a plasmid had no effect on protein synthesis in cells infected with *wt* VV (Fig. 2, lanes 5 and 6). Similar results were seen when replication of VV vP1080 was analyzed. Transfection with pMT-E3L led to an approximately 1,000-fold increase in replication of VV vP1080 (Fig. 4), to a level within 25% of the *wt* VV replication level.

We have previously characterized a number of E3L mutants in terms of the ability of their encoded proteins to bind to dsRNA and inhibit PKR in vitro (7). Deletion of 26 C-terminal amino acids from the E3L-encoded proteins destroyed the ability of the proteins to bind to dsRNA and inhibit PKR (7). Expression of this gene (Δ26C, previously called E3L/3-5 [7]) from pMT could not detectably rescue protein synthesis in VV vP1080-infected cells (Fig. 3, lane 6) and did not rescue replication of VV vP1080 to a greater extent than did the parental plasmid, pMT (Fig. 4). Deletion of 83 N-terminal amino acids from the E3L-encoded proteins did not affect binding to dsRNA or inhibition of PKR (7). Expression of this gene (Δ83N, previously called E3L/AatII [7]) from pMT rescued VV vP1080 protein synthesis (Fig. 3, lane 7) and replication of VV vP1080 (Fig. 3) nearly as efficiently as *wt* E3L. Mutation of glycine 164 or alanine 174 to valine (G164V and A174V, re-

TABLE 1. dsRNA binding, nuclear localization, and rescue of protein synthesis and virus replication

| Protein | dsRNA binding | Nuclear localization | Rescue of: Protein synthesis | Rescue of: Virus replication |
|---|---|---|---|---|
| E3L | +[a] | + | + | + |
| Δ83N | +[a] | − | + | + |
| Δ7C | ±[a] | +[b] | ± | ± |
| Δ26C | −[a] | NT[c] | − | − |
| G164V | −[a] | NT | − | − |
| G164A | +[a] | NT | + | + |
| A174V | −[a] | NT | − | − |
| A174G | +[a] | NT | + | + |
| Human TRBP[d] | +[e] | +[e] | +[e] | +[e] |
| Reovirus σ4 | +[f] | +[b] | +[g] | +[g] |

[a] Data from reference 7.
[b] Data not shown.
[c] NT, not tested.
[d] TRBP, TAR/Rev response element-binding protein.
[e] Data from reference 15.
[f] Data from reference 12.
[g] Data from reference 2.

spectively) destroyed the ability of the E3L-encoded proteins to bind dsRNA and inhibit PKR (7). Neither mutation allowed rescue of VV vP1080 protein synthesis (Fig. 3, lanes 8 and 12, respectively) or rescue of VV vP1080 replication (Fig. 4). Mutation of glycine 164 to alanine (G164A) or alanine 174 to glycine (A174G) did not appreciably affect dsRNA binding or PKR inhibition (7). Both G164A and A174G rescued VV vP1080 protein synthesis (Fig. 3, lanes 9 and 11) and VV vP1080 replication (Fig. 4). Finally, deletion of seven C-terminal amino acids (Δ7C, previously called E3L/2-2 [7]) reduced the affinity of the encoded proteins for dsRNA nearly 100-fold in comparison with *wt* E3L (7). Expression of this gene from pMT rescued VV vP1080 protein synthesis poorly (Fig. 3, lane 14) and rescued replication of VV vP1080 10-fold less efficiently than *wt* E3L (Fig. 4). For each of the mutants analyzed, E3L-encoded proteins were expressed in HeLa cells at comparable levels (data not shown), suggesting that differences in efficiency of rescue were not due to differences in levels of expression.

The E3L-encoded proteins are present both in the nucleus and in the cytoplasm of infected and transfected cells (22) (Fig. 5A). In order to determine if any of the mutations tested led to proteins with altered localization in the cells, we visualized several of these proteins by indirect immunofluorescence at 24 h posttransfection, using monospecific anti-E3L serum (18). Deletion of 83 N-terminal amino acids prevented localization of the E3L-encoded proteins to the nucleus (Fig. 5B). Since Δ83N bound to dsRNA as efficiently as *wt* E3L and rescued virus replication as efficiently as *wt* E3L, it appears that nuclear localization of E3L-encoded proteins is not necessary for replication of VV in HeLa cells.

All results for dsRNA binding, nuclear localization, and rescue of VV vP1080 protein synthesis and replication are summarized in Table 1. The results suggest that the ability of mutants of E3L to rescue VV vP1080 protein synthesis and replication correlates with their ability to bind dsRNA. All mutations that destroyed the ability of the E3L-encoded proteins to bind dsRNA destroyed the ability to rescue VV vP1080 protein synthesis and replication. Mutant Δ7C, which bound dsRNA with reduced affinity, rescued VV vP1080 protein synthesis and replication poorly. In addition, replication of VV vP1080 could be rescued by several related (group C rotavirus NSP3 protein [14] and human TAR/Rev response element-binding protein [15]) and unrelated (reovirus σ3 protein [2, 12]) dsRNA-binding proteins. In the case of σ3, which has no sequence homology to E3L, the ability of mutants to bind to dsRNA correlated with their ability to rescue protein synthesis in VV vP1080-infected HeLa cells (2). For the group C rotavirus NSP3 protein, a 69-amino-acid peptide containing the dsRNA-binding motif is sufficient to rescue replication of VV vP1080 in HeLa cells (17). All of these results suggest that the sole function of E3L in promoting replication of VV in HeLa cells is binding dsRNA.

The ability to localize to the nucleus did not appear necessary for rescue of VV vP1080 replication, since Δ83N, which did not localize to the nucleus, rescued replication of VV vP1080 nearly as efficiently as *wt* E3L did. It is somewhat surprising that Δ83N rescued replication of VV vP1080 nearly as well as *wt* E3L. The entire E3L gene is well conserved between ectromelia virus (5), VV (11), and variola virus (16), suggesting that the entire protein is required for some function in these viruses. However, the N-terminal 45% of the protein appears dispensable for replication in HeLa cells and dispens-

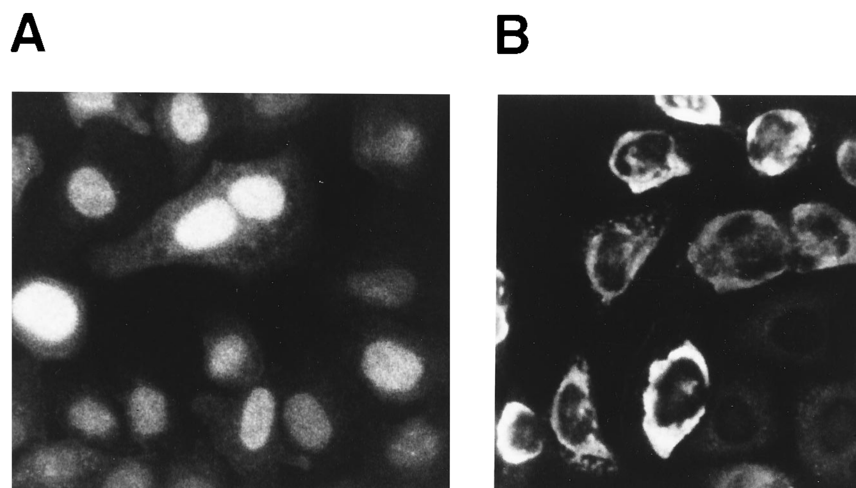


FIG. 5. Detection of E3L-encoded proteins by indirect immunofluorescence. Localizations of *wt* protein (A) and mutant protein encoded by Δ83N (B) are shown.

able for interferon resistance in RK-13 cells (17). It is possible that this region of the protein is required for replication in some other cells or that this portion of the protein provides a subtle advantage not apparent in our transient-transfection assay. Resolution of this apparent conundrum will require a careful comparison of *wt* VV with VV in which the E3L gene has been replaced by Δ83N.

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