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Distinct Patterns of IFN Sensitivity Observed in Cells Infected with Vaccinia K3L⁻ and E3L⁻ Mutant Viruses

ELIZABETH BEATTIE,*,[1] ENZO PAOLETTI,† and JAMES TARTAGLIA†,[2]

*Department of Biomedical Sciences, School of Public Health, University at Albany, Albany, New York 12222; and †Virogenetics Corporation, 465 Jordan Road, Rensselaer Technology Park, Troy, New York 12180

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Recent results have implicated a role for both the VV K3L- and E3L-encoded gene products in conferring VV with an IFN-resistant phenotype (Beattie *et al., Virology* 183, 419–422, 1991; Beattie *et al., J. Virol.* 69, 499–505, 1995). As a means of further establishing the mechanisms by which these functions mediate this process in VV-infected cells, we have further assessed the IFN phenotype in K3L⁻ (vP872) and E3L⁻ (vP1080) virus-infected cells. Biochemical and molecular biological analyses were performed comparing the effects of IFN on wild-type as well as K3L⁻ and E3L⁻ virus-infected cells. Expression analyses of the K3L and E3L gene products revealed that both are evidenced in virus-infected cells as early as 0.5 hr postinfection. E3L expression, however, appears more prolonged, in that it was detectable between 3 to 4 hr postinfection while K3L was undetectable after 3 hr postinfection. Despite having similar expression profiles at early times postinfection, a pronounced sensitivity of protein synthesis to IFN was observed by 30 min postinfection in VV K3L⁻ virus-infected cells, whereas IFN sensitivity was not observed in VV E3L⁻-infected cells until 2 hr postinfection. Subsequent analyses of the IFN-induced antiviral pathways in VV-infected cells demonstrated that the K3L gene product does not contribute to the previously identified *s*pecific *k*inase *i*nhibitory *f*actor (SKIF) activity but does reduce the level of phosphorylated eIF-2α in VV-infected cells. Interestingly, the IFN-induced 2′,5′-oligoadenylate synthetase-mediated antiviral pathway was active in VV K3L⁻-infected cells and not in wild-type virus-infected cells. Collectively these results suggest that the K3L⁻- and E3L⁻-encoded products abrogate the antiviral effect of IFN at distinct levels.

INTRODUCTION

Early studies to assess the antiviral activity of interferon-α/β (IFN-α/β) on vaccinia virus (VV) established that this virus was relatively resistant to this antiviral agent in a number of cell systems (Youngner *et al.*, 1972; Paez and Esteban, 1984a,b; Rice and Kerr, 1984; Rice *et al.*, 1984). Recently, we have shown the involvement of the VV open reading frames (ORFs), E3L and K3L, in conferring this IFN-resistant phenotype (Beattie *et al.*, 1991, 1995). Based upon the functional property of the E3L-specified gene product as a dsRNA binding protein and the significant sequence similarity of the K3L ORF to the amino terminal portion of eukaryotic initiation factor 2α (eIF-2α), the proposed mechanism leading to the abrogation of the IFN-mediated antiviral activities involves the down-regulation of PKR activation and the subsequent phosphorylation of eIF-2α. Such a mechanism has been shown in other viral systems to interfere with the antiviral activities mediated by IFN (reviewed by Katze, 1993).

Results from several studies have substantiated the proposed mechanism by which the K3L- and E3L-encoded products lead to the down-regulation of PKR activity and hence the ability of VV to replicate in IFN-treated cells. Specific deletion of the K3L or the E3L ORFs from VV renders the virus exquisitely sensitive to IFN (Beattie *et al.*, 1991, 1995). The IFN-resistant phenotype was restored either by reinsertion of the K3L ORF under the control of its natural promoter at a distal genomic locus of a K3L⁻-deleted virus (unpublished observations) or by insertion and expression of the sequences encoding a heterologous dsRNA binding protein, reovirus σ3, into the E3L⁻ mutant virus (Beattie *et al.*, 1995). Additionally, *in vitro* assays and transient expression assays in COS-1 cells have shown that both K3L and E3L reduce PKR activity and the eIF-2α phosphorylation state, as well as providing translational stimulatory activity (Davies *et al.*, 1992, 1993; Chang *et al.*, 1992; Carroll *et al.*, 1993).

In this communication, we present results from initial investigations which attempt to address the significance of the apparent multiple mechanisms evolved by VV to down-regulate PKR activation. Together, the results suggest that the E3L- and K3L-encoded functions are required at distinct levels to abrogate the IFN-mediated antiviral activities by VV.

[1] Present address: Department of Microbiology, University of Washington, Seattle, WA 98195.

[2] To whom correspondence should be addressed. Fax: (518) 283-0936.

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MATERIALS AND METHODS

Cells and viruses

Tissue culture medium and supplements for mammalian cell culture were obtained from Gibco-BRL (Grand Island, NY). Mouse L929 cells (ATCC No. CCl1) and Vero cells (ATCC No. CCL81) were propagated as described (Tartaglia *et al.*, 1992).

The derivation of wild-type VC-2 and K3L-minus deletion mutant vP872 are described elsewhere (Beattie *et al.*, 1991; Tartaglia *et al.*, 1992). K3L-reconstituted virus, vP1046, was derived as follows. The K3L coding sequences and 215 bp of upstream sequence (Goebel *et al.*, 1990a,b) were generated by PCR using oligonucleotide primers K3L52 (5′ ATC GAT ATT TTT ATG CGT GAT TGG 3′) and K3LHD (5′ ATC ATC AAG CTT TTA TTG ATG TCT ACA CAT CC 3′) and plasmid template pSD407VC, containing the vaccinia virus *Hin*dIII K fragment. The resultant 0.5-kbp fragment was ligated into plasmid pSD541VC digested with *Sma*I. pSD541VC is a vaccinia virus insertion plasmid containing VV A type inclusion (ATI) flanking sequences (ORFs A24R and A27R) which will result in a recombinant deleted of the ATI locus (Goebel *et al.*, 1990a). The resultant plasmid, pK3LGP, was used in standard *in vivo* recombination protocols (Perkus *et al.*, 1993). Recombinants were screened by hybridization to radiolabeled K3L-specific probe, as previously described (Perkus *et al.*, 1993) and the selected recombinant, designated vP1046, was verified by restriction analyses (data not shown).

Interferon sensitivity studies

Mouse IFN-α/β was purchased from Lee Biomolecular Research Laboratories, Inc. (San Diego, CA). The interferon sensitivity of wild-type (VC-2), K3L-minus deletion mutant (vP872), and K3L-reconstituted virus (vP1046) was determined as previously described (Beattie *et al.*, 1991). Briefly, confluent monolayers of mouse L929 cells were pretreated with various amounts of mouse IFN-α/β for 24 hr. Cells were then mock-infected, or infected with VC-2, vP872, or vP1046 at an m.o.i. of 10. Monolayers were pulsed with [^{35}S]methionine (E.I. DuPont deNemours & Co., Boston, MA) from 7 to 8 hr postinfection (hpi) in methionine-free medium (Gibco-BRL, Grand Island, NY). Protein concentrations were determined using the BioRad protein assay kit (BioRad Laboratories, Richmond, CA). Equivalent quantities of total protein were fractionated by SDS–PAGE and visualized by fluorography, as previously described (Beattie *et al.*, 1991).

To determine the time of IFN-mediated inhibition of viral protein synthesis, IFN-treated L929 cells were prepared and infected as above. Monolayers were pulsed for 1 hr with [^{35}S]methionine in methionine-free medium, beginning at the times indicated, with the exception of the first pulse which was for 30 min. Protein concentrations were determined as above and equivalent quantities of total protein were fractionated by SDS–PAGE and visualized by fluorography.

Detection of kinase inhibitory activity present in extracts from virus-infected L929 cells

Determination of the presence of PKR kinase inhibitory activity was determined as described (Watson *et al.*, 1991). Briefly, cell lysates of virus-infected L929 cells were harvested at 4 hpi and solubilized in NP-40 lysis buffer. Extracts of uninfected cells pretreated with 100 units/ml mouse IFN-α/β were used as a source of PKR. Kinase assays were performed by mixing equivalent volumes of IFN-treated and viral lysates in the presence of poly(rI)–poly(rC) and [γ-^{32}P]ATP. Lysates were fractionated by SDS–PAGE and phosphorylated proteins were visualized by autoradiography.

Determination of the phosphorylation state of eIF-2α in virus-infected cells

Sixty-millimeter dishes of mouse L929 cells pretreated with 0 or 100 units/ml mouse IFN-α/β were mock-infected or infected with virus at an m.o.i. of 10. Plates were harvested at 4 hpi by scraping cells into the medium. The cells were washed once with PBS and resuspended in 300 μl PBS–P (PBS containing 50 m*M* NaF, 10 m*M* 2-Aminopurine, 1 m*M* Prefabloc ((4-(2-aminoethyl)-benzenesulfonylfluoride, hydrochloride); Boehringer Mannheim Biochemicals, Indianapolis, IN) and 0.21 mg/ml aprotinin). Samples were prepared as published (Scorsone *et al.*, 1987; Wong *et al.*, 1982) except that solubilization was for 30 min at 37°C with periodic mixing. Run conditions for vertical slab isoelectric focusing (VSIEF) and visualization of eIF-2α were as published (Scorsone *et al.*, 1987; Wong *et al.*, 1982).

Activation of 2-5A/RNase L pathway

Confluent monolayers of mouse L929 cells (10^7 cells/100 mm dish) were pretreated with 0, 10, 100, 1000, or 5000 units/ml mouse IFN-α/β, as described above. After 24 hr, the cells were mock-infected or infected with vaccinia virus at an moi of 10. At 7 hpi, total RNA was isolated using the guanidinium isothiocyanate method (Kingston *et al.*, 1991). Equivalent volumes of total RNA were fractionated by formaldehyde–agarose electrophoresis and transferred to Hybond-N membrane (Amersham Corp., Arlington Heights, IL) as per the manufacturer's specifications. Filters were probed with a ^{32}P-labeled 18S-specific probe (generous gift of Dr. Ira G. Wool, University of Chicago) as previously described (Beattie *et al.*, 1991).

Immunoprecipitation

To generate K3L-specific antisera, NM522 cells transformed with plasmid pGEX–K3L were induced to overex-

press the K3L–GST (glutathione-*S*-transferase) fusion protein and harvested as described (Smith and Corcoran, 1990). K3L–GST fusion protein was adjusted to 1 mg/ml in elution buffer and stored at −85°. Rabbit number A334 (antisera generated henceforth referred to as A334) was injected by the subcutaneous route with 200 μg K3L–GST in a polyacrylamide gel slice. The rabbit was boosted at Weeks 3, 6, and 9 using the same immunogen preparation. The rabbit was boosted with 1 mg K3L–GST fusion protein in complete Freund's adjuvant on Week 16 and with 1 mg K3L–GST fusion protein in incomplete Freund's adjuvant on Weeks 24 and 28. Rabbit anti-E3L was kindly provided by Dr. Bertram Jacobs (Arizona State University, Tempe, AZ).

Immunoprecipitation reactions were performed as described (Harlow and Lane, 1988; Tartaglia *et al.*, 1992) using stringent lysis buffer (1%, Triton X-100, 1% sodium deoxycholate, 1% NP-40, 10 m*M* Tris, pH 7.4, 150 m*M* NaCl, 1 m*M* EDTA, 0.01% sodium azide, 0.2 mg/ml PMSF, 42 μg/ml aprotinin). To increase the specificity of the K3L immunoprecipitation reaction, after washing the protein A–Sepharose–A334 beads (following absorption with labeled lysates), an additional step was included. The supernatant was aspirated from the beads and bound protein was eluted from the beads in 100 μl 1% SDS as published (Firestone and Winguth, 1990). The eluate was added to a fresh aliquot of SPA–A334 beads along with 1 ml stringent lysis buffer and the immunoprecipitation steps were repeated. Immunoprecipitates were fractionated by SDS–tricine–PAGE (Schägger and Von Jagow, 1987) and visualized by fluorography.

RESULTS

Temporal analyses of VV K3L- and E3L-specified gene expression

As a prelude to further understanding the multiple mechanisms evolved by VV leading to an IFN-resistant phenotype, the kinetics of K3L gene expression was investigated. To assess expression at the protein level, immunoprecipitation analyses were performed using a K3L-specific antiserum preparation derived and utilized as described under Materials and Methods. In short, Vero cell monolayers were mock-infected (Fig. 1A; lanes 1–4), infected with wild-type VV, VC-2 (Fig. 1A; lanes 5–8), or with the K3L$^-$ VV mutant virus, vP872 (Fig. 1A; lanes 9–12). Samples were [^{35}S]methionine pulse-labeled at 0 hpi (Fig. 1A; lanes 1, 5, and 9), 0.5 hpi (Fig. 1A; lanes 2, 6, and 10), 1 hpi (Fig. 1A; lanes 3, 7, and 11), and 3 hpi (Fig. 1A; lanes 4, 8, and 12) for 0.5 hr. Following the pulse period, cell lysates were harvested and analyzed by immunoprecipitation using the K3L-specific antiserum.

No K3L-specific protein species were precipitated from lysates derived from mock-infected (Fig. 1A; lanes 1–4) or vP872-infected (Fig. 1A; lanes 9–12) cells. Conversely, a K3L-specific protein species with an apparent molecular weight of approximately 10 kDa was immunoprecipitated from lysates derived from wild-type VV (VC-2)-infected cells pulse-labeled at 0 to 0.5 hpi (Fig. 1A; lane 5), 0.5 to 1 hpi (Fig. 1A; lane 6), and 1 to 2 hpi (Fig. 1A; lane 7) but not 3 to 4 hpi (Fig. 1A; lane 8). The apparent size of this protein species corresponds to the molecular mass derived from the deduced amino acid sequence of the K3L ORF of approximately 10 kDa (Goebel *et al.*, 1990a,b; Beattie *et al.*, 1991). Results from expression

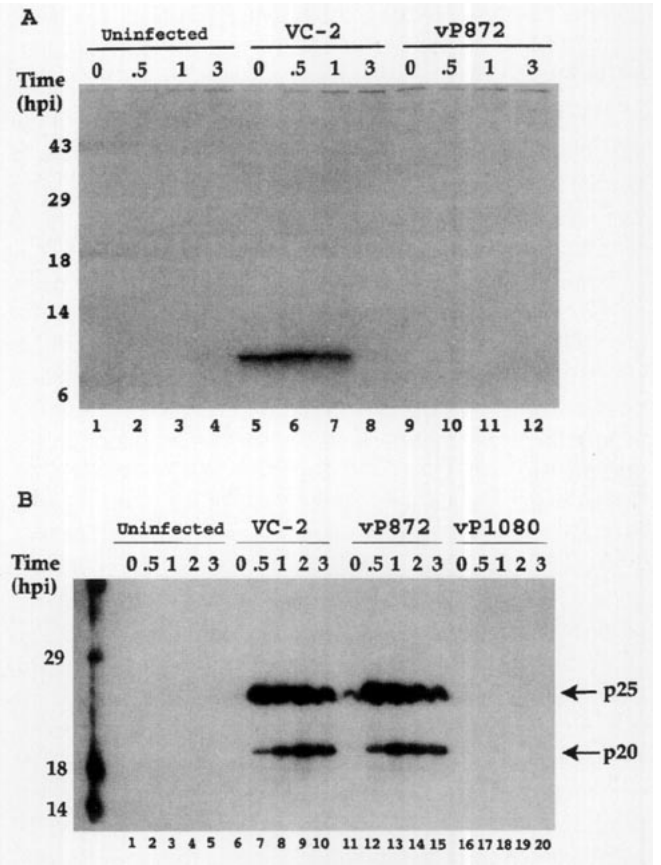

FIG. 1. Temporal expression of the VV K3L and E3L gene products. (A) K3L expression. Vero cells were mock-infected (uninfected; lanes 1–4), or infected with wild-type (VC-2; lanes 5–8), or K3L-minus virus (vP872; lanes 9–12). Monolayers were pulsed with [^{35}S]methionine at 0–30 min (lanes 1, 5, and 9), 30–60 min (lanes 2, 6, and 10), 1–2 hr (lanes 3, 7, and 11), or 3–4 hr (lanes 4, 8, and 12). Lysates were immunoprecipitated with K3L-specific antisera, A334, fractionated by SDS–tricine electrophoresis, and visualized by fluorography (described under Materials and Methods). The migration positions of ^{14}C-labeled molecular weight standards (Bethesda Research Laboratories, Gaithersburg, MD) are shown to the left. (B) E3L expression. Vero cells were mock-infected (uninfected; lanes 1–5), or infected with wild-type (VC-2; lanes 6–10), K3L-minus virus (vP872; lanes 11–15), or E3L-minus virus (vP1080; lanes 16–20). Monolayers were pulsed with [^{35}S]protein-labeling mix (EXPRE^{35}S; DuPont deNemours & Co., Boston, MA) for 30 min, starting at the indicated times. Lysates were immunoprecipitated with E3L-specific antisera, A370, fractionated by SDS–PAGE, and visualized by fluorography. The migration positions of the E3L-specified p25 and p20 species are indicated on the right and ^{14}C-radiolabeled molecular weight standards are indicated on the left.

analyses at the RNA level paralleled those at the protein level, in that K3L expression appears limited to immediate-early to early times postinfection (Fig. 1A; unpublished results).

Whereas K3L expression was found to be limited to between 0 to 2 hpi (Fig. 1A), E3L-specified expression was observed to occur over a more prolonged time period p.i. E3L-specific protein expression was assessed in a similar manner as for the K3L gene product using an E3L-specific antiserum preparation (described under Methods and Materials). E3L-specified protein expression was assessed in mock-infected Vero cell lysates (Fig. 1B; lanes 1–5) and Vero cell lysates derived following infection with wild-type VV, VC-2 (Fig. 1B; lanes 6–10), the K3L$^-$ VV mutant, vP872 (Fig. 1B; lanes 11–15), or an E3L$^-$ mutant VV, vP1080 (Fig. 1B; lanes 16–20). Infected cell cultures were pulse-labeled with [^{35}S]methionine for 0.5 hr at 0, 0.5, 1, 2, and 3 hpi. As observed in Fig. 1B, nascent E3L protein expression (both the p20 and p25 species as described in Watson *et al.,* 1991 and Yuwen *et al.,* 1993) was detected in wild-type VV (lanes 6–10) and vP872-infected cell lysates (lanes 11–15) immediately postinfection, increased in level between 0.5 to 1 hpi and plateaued by 2 hpi. Some slight dimunition in nascent E3L-specific protein synthesis was observed at 3 hpi (Fig. 1B; lanes 10 and 15). These results corroborate and extend previous results relating to E3L expression (Watson *et al.,* 1991) which demonstrated expression as early as 2 hpi and as late as 12 hpi. RNA analyses of the E3L transcription unit also demonstrated a parallel between RNA and protein expression (data not shown).

Temporal analyses of IFN-mediated protein synthesis inhibition in VV E3L- and K3L-mutant-infected cells

Previous studies with the VV K3L$^-$ and E3L$^-$ deletion mutants (vP872 and vP1080, respectively) had focused on the protein synthesis inhibitory activity of IFN treatment at late times (7–8 hr) p.i. (Beattie *et al.,* 1991, 1995). In order to determine the effects of IFN treatment on early viral protein synthesis in vP872 and vP1080 virus-infected cells, monolayers of L929 cells pretreated with mouse IFN-α/β were infected with wild-type VC-2 (Fig. 2A), K3L$^-$ (vP872) (Fig. 2B), or E3L$^-$ (vP1080) virus (Fig. 2C) and pulsed with [^{35}S]methionine at various times postinfection. Equivalent quantities of total protein were fractionated by SDS–PAGE and radiolabeled proteins visualized by fluorography. Wild-type VV (VC-2)-infected cells displayed a typical pattern of viral protein synthesis in the presence or absence of IFN treatment at all times analyzed (Fig. 2A). Protein profiles of lysates derived from vP872-infected cells were similar to wild-type VV in the absence of IFN treatment (Fig. 2B; lanes 1, 4, 7, 10, and 13). Cells infected with vP872 demonstrated a pronounced sensitivity to IFN treatment by 30 min postinfection (Fig. 2B; lanes 2 and 3). These results suggested that the K3L-encoded function is required immediately postinfection to abrogate the antiviral effects of IFN. Interestingly, cells infected with the E3L- VV, vP1080, did not demonstrate a pronounced IFN sensitivity until 2 hpi (Fig. 2C; lanes 8 and 9). These results imply that a temporal distinction exists for the requirements of K3L and E3L in the IFN-resistant phenotype of VV.

Assessment of the involvement of the VV K3L- and E3L-specified protein species in PKR down-regulation

Due to the observations that K3L and E3L protein expression had similar temporal profiles, in that both were expressed at immediate-early times p.i., the distinct time of IFN-mediated inhibition of K3L-minus and E3L-minus VV may not be attributable to differential expression patterns (Figs. 1 and 2). These results collectively suggest that the K3L- and E3L-specified gene products act mechanistically at distinct levels to abrogate the antiviral effects of IFN. To further address this issue, we investigated the effect of K3L- and E3L-specific deletion on IFN-induced antiviral mechanisms in virus-infected cells. VV has been shown to encode an activity, designated SKIF, which blocks PKR activation in a stoichiometric manner (Whitaker-Dowling and Youngner, 1984). Results from previous analyses utilizing lysates derived from vP1080 virus-infected cells demonstrated that the E3L-specified gene product was essential for expression of SKIF activity (Beattie *et al.,* 1995), supporting the identity of the E3L gene product as SKIF.

To determine whether K3L contributes to VV-encoded SKIF activity, lysates derived from vP872-infected cells were tested for PKR kinase inhibitory activity (SKIF). As shown in Fig. 3, utilizing lysates derived from uninfected cells PKR was autophosphorylated in the presence of 1 μg dsRNA/ml (Fig. 3; lanes 1–4). In contrast, lysates derived from wild-type VV (VC-2)-infected cells required the addition of increased amounts of dsRNA to detect a similar level of PKR autophosphorylation (Fig. 3; lanes 5–9). Significantly, lysates derived from vP872 virus-infected cells showed similar levels of SKIF activity as wild-type virus-infected cells (Fig. 5; lanes 10–14), suggesting that K3L does not function in a manner previously demonstrated for SKIF activity. The previous identification of the E3L product as SKIF was corroborated in this experiment where the specific deletion of E3L (vP1080) eliminated SKIF activity in VV-infected cell lysates (Fig. 3; lanes 15–19).

The early time of IFN-mediated inhibition of vP872 suggested that the K3L-specified gene product functions immediately postinfection to evade antiviral activities imparted by IFN treatment. Lysates utilized for the above experiment were isolated at 4 hpi, a time when SKIF activity was reported to be high (Watson *et al.,* 1991; Whitaker-Dowling and Youngner, 1984). It was therefore possible that the

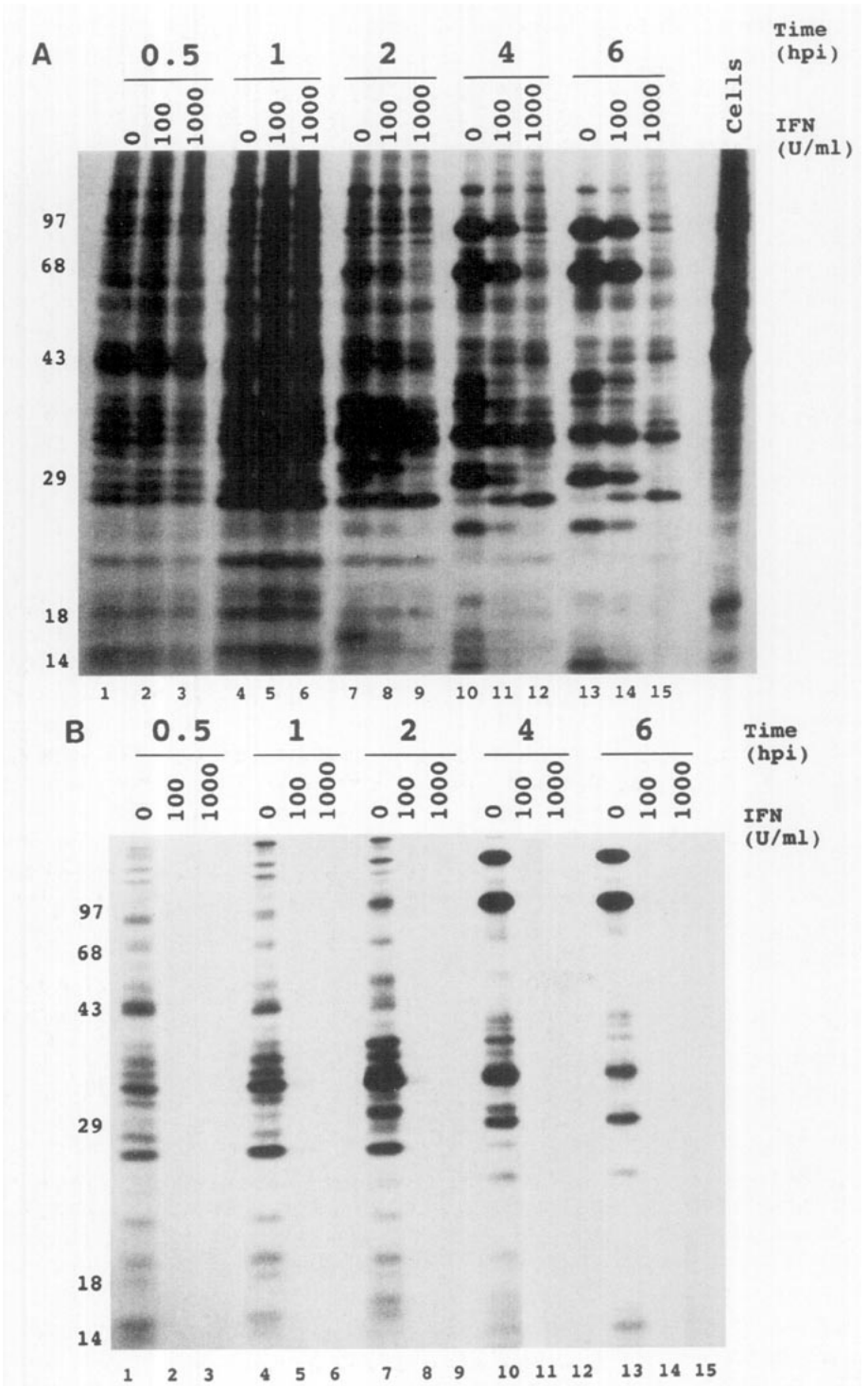

FIG. 2. Time of interferon-mediated inhibition of viral protein synthesis. Monolayers of L929 cells were pretreated with the indicated amounts of mouse IFN-α/β and infected with wild-type VC-2 (A), K3L-minus vP872 (B), or E3L-minus vP1080 (C). Monolayers were pulsed with [^{35}S]methionine at 0.5–1 hpi (lanes 1–3), 1–2 hpi (lanes 4–6), 2–3 hpi (lanes 7–9), 4–5 hpi (lanes 10–12), or 6–7 hpi (lanes 13–15). Equivalent quantities of total protein were fractionated by SDS–PAGE and visualized by fluorography. The migration positions of ^{14}C-labeled molecular weight standards (Bethesda Research Laboratories, Gaithersburg, MD) are shown to the left.

comparatively late time of harvest obscured any kinase-inhibitory activity contributed by K3L due to expression of other IFN-resistance functions which are expressed at later times. To test this possibility, lysates from virus-infected *cells were isolated at 1, 2, 3, and 4 hpi and PKR kinase* inhibitory assays were performed as above. Results from such experiments demonstrated that SKIF activity was expressed by 1 hpi and reached a maximum at 3–4 hpi in

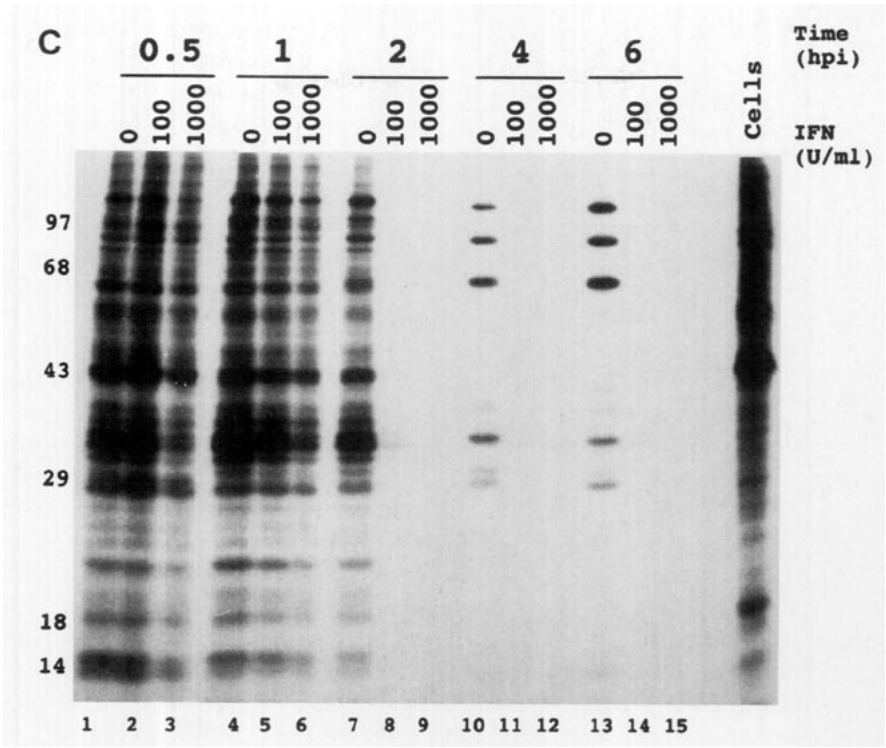

FIG. 2—*Continued*

lysates derived from VC-2 and vP872 virus-infected cells (data not shown). No difference in SKIF activity was detected utilizing lysates derived from VC-2 and vP872 virus-infected cells at any time, suggesting that the K3L-specified gene product does not contribute to the SKIF activity of VV. In contrast, there was no detectable SKIF activity in lysates derived from E3L$^-$ mutant (vP1080) virus-infected cells and by 3 hpi the addition of exogenous dsRNA was not required to detect PKR autophosphorylation (data not shown).

Phosphorylation state of eIF-2α in IFN-treated, vaccinia virus-infected cells

The demonstration that K3L has sequence similarity to eIF-2α suggested that it could function as a PKR pseu-

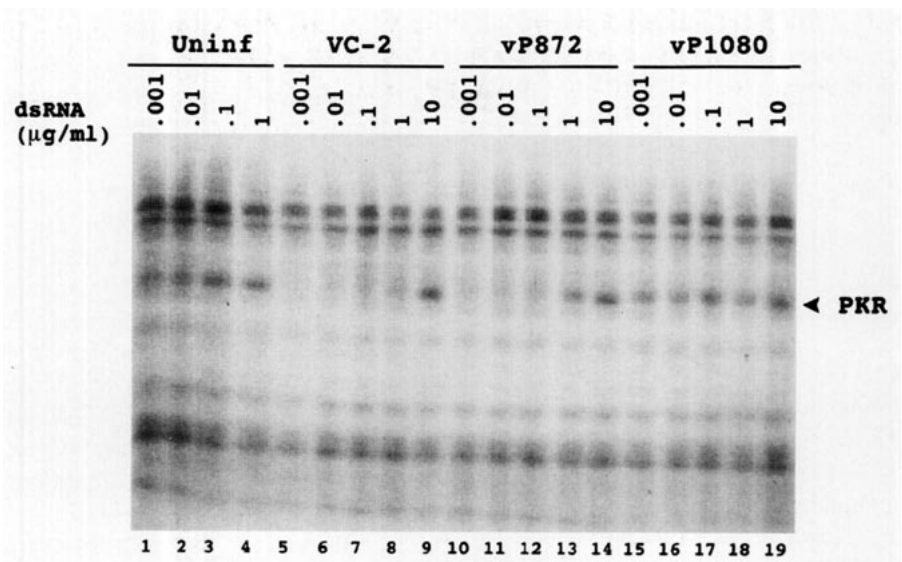

FIG. 3. Analyses of PKR kinase-inhibitory activity in VV-infected cells. Kinase inhibitory assays were performed using lysates from mock-infected or virus-infected L929 cells harvested at 4 hpi. Lysates from mock-infected (uninfected; lanes 1–4), wild-type VV-, VC-2-infected (lanes 5–9), K3L$^-$ VV, vP872 (lanes 10–14), or E3L$^-$ VV, vP1080 (lanes 15–19) were mixed with lysates from uninfected, IFN-treated cells in the presence of the indicated concentration (μg/ml) of dsRNA (poly(rI)–poly(rC)) and [γ-^{32}P]ATP. Reactions were fractionated by SDS–PAGE and visualized by autoradiography. The position of phosphorylated PKR is indicated.

TABLE 1

Phosphorylation State of eIF-2α in VV-Infected Cells

Sample	IFN (IU/ml)	1 hpi	4 hpi
Uninfected	0	10	22
	100	11	21
VC-2	0	9	16
	100	6	18
vP872	0	24	32
	100	25	46
vP1046	0	12	16
	100	9	18
vP1080	0	11	34
	100	12	43

Note. Evaluation of the phosphorylation state of eIF-2α in VV-infected cells was performed as described under Materials and Methods. Autoradiograms were analyzed using a PDI Model 35 DNA densitometer and Quantity One program (PDI, Huntington Station, NY). These data are presented as percentage of total eIF-2α that exists in phosphorylated state.

dosubstrate (Beattie *et al.*, 1991). Therefore, although the K3L-specified gene product does not inhibit *in vivo* PKR activation, it could prevent adverse effects of PKR activation by blocking downstream phosphorylation of eIF-2α. To test this possibility, the phosphorylation state of eIF-2α in virus-infected cells was determined. It was of additional interest to determine the kinetics of IFN-dependent eIF-2α phosphorylation in vP872-infected cells based on the early time of IFN-mediated inhibition of viral protein synthesis in vP872-infected cells (Fig. 2B).

Monolayers of L929 cells pretreated with 0 or 100 units/ml mouse IFN-α/β were mock-infected or infected with virus at an m.o.i. of 10. Lysates were prepared at 1 and 4 hpi and fractionated by vertical slab isoelectric focusing (VSIEF) (Scorsone *et al.*, 1987; Wong *et al.*, 1982). Proteins were transferred to nitrocellulose and phosphorylated and nonphosphorylated eIF-2α species were detected by Western blot analyses using a monoclonal antiserum specific for mouse eIF-2α (generous gift of Dr. E. C. Henshaw, University of Rochester). Autoradiograms were subjected to laser densiometric analyses, to quantitate the percentage of phosphorylated eIF-2α species. The results are summarized in Table 1.

Consistent with K3L serving as a PKR pseudosubstrate, the specific deletion of the K3L ORF, resulting in deletion mutant vP872, led to enhanced phosphorylation of eIF-2α (Table 1). This effect is detected by 1 hpi and, interestingly, is not significantly enhanced at this time point by IFN treatment. However, at 4 hpi, eIF-2α phosphorylation in vP872-infected cells appears to be augmented by IFN treatment. This result was not unexpected as there are elevated dsRNA levels as well as activated PKR at late times postinfection in VV-infected cells (Bayliss and Condit, 1993; Colby *et al.*, 1971; Hovanessian *et al.*, 1987). Additionally, K3L-reconstituted virus, vP1046, behaves in a similar manner as wild-type virus (Table 1) suggesting that it is the specific deletion of the K3L ORF which leads to enhanced eIF-2α phosphorylation in vP872 virus-infected cells. Further, results from a sample assessed in triplicate demonstrated variation within the experiment to be low (±4%). This strongly suggests that the differences noted between the eIF-2α phosphorylation state in K3L$^-$ and wild-type VV-infected cells are, indeed, significant.

The effect of the specific deletion of the E3L ORF on eIF-2α phosphorylation was also determined. As with K3L-minus virus, vP872, enhanced phosphorylation of eIF-2α was observed in E3L$^-$ mutant virus (vP1080)-infected cells. However, enhanced eIF-2α phosphorylation was not detected in vP1080-infected cells until 4 hpi and was further augmented by IFN treatment (Table 1). These results further define the temporal distinction between the roles for the VV K3L and E3L gene products in abrogating the antiviral activities mediated by IFN.

Activation of the 2′,5′-oligoadenylate synthetase pathway in VV-infected cells

Previous studies suggested that VV encodes an activity (activities) that prevents activation of the 2′,5′-oligoadenylate synthetase (2-5A) pathway (Paez and Esteban, 1984b) and the action of RNase L (Rice *et al.*, 1984). We were interested in determining whether the K3L-specified gene product contributes to this activity. Activation of the 2-5A pathway in cells can be detected by analyzing degradation of the 18S and 28S ribosomal RNA (rRNA) species into characteristic, discrete fragments (Silverman *et al.*, 1983; Wrescher *et al.*, 1981). We assessed 2-5A activity in cells infected with VC-2, vP872, and the K3L reconstituted virus, vP1046, by assessing 18S and 28S rRNA integrity. L929 cells pretreated with increasing amounts of IFN were mock- or virus-infected and total RNA was isolated at 7 hpi.

In mock-infected (Fig. 4; lanes 1–5) or wild-type VC-2-infected cells (Fig. 4; lanes 6–10), there was no detectable rRNA degradation in the presence or absence of IFN treatment. In marked contrast, deletion of the K3L ORF (vP872) resulted in IFN-mediated rRNA degradation (Fig. 4; lanes 11–14). Reinsertion of the K3L ORF into K3L-minus virus (vP1046) restored rRNA stability (Fig. 4; lanes 15–19), suggesting a role for the K3L-specified gene product in inhibiting the IFN-induced 2-5A pathway. Similar results were observed when RNA samples were prepared as early as 1 hpi (data not shown).

DISCUSSION

The studies presented in this communication have further distinguished the roles of the K3L- and E3L-specified gene products in mediating IFN resistance in VV-infected cells. Clearly, the results have corroborated previous re-

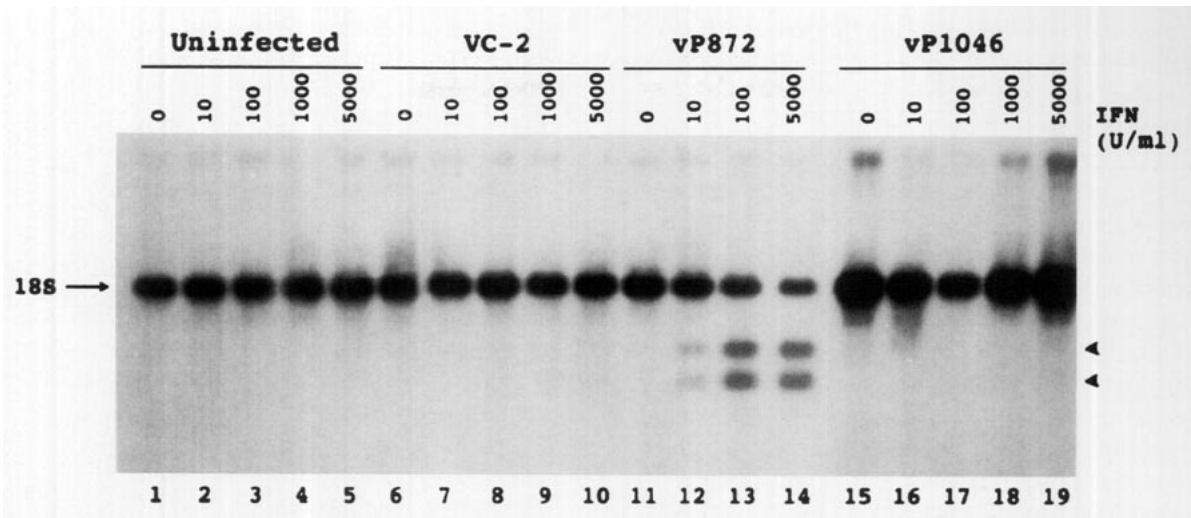

FIG. 4. Activation of the 2-5A system in vaccinia virus-infected cells. Monolayers of L929 cells were pretreated with the indicated amounts of mouse IFN-α/β and infected with virus as described under Materials and Methods. Total RNA was extracted from mock-infected cells (uninfected; lanes 1–5), or cells infected with wild-type (VC-2; lanes 6–10), K3L-minus (vP872; lanes 11–14), or K3L-reconstituted virus (vP1046; lanes 15–19). Equivalent volumes of total RNA were fractionated by formaldehyde–agarose gel electrophoresis, transferred to Hybond-N membrane, and probed with 18S rRNA-specific probe (generously provided by Dr. Ira G. Wool) as described under Materials and Methods. The migration position of intact 18S rRNA is indicated on the left and the characteristic, discrete 18S rRNA degradation products are indicated by arrowheads on the right.

sults which demonstrated that specific deletion of either the K3L or E3L ORF renders VV exquisitely sensitive to the antiviral effects of IFN (Beattie *et al.*, 1991, 1995). Further analyses of cells infected with these VV mutants specifically deleted of the K3L or E3L ORFs (vP872 and vP1080, respectively) have revealed a temporal distinction for the requirement of these gene products in mediating an IFN-resistant phenotype (Fig. 2). Interestingly, this differential temporal requirement occurs despite both encoded gene products displaying similar expression kinetics, in that both are expressed at immediate-early to early times postinfection (Figs. 1A and 1B).

The best characterized VV-mediated anti-IFN function is the PKR kinase-inhibitory activity, designated SKIF, which functions by increasing the effective concentration of dsRNA required for kinase activation (Whitaker-Dowling and Youngner, 1984). This was likely to be an important mechanism whereby VV evades the antiviral effects of IFN, since high levels of dsRNA (Bayliss and Condit, 1993; Duesberg and Colby, 1969) and activated PKR occur in VV-infected cells at late times postinfection (Hovanessian *et al.*, 1987). To investigate the potential involvement of the K3L- and E3L-encoded proteins in this previously described SKIF activity, *in vitro* PKR kinase-inhibitory assays were performed. In confirmation of *in vitro* studies demonstrating the ability of the E3L-encoded gene product to prevent PKR activation (Davies *et al.*, 1993), vP1080, specifically deleted of the E3L ORF, lacked the PKR kinase-inhibitory activity characteristic of wild-type virus-infected cells (Fig. 3; lanes 15–19; Beattie *et al.*, 1995). However, whereas vP1080 lacked PKR kinase-inhibitory activity, specific deletion of the K3L ORF did not diminish the production of SKIF activity in lysates derived from virus-infected cells. Further, the levels of SKIF activity in vP872 virus-infected cells were similar to those of wild-type virus-infected cells at all times tested between 1 and 4 hpi (Fig. 3; lanes 5–14; unpublished results), supporting the lack of SKIF activity for the K3L-specified gene product.

The results illustrating that the K3L-specified gene product did not significantly inhibit activation of PKR in VV-infected cells is in contrast to studies which showed that plasmid-borne expression of K3L prevented PKR activation (Carroll *et al.*, 1993; Davies *et al.*, 1992). This discrepancy most likely reflects the different assay systems employed which measured the effect of significantly different K3L steady-state level on PKR activation. The studies in which K3L was shown to prevent PKR activation utilizing plasmid-borne K3L resulted in steady-state levels that were approximately one-third that of eIF-2α (Carroll *et al.*, 1993). Based on our studies with VV-expressed K3L, we have estimated that the K3L steady-state level in virus-infected cells is no more that 0.02% that of eIF-2α (unpublished results). These results suggest that the studies described previously utilized K3L steady-state levels that were not in a physiological range, and therefore may not be representative of *in vivo* conditions.

The K3L- and E3L-specified gene products appear to act at different levels to abrogate the antiviral effects of IFN. Consistent with this notion is the differential time of IFN-mediated inhibition and the requirement for both gene products to mediate an IFN-resistant state. Further, the E3L-specified gene product has all the characteristics of the activity previously designated SKIF, and likely contributes to the VV IFN-resistant phenotype by preventing PKR activation in virus-infected cells.

Based on the sequence similarity of K3L to eIF-2α, it was proposed that K3L functions as a PKR pseudosubstrate, thus preventing phosphorylation of eIF-2α and the resultant cessation of protein synthesis (Beattie *et al.*, 1991). Consistent with this notion, we have demonstrated that the phosphorylation state of eIF-2α was increased twofold in K3L-minus mutant (vP872) virus-infected cells compared to wild-type virus infected cells (Table 1). Further, others have shown in *in vitro* assays that, indeed, the K3L gene product binds to PKR (Carroll *et al.*, 1993). It is conceivable that the K3L gene product modulates the eIF-2α phosphorylation state at early times postinfection, which either directly or indirectly interferes with IFN-mediated antiviral activities. Interestingly, at 1 hpi an enhanced level of phosphorylated eIF-2α was observed in K3L$^-$ VV (vP872)-infected cells both in the presence and absence of IFN treatment (Table 1). From this, it can be inferred that K3L has an indirect role in the IFN-sensitive phenotype of VV. It is tempting to speculate that K3L is acturally involved in the optimal translation of viral protein species at early times postinfection, with a subset of these viral products having a direct role in abrogating the IFN-mediated antiviral activities.

In support of this hypothesis, viruses expressing the K3L ORF showed no detectable increase in eIF-2α phosphorylation at 1 and 4 hpi relative to uninfected cells (Table 1). In contrast, in K3L-minus virus-infected cells an increased level of eIF-2α phosphorylation was observed at both times. Interestingly, even in the absence of IFN treatment, virus-specified protein synthesis was reduced, but not eliminated in vP872-infected cells (Fig. 2B). This could explain the nonessential nature of K3L to VV replication in, at least, a limited number of cells systems tested (i.e., L929, chick embryo fibroblasts, HeLa). The dimunition in virus-specific protein synthesis in the absence of IFN, however, was not observed at early times in cells infected with the E3L$^-$ (K3L$^+$) mutant virus (vP1080) or a K3L reconstituted VV (vP1046) (Fig. 2C; unpublished results). In E3L-minus virus-infected cells, reduced levels of viral-specified protein synthesis was observed in the absence of IFN at later times postinfection (4 hpi), but not between 0 and 2 hpi. Mechanistically, this most likely can be attributed to PKR activation, as suggested by the ability of lysates derived from vP1080 virus-infected cells to activate PKR in the absence of activator dsRNA (Fig. 3) and the increased level of eIF-2α phosphorylation (Table 1) at these later times postinfection. Modulation of eIF-2α phosphorylation levels by the K3L gene product occurring at early times postinfection in VV-infected cells may, therefore, be involved in the optimal translation of viral mRNA species. Further, such dramatic effects by small changes in the phosphorylation state of eIF-2α on protein synthesis would be predicted from the mechanisms governing eukaryotic translation initiation (reviewed by Kauffman, 1994; Ramaiah *et al.*, 1994).

Another intriguing observation made in the present study was the pronounced rRNA degradation, characteristic for the activation of the 2-5A system (Silverman *et al.*, 1983; Wreschner *et al.*, 1981) in IFN-treated cells infected with the K3L$^-$ VV mutant (vP872) treated with IFN. This observation was made at both early (1 hr) and late times (7 hr) postinfection (Fig. 4; unpublished results). Whereas the ability of the E3L-specified gene product to prevent 2-5A-mediated rRNA degradation is functionally linked to its dsRNA binding activity (Beattie *et al.*, 1995), the mechanism whereby the K3L product inhibits the 2-5A pathway remains unknown. One potential mechanism can be drawn from the above provocation concerning K3L-mediated effects on eIF-2α phosphorylation at immediate-early to early times postinfection. From this, one can consider that K3L is required for optimal expression of protein species that allow VV to evade the antiviral activities imparted by the 2′,5′-oligoadenylate synthetase or a related pathway at such times postinfection.

In summary, the VV K3L- and E3L-specified gene products are involved in the IFN-resistant phenotype of VV. Both gene products have been found to be expressed at immediate-early to early times postinfection. Despite being simultaneously expressed in VV-infected cells, the temporal requirement for K3L and E3L differ significantly with respect to abrogating IFN-mediated antiviral activities. Further, since both activities presumably function by down-regulating PKR activity and/or downstream events, K3L and E3L most likely represent viral functions critical for optimizing VV/host cell interactions. To further support this notion, specific deletion of E3L from VV results in a conditional-lethal phenotype, whereby the virus productively replicates on RK-13 and primary chick embryo fibroblasts but not Vero or HeLa cell substrates (Beattie *et al.*, 1995; Beattie *et al.*, manuscript in preparation).

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