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The Vaccinia Virus K3L Gene Product Potentiates Translation by Inhibiting Double-Stranded-RNA-Activated Protein Kinase and Phosphorylation of the Alpha Subunit of Eukaryotic Initiation Factor 2

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Interferon resistance of vaccinia virus is mediated by specific inhibition of phosphorylation of the alpha subunit of eukaryotic initiation factor 2 (eIF-2α) by the double-stranded-RNA-activated (DAI) protein kinase. Vaccinia virus encodes a homolog of eIF-2α, K3L, the deletion of which renders the virus sensitive to interferon treatment. We have studied the mechanism by which this protein product elicits interferon resistance in a transient DNA transfection system designed to evaluate regulators of eIF-2α phosphorylation. In this system, translation of a reporter gene mRNA is inefficient because of eIF-2 phosphorylation mediated by the DAI protein kinase. Cotransfection of the K3L gene enhances translation of the reporter mRNA in this system. The K3L protein inhibits eIF-2α phosphorylation and DAI kinase activation, apparently without being phosphorylated itself. Inhibition of protein synthesis, elicited by expression of a mutant Ser-51→Asp eIF-2α designed to mimic a phosphorylated serine, is not relieved by the presence of K3L, suggesting that K3L cannot bypass a block imposed by eIF-2α phosphorylation. The results suggest that K3L acts as a decoy of eIF-2α to inhibit DAI kinase autophosphorylation and activation. Another vaccinia virus gene product, K1L, which is required for growth of vaccinia virus on human cells, does not enhance translation in this assay.

Higher eukaryotic organisms have a variety of specific and nonspecific defenses against viral invaders. In animal cells, viral replication may be limited through the action of interferon (32). Two interferon-regulated pathways of translational control, both activated by double-stranded RNA produced during viral infections, have been discovered. One pathway involves the interferon-inducible double-stranded-RNA-activated (DAI) protein kinase, and the other involves the 2-5A [ppp(A2′p)nA] synthetase. Activated DAI kinase phosphorylates the alpha subunit of the eukaryotic translation initiation factor 2 (eIF-2α), leading to inhibition of protein synthesis at the level of initiation. In contrast, 2-5A activates a latent endo-RNase that prevents protein synthesis by cleavage of mRNA and rRNA. Some viruses, however, have evolved mechanisms that counteract the interferon response of the host (35). Thus, adenovirus encodes an RNA molecule, the adenovirus-associated RNA I (VAI), that interferes with the cellular antiviral response by binding to and inhibiting DAI protein kinase (24, 28). This mechanism allows adenovirus to synthesize virus-specific polypeptides in interferon-treated cells.

Vaccinia virus also displays resistance to interferon and can even rescue other interferon-sensitive viruses (38), despite the production of large amounts of double-stranded RNA late in infection (5, 10). This resistance depends, at least in part, on inhibition of DAI kinase (1, 7, 29, 31, 42). Although neither the identity nor the mode of action of this putative kinase inhibitor was known, it was proposed to be a protein that stoichiometrically interacts with double-stranded RNA (42). A recently sequenced segment of the vaccinia virus genome with an 88-amino-acid open reading frame, initially referred to as K2L (6) but renamed K3L (17), has 28% identity with eIF-2α (4). Interestingly, the region of overlap with the considerably larger eIF-2α protein included the DAI protein kinase phosphorylation site at residue 51. A virus with a deletion of the K3L open reading frame had significantly increased sensitivity to interferon (4).

The object of the present study was to determine whether the product of the K3L open reading frame inhibits DAI kinase inactivation of eIF-2α. Transfection systems that permit the measurement of the effect of cloned gene products on the activation of DAI kinase were previously described (2, 22). Transient transfection of cells with an expression vector encoding a reporter gene resulted in DAI kinase activation, eIF-2α phosphorylation, and inhibition of translation of the reporter mRNA derived from the expression vector. In this assay system, adenovirus VAI RNA (2, 22), reovirus sigma 3 (15), and treatment of cells with the DAI kinase inhibitor 2-aminopurine (22) stimulated protein synthesis as a consequence of inhibition of eIF-2α phosphorylation. In addition, expression of a nonphosphorylatable form of eIF-2α (Ser-51→Ala) had a similar effect (21). Further experiments demonstrated that the nonphosphorylated form of eIF-2α did not directly inhibit DAI kinase autophosphorylation and activation but likely acted by forming a nonphosphorylatable eIF-2 heterotrimer (9). In this report, we show that expression of the vaccina virus K3L gene also acts to inhibit eIF-2α phosphorylation in transfected cells and prevents inhibition of protein synthesis. The K3L gene product acts through inhibition of DAI kinase autophosphorylation and activation.

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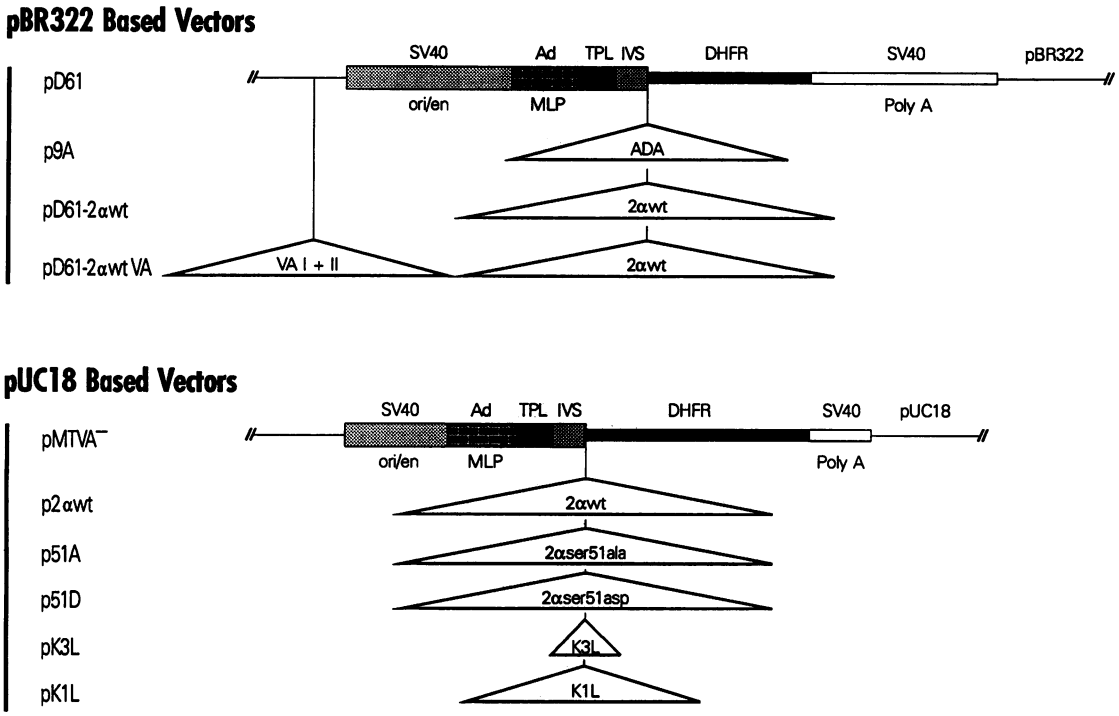

FIG. 1. Expression vectors used in this study. All expression vectors utilize the same control elements for transcription initiation and mRNA processing (21). These include the SV40 enhancer and origin of replication (SV40 ori/en; the 343-bp *Hin*dIII-*Pvu*II fragment in pUC18-based vectors or a 685-bp *Ava*II E fragment in pBR322-based vectors), the adenovirus major late promoter (Ad MLP; a 261-bp fragment from the *Xho*I site [15.83 map units] to the 5′ cap site [16.55 map units]), an untranslated region from the tripartite leader from adenovirus late mRNAs (TPL; 180 bp of the first two leaders and two-thirds of the third leader from adenovirus mRNAs), a small intron (IVS; a hybrid intron containing the 5′ splice site from the first leader of the adenovirus major late mRNAs and a 3′ splice site from an immunoglobulin gene), an *Eco*RI cloning site for insertion of foreign coding regions, a DHFR coding region, and the SV40 early polyadenylation signal (a 237-bp *Bcl*I [position 2270]-to-*Bam*HI [2533] fragment in the pUC18-based vectors and an 800-bp *Bcl*I [2270]-to-*Pst*I [1988] fragment in the pBR322-based vectors). The pBR322-based vectors produce an mRNA which activates DAI kinase and is not efficiently translated unless eIF-2α phosphorylation is inhibited (21). The pUC18-based vectors yield mRNAs which do not significantly activate DAI kinase and which are efficiently translated. For the studies presented here, the murine ADA (1.2 kb) or the human eIF-2α cDNA (1.6 kb) was cloned into the *Eco*RI site in pD61. A derivative of pD61-2αwt which also contains the adenovirus VAI and VAII RNA genes was also derived (see Materials and Methods). Wild-type eIF-2α cDNA as well as two mutants (Ser-51→Ala and Ser-51→Asp) were cloned into the pMTVA$^-$ vector. The vaccinia virus coding regions for K3L and K1L were cloned into the pMTVA$^-$ vector to yield pK3L and pK1L. Insertion of a coding region into these vectors reduces DHFR translation at least 100-fold (23).

MATERIALS AND METHODS

Vector constructions. All of the vectors contain the same transcription unit utilizing the adenovirus major late promoter and simian virus 40 (SV40) enhancer element for transcription initiation (Fig. 1). In addition, the vectors contain the SV40 origin for replication in COS-1 cells. The dihydrofolate reductase (DHFR) expression plasmids pD61, pMT2, and pMT$_2$VA$^-$ (herein called pMTVA$^-$) (21) and the adenosine deaminase (ADA) expression plasmid p9A (22) were previously described. The eIF-2α expression vectors encoding the wild type (peIF2αwtVA$^-$, herein called p2αwt), the Ser-51→Ala mutant (p51A VA$^-$, herein called p51A), and the Ser-51→Asp mutant (p51D VA$^-$, herein called p51D) have been described elsewhere (22). The *Eco*RI fragment encoding eIF-2α wild type was subcloned into the unique *Eco*RI site of p91023(B) (44) to derive pD61-2αwtVA. pD61-2αwtVA was then digested with *Bgl*II and *Eco*RV, and the 5.8-kb fragment containing eIF-2α was ligated to the *Bgl*II-*Eco*RV 2.1-kb fragment of pD61 to derive pD61-2αwt. pD61-2αwt is identical to pD61-2αwtVA, except it lacks the VAI and VAII RNA genes. pD61 and p9A have a pBR322 backbone and encode tetracycline resistance, whereas the pMTVA$^-$ derivatives have a pUC18 backbone and encode ampicillin resistance. Transfection of pD61 or p9A, and not the pMTVA$^-$ derivatives, into COS-1 cells results in DAI kinase activation and eIF-2α phosphorylation (21). The mRNAs derived from pD61 and p9A are not translated efficiently unless eIF-2α phosphorylation is inhibited.

For expression of K3L, the coding region from vaccinia virus strain WR was isolated by polymerase chain reaction amplification using the primers 5′-GGGGCCATGGTTGC ATTTTGTTATTCG-3′ and 5′-GGGGGGATCCTATTGA TGTCTACACAT-3′. The 266-bp fragment was subcloned into pTM1 (27) between the *Nco*I and *Bam*HI restriction sites. DNA sequence analysis confirmed that the 266-bp insert had a sequence identical to that previously described (17). pTM1 was linearized with *Nco*I, treated with the Klenow fragment of DNA polymerase I, and ligated to *Eco*RI adapters. The DNA was then digested with *Xho*I to liberate the 266-bp K3L coding fragment. This *Eco*RI-*Xho*I fragment was subcloned into pMT21 (21), a derivative of pMT2, to yield pMT21/K3L. In order to clone K3L into a

VAI-deficient expression plasmid, an *Eco*RI-*Hpa*I fragment from pMT21/K3L was subcloned into pMTVA$^-$ digested with *Eco*RI and *Hpa*I. The resulting expression vector was designated pK3L.

The K1L coding region from vaccinia virus strain WR was isolated by polymerase chain reaction amplification using the primers 5′-CGGAATTCACCATGGATCTGTCACGAATT AATAC-3′ and 5′-GCGGAATTCTTAGTTTTTCTTTACA CAATTC-3′. The 1.0-kb fragment was cloned into the *Eco*RI site of pMTVA$^-$, and independent isolates were designated pK1La and pK1Lb. DNA sequences of the polymerase chain reaction-amplified DNA were confirmed to be correct by direct DNA sequencing (34) and agree with the previously published sequence of K1L (3).

DNA transfection and analysis of expression. COS-1 monkey kidney cells were transfected by the DEAE-dextran procedure with the addition of a chloroquin treatment as described previously (20). After 42 h, cells were labeled with [^{35}S]methionine (100 μCi/ml; 1,000 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) for 30 min in methionine-free minimal essential medium. To monitor in vitro phosphorylation of eIF-2α, COS-1 cells were labeled at 42 h posttransfection with 2 ml of $^{32}PO_4$ (200 μCi/ml) for 4 h in phosphate-free medium. Cell extracts were prepared by lysis in Nonidet P-40 lysis buffer as described elsewhere (21) and analyzed by reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (25), either before or after immunoprecipitation. Rabbit polyclonal anti-ADA antibody was kindly provided by Rodney Kellems (Baylor University, Houston, Tex.). Anti-eIF-2α polyclonal antibody was kindly provided by John W. B. Hershey (University of California, Davis). Quantitative immunoprecipitations with excess antibody were performed as described previously (21). Gels were fixed in 40% methanol–10% acetic acid, prepared for fluorography by treatment with En^{3}Hance (New England Nuclear Corp.), and dried. Dried gels were autoradiographed with Kodak XAR-5 film with a Dupont Cronex Lightning-Plus screen. Protein levels were quantitated by visual comparison of band intensities from multiple autoradiograms of different exposure times. Phosphorylation of eIF-2α was quantitated with an LKB UltroScan XL laser densitometer (Pharmacia Inc.).

Steady-state levels of eIF-2α were measured in cell extracts obtained as described above. Samples of cell extract protein (0.5 μg) were resolved by reducing SDS-PAGE, electroblotted to nitrocellulose, and analyzed by the ECL Western immunoblotting detection system as described by the supplier (Amersham Corp.). The primary rabbit anti-eIF-2α antibody was diluted 1:5,000, and the secondary anti-horseradish peroxidase conjugated to goat anti-rabbit immunoglobulin was used at a 1:5,000 dilution.

Total RNA was prepared by guanidine thiocyanate extraction (8) and analyzed by Northern blot (RNA blot) hybridization (39) following electrophoresis on formaldehyde-formamide denaturing agarose gels as described elsewhere (13). Hybridization was performed with a DHFR probe prepared by ^{32}P-dCTP labeling by random priming with oligonucleotides as described by the supplier (Pharmacia Inc.).

DAI kinase assay. In vitro DAI kinase assays were performed as described previously (33). Poly(rI-rC) (250 ng/ml) was used to activate DAI kinase in this assay. Partially purified DAI kinase was kindly provided by T. Maniatis (Harvard University, Cambridge, Mass.). Reaction products were analyzed by SDS-PAGE.

RESULTS

Expression of K3L stimulates translation of mRNA transcribed from a cotransfected plasmid. The expression vectors utilized in this study are depicted in Fig. 1. A 266-bp fragment of vaccinia virus DNA encoding the eIF-2α homolog K3L was cloned into the expression vector pMTVA$^-$ (see Materials and Methods). Expression of K3L was evaluated by transfection of COS-1 cells and examination of total protein synthesis by [^{35}S]methionine pulse-labeling and analysis of labeled proteins in cell extracts by SDS-PAGE. Results show that in comparison with the original vector, pMTVA$^-$, which directed the synthesis of significant amounts of DHFR migrating at 20 kDa (Fig. 2A, lane 2), transfection of pK3L directed the synthesis of an 11-kDa protein, the size expected from the amino acid-coding region (Fig. 2A, lane 3).

We next asked whether expression of K3L can substitute for the VAI RNA requirement to enhance translation of plasmid-derived mRNA. The transfection system depends on the cotransfection of two plasmids, one of which represents a reporter gene and the second of which encodes a gene product that potentially affects translation, i.e., the effector gene. The two vectors have identical transcription units described in the legend to Fig. 1 but differ within their plasmid backbones, one containing a pBR322-based backbone and the other containing a pUC18-based backbone. The remarkable feature of this system which allows the constructs to be distinguished is that all mRNAs derived from the pBR322 vectors elicit DAI kinase activation and eIF-2α phosphorylation whereas the pUC18 vectors do not. Transfection into COS-1 cells of the pBR322-based expression vector results in DAI kinase activation and eIF-2α phosphorylation and yields an mRNA which is translated inefficiently unless eIF-2α phosphorylation is inhibited (21). In contrast, transfection of the pUC18-based expression vectors do not significantly activate DAI kinase, and the transcribed mRNA derived is translated efficiently (21). Surprisingly, cotransfection of these two vectors into the same cell results in inefficient translation of mRNA derived from the pBR322 vector, while the mRNA from the pUC18 vector is efficiently translated. At present we do not understand the mechanism for the *cis*-acting effect mediating reduced translation of the pBR322-based vector, but we have speculated that this occurs through localized activation of DAI kinase on the mRNA derived from the pBR322-based vector (21, 22). In the cotransfection experiments described below, the reporter gene (DHFR, ADA, or eIF-2α) was expressed from the pBR322-derived vector pD61 while the effector genes (K3L, K1L, eIF-2αwt, 2α51A, and 2α51D) were expressed from the pUC18-derived vector pMTVA$^-$. By cotransfection of the DHFR or ADA reporter gene in pD61 with the effector gene in pMT2VA$^-$, it was possible to monitor the ability of the effector gene product to affect DHFR translation from pD61 without altering the synthesis of the effector gene.

To monitor the effect of K3L on translation, pK3L was cotransfected with either pD61 or p9A, 42 h later cells were labeled with [^{35}S]methionine, and cell extracts were prepared for analysis of protein synthesis by SDS-PAGE. As a control, pD61 was cotransfected with either p2αwt (encoding wild-type eIF-2α) or p51A (encoding a Ser-51→Ala mutant which is resistant to DAI kinase-mediated phosphorylation). Expression of p2αwt does not affect DHFR translation, whereas expression of mutant eIF-2α from p51A can enhance DHFR translation from pD61 (21). Cotransfection

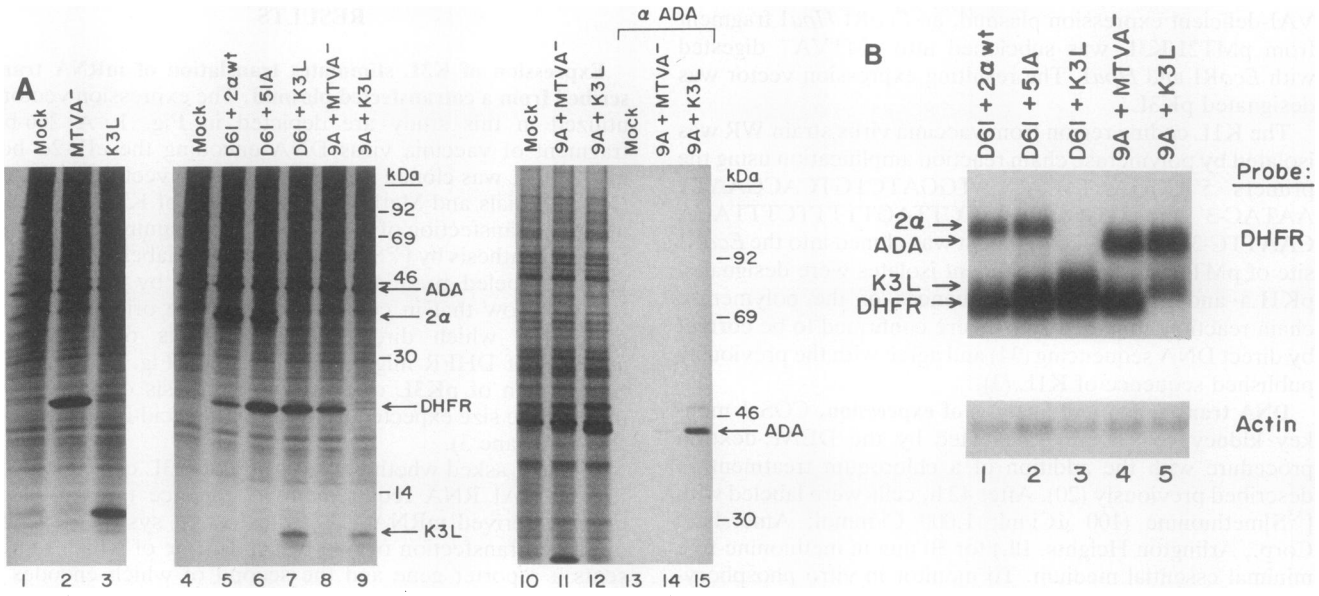

FIG. 2. Expression of K3L enhances translation of plasmid-derived mRNA. COS-1 cells were transfected with the indicated plasmid DNAs as described in Materials and Methods. For cotransfections, equal amounts of the two plasmid DNA were used (8 μg of each plasmid per 4 ml per 10-cm plate). After 42 h cells were labeled with [^{35}S]methionine and harvested for analysis of protein synthesis by SDS-PAGE (A). For analysis of ADA synthesis, cell extracts were immunoprecipitated with anti-murine ADA polyclonal antibody (lanes 13 to 15). DHFR synthesis was analyzed by electrophoresis on 15% polyacrylamide gels (lanes 1 to 9), and ADA synthesis was analyzed by electrophoresis on a 10% polyacrylamide gel (lanes 10 to 15). In parallel, RNA was harvested for Northern blot analysis by hybridization to a DHFR probe (B). A duplicate blot was hybridized to an actin probe. Plasmid DNAs used for each transfection are indicated above the lanes. Migrations of eIF-2α (2α), DHFR, K3L, and ADA are shown.

of pD61 with p2αwt resulted in detectable levels of DHFR and eIF-2α synthesis (Fig. 2A, lane 5). Cotransfection of pD61 with p51A increased DHFR translation eightfold (Fig. 2A, lane 6) above that observed with pD61 plus p2αwt. eIF-2α translation was not dramatically affected, since the mRNA derived from the pMTVA$^-$ vector does not activate DAI kinase (21). Cotransfection of pD61 with pK3L increased DHFR translation fivefold compared with that in the control (Fig. 2A, compare lanes 5 and 7). ADA translation from p9A was also increased in the presence of K3L compared with that in the control DHFR expression vector pMTVA$^-$ (Fig. 2A, lanes 8 and 9). This increase was more clearly demonstrated by SDS-PAGE on a lower percentage gel in which the migration of ADA allowed distinction from cellular actin (Fig. 2A, lanes 10 and 11). The 10-fold increase in ADA expression was determined by quantitative immunoprecipitation of the samples and comparison of band intensities upon SDS-PAGE (Fig. 2A, lanes 14 and 15). The differences in DHFR and ADA protein synthesis did not result from differences in mRNA levels as evaluated by Northern blot analysis. Since DHFR sequences are present within all mRNAs derived from the plasmids used in these experiments, hybridization to a DHFR probe detected two plasmid-derived mRNAs in the cotransfected cells (Fig. 2B). Each mRNA species was derived from one of the two cotransfected plasmids. Northern blot analysis detected roughly similar levels of eIF-2α, ADA, or DHFR mRNA species in the cotransfection experiments. Although the DHFR mRNA in p51A- and pK3L-cotransfected cells showed a slight increase, the difference could not account for the five- to eightfold increase in DHFR translation. It was not possible to accurately quantitate K3L mRNA in this analysis since it migrated just above the DHFR mRNA. These results demonstrate that the increase in ADA and DHFR translation observed by the presence of K3L primarily resulted from increased translational efficiency.

K3L inhibits DAI kinase autophosphorylation and phosphorylation of eIF-2α. The effect of K3L expression on eIF-2α phosphorylation was studied by cotransfection of the K3L expression vector pK3L with a wild-type eIF-2α expression plasmid, pD61-2αwt. Phosphorylation of the overexpressed eIF-2α in the transfected cells was studied after 42 h by monitoring the steady-state eIF-2α level by Western analysis and eIF-2α phosphorylation by incorporation of $^{32}PO_4$. Western blot analysis showed that transfection of pD61-2αwt with the control vector pMTVA$^-$ resulted in an approximately 100-fold increase in eIF-2α steady-state levels compared with that in mock-transfected cells, as determined from a much longer exposure in which the endogenous COS-1 cell eIF-2α was detectable (Fig. 3A, lanes 1 and 2). Cotransfection with pK3L yielded a 4.5-fold increase in the steady-state level of eIF-2α (Fig. 3A, lane 3). The expression and phosphorylation of eIF-2α was also monitored in the presence of adenovirus VAI RNA contained within the expression plasmid in pD61-2αwtVA (a vector identical to pD61-2αwt but also contains the VAI and VAII genes) (Fig. 3A, lanes 4 and 5). Transfection of pD61-2αwtVA yielded a steady-state level of eIF-2α similar to that obtained by cotransfection of pD61-2αwt with pK3L (compare lanes 3 and 4). In addition, cotransfection of pD61-2αwtVA with pK3L did not yield a further increase in eIF-2α (lane 5). Analysis of [^{35}S]methionine-pulse-labeled cells demonstrated that the rate of eIF-2α synthesis was proportional to the steady-state level of eIF-2α (data not shown). This result demonstrated that adenovirus VAI RNA and K3L did not act additively to improve eIF-2α synthesis in this system. Analysis of the $^{32}PO_4$ incorporation into eIF-2α by immunoprecipitation of labeled cell extracts from

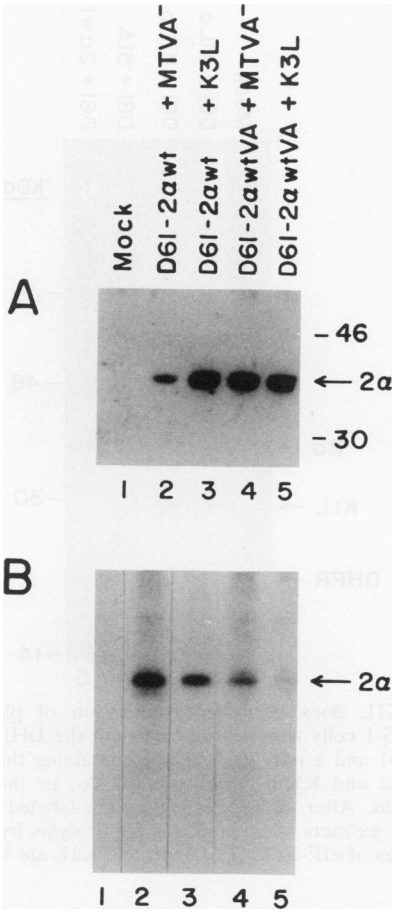

FIG. 3. Phosphorylation status of eIF-2α in the presence of K3L expression. COS-1 cells were transfected with the eIF-2α wild-type expression vector pD61-2αwt or pD61-2αwtVA in the presence of control pMTVA⁻ or pK3L. After 42 h, cell extracts were prepared for Western blot analysis to quantitate levels of eIF-2α (A). In parallel, cells were labeled with $^{32}PO_4$ and extracts were prepared for immunoprecipitation with anti-human eIF-2α as described in Materials and Methods (B). Migration of eIF-2α is indicated (2α).

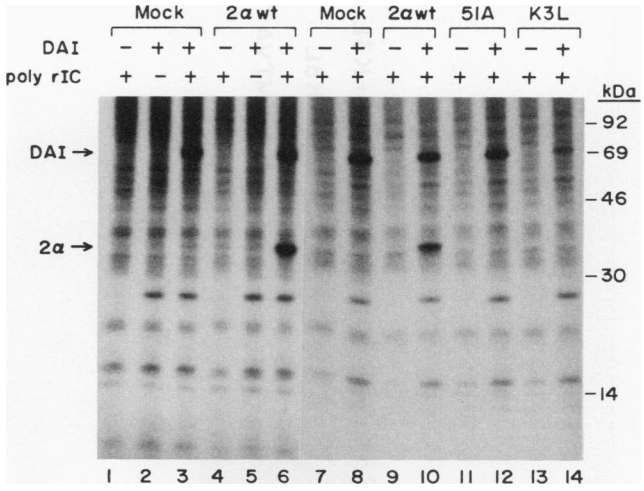

FIG. 4. K3L inhibits autophosphorylation of DAI kinase. COS-1 cells were transfected with the indicated plasmid DNAs, and cell extracts were prepared at 42 h posttransfection as described in Materials and Methods. DAI kinase and [γ-^{32}P]ATP was added to the extracts in the presence or absence of poly(rI-rC). Incorporation into DAI kinase and into eIF-2α was monitored by SDS-PAGE of an aliquot of the reaction mixture. Indicated are the migration locations of eIF-2α (2α) and DAI kinase.

control cells transfected in the absence of VAI or the K3L gene showed significant $^{32}PO_4$ incorporation into eIF-2α compared with that with mock-treated cells (Fig. 3B, lane 2). In the presence of K3L, the level of phosphorylation was reduced 1.5-fold (Fig. 3B, lane 3). Upon correction for the total level of eIF-2α in the cell to evaluate the percentage of eIF-2α phosphorylation, the expression of K3L reduced phosphorylation of eIF-2α sevenfold. Correcting for the steady-state level of eIF-2α, cotransfection with eIF-2α in the presence of VAI RNA reduced the percentage of eIF-2α phosphorylation 10-fold. Interestingly, cotransfection of eIF-2α in the presence of VAI RNA and the K3L gene resulted in a lower level of eIF-2α phosphorylation (25-fold when corrected for the steady-state level of eIF-2α; lane 5), suggesting that VAI RNA and K3L act additively to inhibit eIF-2α phosphorylation. However, this further reduction of eIF-2α phosphorylation did not result in a further increase in translation (Fig. 3A, lane 5, and data not shown).

The effect of K3L expression on autophosphorylation and activation of DAI kinase in vitro was studied by incubating extracts from transfected cells with a partially purified preparation of DAI kinase and measuring incorporation of $^{32}PO_4$ into DAI kinase. Addition of DAI kinase to cell extracts from mock-transfected cells resulted in the phosphorylation of DAI kinase only if poly(rI-rC) was included in the reaction (Fig. 4, compare lanes 2 and 3). In the absence of added DAI kinase, phosphorylation of endogenous DAI kinase was not detected, likely because of the insensitivity of this assay. Addition of DAI kinase and poly(rI-rC) to extract from cells transfected with p2αwt resulted in the phosphorylation of eIF-2α in addition to DAI kinase (Fig. 4, lanes 6 and 10), and this phosphorylation of eIF-2α was poly(rI-rC) dependent (Fig. 4, lanes 5 and 6). Addition of DAI kinase to extract from cells transfected with Ser-51→Ala mutant eIF-2α (p51A) did not result in phosphorylation of eIF-2 (Fig. 4, lane 12). Most importantly, addition of DAI kinase to extract from cells transfected with K3L reduced DAI kinase autophosphorylation (Fig. 4, lane 14). Although the reduction in DAI kinase autophosphorylation was small, it was reproducible in several independent experiments. $^{32}PO_4$ incorporation was not detected in any polypeptide migrating at 11 kDa, the position at which K3L migrates. This analysis shows that expression of K3L can directly or indirectly inhibit DAI kinase autophosphorylation and suggests that the mechanism does not involve direct phosphorylation of K3L.

Expression of K3L does not bypass inhibition mediated by eIF-2α phosphorylation. It is possible that K3L facilitates mRNA translation initiation by bypassing the requirement for functional eIF-2. We evaluated whether K3L could bypass translation inhibition mediated by eIF-2α phosphorylation by asking whether K3L expression could alleviate the translation inhibition mediated by expression of Ser-51→Asp mutant eIF-2α. Expression of the aspartic acid 51 mutant of eIF-2α inhibits protein synthesis, likely as a consequence of mimicking the charge of the phosphorylated eIF-2α (9, 21). Thus, translation of Ser-51→Asp mutant eIF-2α is significantly inhibited compared with that of wild-type eIF-2α. We asked whether expression of K3L could restore translation of the Ser-51→Asp mutant of eIF-2α.

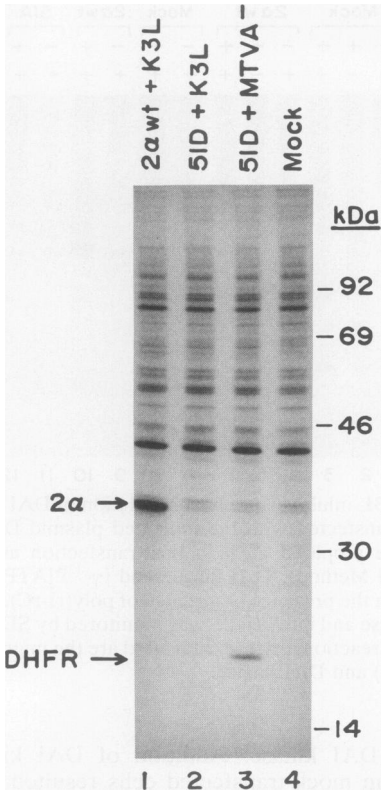

FIG. 5. K3L cannot bypass inhibition of protein synthesis mediated by Ser-51→Asp mutant eIF-2α. COS-1 cells were cotransfected with the indicated plasmid DNAs, and at 42 h posttransfection cells were labeled with [^{35}S]methionine and cell extracts were prepared for SDS-PAGE.

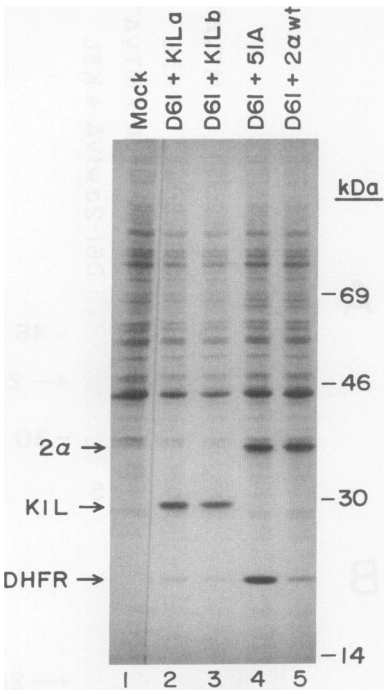

FIG. 6. K1L does not affect translation of plasmid-derived mRNA. COS-1 cells were transfected with the DHFR expression plasmid pD61 and a pMTVA$^-$ vector containing the K1L coding region (K1La and K1Lb), wild-type eIF-2α, or the Ser-51→Ala mutant eIF-2α. After 42 h, cells were pulse-labeled with [^{35}S]methionine and extracts were prepared for analysis by SDS-PAGE. The mobilities of eIF-2α (2α), DHFR, and K1L are indicated.

Cells were cotransfected with wild-type eIF-2α (p2αwt) or Ser-51→Asp eIF-2α mutant (p51D) in the presence of pK3L or pMTVA$^-$ for control. Cotransfection of p2αwt with pK3L resulted in a significant amount of eIF-2α translation (Fig. 5, lane 1). In contrast, eIF-2α synthesis was not detected upon cotransfection of p51D with vector pMTVA$^-$ (Fig. 5, lane 3). Polypeptide synthesis detected in the presence of p51D transfection in lane 3 likely resulted from cells that did not receive p51D DNA. Cotransfection with pK3L did not increase eIF-2α Ser-51→Asp expression to detectable levels (Fig. 5, lane 2). This suggests that K3L cannot bypass the translation inhibition mediated by mutant Ser-51→Asp eIF-2α expression.

Expression of K1L does not enhance translation. Vaccinia virus encodes several genes which are required for growth on human cells (30). We evaluated whether the K1L gene product could potentiate translation in the transfection assay. The coding region of K1L was isolated by polymerase chain reaction amplification and cloned into pMTVA$^-$ to derive pK1L. Two independent isolates of pK1L or control expression plasmids encoding wild-type or Ser-51→Ala eIF-2α contained within pMTVA$^-$ were cotransfected with pD61. After 42 h, cells were labeled with [^{35}S]methionine and cell extracts were prepared for SDS-PAGE. Transfection of either K1L expression vector directed the synthesis of the designated 29-kDa product, the size expected from the open reading frame. Expression of K1L (Fig. 6, lanes 2 and 3), or wild-type eIF-2α (Fig. 6, lane 5) did not enhance the translation of DHFR mRNA from the cotransfected pD61 plasmid compared with cotransfection with the Ser-51→Ala mutant eIF-2α expression plasmid (Fig. 6, lane 4). Analysis of mRNA by Northern blot analysis demonstrated that cotransfection of pK1L did not alter the level of DHFR mRNA expressed from pD61 (data not shown). Thus, in contrast to K3L, the K1L gene product did not influence the translation of DHFR in this system.

DISCUSSION

Interferon-resistant viruses have evolved mechanisms that overcome the interferon-induced cellular defense. The best-characterized mechanism for this resistance is a virus-encoded activity which inhibits the ability of the interferon-induced DAI kinase to phosphorylate eIF-2α. Phosphorylation of eIF-2α interferes with the ability of eIF-2 to recycle and promote another round of joining the 60S ribosome to the 40S-mRNA complex and thus inhibits protein synthesis at the level of initiation (19). Adenovirus inhibits DAI activation by directing expression of an adenovirus RNA polymerase III gene product (VAI) which binds DAI kinase and prevents its autophosphorylation and activation (24, 26, 28, 40). Adenovirus VAI RNA gene deletion mutants express late viral mRNAs, but both host and viral mRNA translation are inhibited as a consequence of eIF-2α phosphorylation (36). Expression of a nonphosphorylatable eIF-2α subunit can complement the growth of adenovirus VAI RNA gene deletion mutants (12). Interferon resistance of reovirus is encoded by the S4 gene which directs the synthesis of a polypeptide (sigma 3) that can bind double-stranded RNA and prevent activation of DAI kinase (19).

The activity of VAI and sigma 3 have been studied in a transfection system in which the DAI kinase inhibitory effects can be monitored by enhancing translation from plasmid-derived mRNA (2, 15, 22). Although interferon resistance in vaccinia virus was characterized early (38), the mechanism of resistance has not been fully elucidated.

Recently, an eIF-2α homolog was identified within the vaccinia virus genome (17). This prompted us to determine whether this protein, K3L, may affect eIF-2α phosphorylation under defined conditions. The results show that expression of K3L can potentiate translation of a plasmid-derived mRNA to a degree similar to that observed for expression of adenovirus VAI RNA or for expression of a nonphosphorylatable mutant of eIF-2α. The translation stimulation observed correlated with a reduced level of eIF-2α phosphorylation in the transfected cells. In addition, assay of DAI kinase activity in vitro demonstrated that extracts of K3L-transfected cells exhibit an activity that reduces the autophosphorylation of DAI kinase. Although the effect of VAI and K3L was not additive for the translation enhancement, the expression of both products was additive for the inhibition of eIF-2α phosphorylation. The K3L gene product could not bypass translation inhibition mediated by Ser-51→Asp mutant eIF-2α expression, suggesting that it does not promote ribosome binding to mRNA independent of the presence of phosphorylated eIF-2α. The K3L gene product is homologous to eIF-2α over the site of serine 51 phosphorylation, although the serine residue is not conserved (4). In the in vitro DAI kinase assay, there was no detectable phosphorylation of K3L. Thus, it does not appear that K3L acts as a substrate for DAI kinase. Immunoprecipitation of DAI kinase with specific antibody did not detect coprecipitation of K3L (data not shown), suggesting that a stable complex between K3L and DAI kinase may not exist. On the basis of its homology with the phosphorylation site of eIF-2α, we speculate that, in contrast to VAI which competes with double-stranded RNA, K3L functions by interacting with the eIF-2α-binding site on DAI kinase and inhibiting DAI kinase autophosphorylation and activation. This mechanism for stimulation of translation may be significantly different from that proposed for nonphosphorylatable mutants of eIF-2α (9). The data presented here provide a mechanism to explain the recent finding that deletion of the K3L gene from vaccinia virus reduces interferon resistance (4).

The ability of vaccinia virus to grow in interferon-treated cells and its ability to rescue other viruses which are sensitive to inhibition by interferon has been correlated with the induction of a virus-encoded factor, likely a protein that prevents activation of DAI kinase (31, 41–43). This activity has been termed SKIF for specific kinase inhibitory factor (1), although the gene has not been identified to date. The molecular mass of SKIF, approximately 25 kDa, is not consistent with it being encoded by K3L. Thus, SKIF may represent a second mechanism within vaccinia virus to ensure interferon resistance. Whereas SKIF functions by interacting with double-stranded RNA (1, 42), K3L may act through direct interaction with DAI kinase, although to date we have not been able to detect a DAI-K3L complex.

Abortive infection by vaccinia virus is characterized by a general inhibition of cellular and viral protein synthesis before the late phase of infection (11). This phenotype is similar to that observed for interferon-resistant viruses which have defects in interferon resistance. Several vaccinia virus host range genes which are required for translation of mRNA at late times after infection in specific host cells have been characterized. Vaccinia virus infection of CHO cells is blocked by the inability to translate late viral mRNAs in the late stages of the infectious cycle. A cowpox 77-kDa polypeptide alleviates this block in CHO cells (37). A vaccinia virus 29-kDa gene product, K1L, is required for efficient replication in human cells (14, 16). Because SKIF has a molecular mass of approximately 25 kDa (39), close to that encoded by the K1L vaccinia gene product, we tested whether K1L could inhibit eIF-2α phosphorylation to result in enhanced translation in our transfection system. The results show that K1L does not enhance translation and thus is likely not directly involved in mediating interferon resistance through DAI kinase inhibition.

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