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The *E3L* gene of vaccinia virus encodes an inhibitor of the interferon-induced, double-stranded RNA-dependent protein kinase

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ABSTRACT **A vaccinia virus-encoded double-stranded RNA-binding protein, p25, has been previously implicated in inhibition of the interferon-induced, double-stranded RNA-activated protein kinase. In this study, we have identified the vaccinia viral gene (WR strain) that encodes p25. Amino acid sequence analysis of a chymotryptic fragment of p25 revealed a close match to the vaccinia virus (Copenhagen strain) *E3L* gene. The WR strain *E3L* gene was cloned and expressed either in COS-1 cells or in rabbit reticulocyte lysates *in vitro*. A M_r 25,000 polypeptide that could bind to poly(rI)·poly(rC)-agarose and that reacted with p25-specific antiserum was produced in each case. In addition, COS cells expressing *E3L* gene products inhibited activation of the double-stranded RNA-activated protein kinase in extracts from interferon-treated cells. Removal of *E3L*-encoded products by adsorption with anti-p25 antiserum resulted in loss of kinase inhibitory activity. These results demonstrate that the vaccinia virus *E3L* gene encodes p25 and that the products of the *E3L* gene have kinase inhibitory activity. Comparison of the deduced amino acid sequence of the *E3L* gene products with the protein sequence data base revealed a region closely related to the human interferon-induced, double-stranded RNA-activated protein kinase.**

The type I interferons (IFNs) are a group of related proteins that are produced and secreted by mammalian cells in response to various inducers, such as double-stranded (ds)RNA (1) and viral infection (2). IFNs bind to specific receptors on cells and induce an antiviral state. Cells in the antiviral state are resistant to infection by many viruses. At least two IFN-induced enzymatic pathways are involved in establishment of the antiviral state. One of the IFN-induced enzymes is a protein kinase that can autophosphorylate a M_r 67,000 subunit of the enzyme, designated P_1. Autophosphorylation requires binding to dsRNA (3). Autophosphorylated enzyme becomes activated and can phosphorylate exogenous substrates such as eukaryotic protein synthesis initiation factor eIF-2 and histone proteins (4, 5). Phosphorylation of eIF-2 on its α subunit alters its interaction with eIF-2B, leading to inhibition of initiation of translation (6).

Recently, a number of viruses, including adenovirus, reovirus, influenza virus, and vaccinia virus have been shown to induce inhibitors of the P_1/eIF-2α kinase (7–12). For adenovirus, large quantities of adenovirus-associated RNA I (VAI) are synthesized in virally infected cells. The VAI RNA can bind to the P_1/eIF-2α kinase and cause its inactivation (13). The reovirus inhibitor is a dsRNA-binding protein, σ3 (8). This protein appears to inhibit the P_1/eIF-2α kinase by competing for activator dsRNA (8). Both adenovirus and certain strains of reovirus have been shown to be IFN resistant (14, 15). In the case of adenovirus, deletion of the gene for VAI RNA renders the virus sensitive to the antiviral effects of IFN (14). Replication of these deletion mutants can be rescued in a cell line expressing a nonphosphorylatable form of eIF-2α (16). For influenza virus, the kinase inhibitor is apparently a cellular protein, whose mechanism of action has not been well characterized (9).

Vaccinia virus-infected mouse L cells are resistant to IFN treatment (17). Vaccinia virus can also rescue IFN-sensitive viruses, such as vesicular stomatitis virus (VSV) and encephalomyocarditis virus (EMCV), from the antiviral effects of IFN (10, 18). Rescue appears to be at the level of translation of viral RNA to protein (10, 18). It has been suggested that the vaccinia virus inhibitor of the IFN-induced protein kinase may be responsible for IFN resistance of vaccinia virus and for rescue of VSV and EMCV from the antiviral effects of IFN (10, 18). The vaccinia virus kinase inhibitor has been reported to be a protein that interacts in a stoichiometric manner with dsRNA (19). We have previously characterized a M_r 25,000, vaccinia virus-encoded dsRNA-binding protein, p25, that copurifies with and appears to be necessary for kinase inhibitory activity (20). In this paper, we demonstrate that vaccinia virus open reading frame (ORF) *E3L* encodes the dsRNA-binding protein p25 as well as the immunologically related M_r 20,000 polypeptide p20. Furthermore, the results indicate that the *E3L* gene products act as inhibitors of the dsRNA-dependent P_1/eIF-2α kinase. Comparison of the deduced amino acid sequence of the *E3L* ORF with the protein data base revealed a high degree of similarity between a region of the *E3L* gene products and several dsRNA-binding proteins, including the dsRNA-dependent protein kinase (21). This shared motif may be involved in binding these proteins to dsRNA.

MATERIALS AND METHODS

Cells and Virus. Mouse L cells were grown in suspension at 37°C in suspension minimal essential medium (s-MEM) supplemented with 5% fetal calf serum and 50 μg of gentamicin sulfate per ml. HeLa cells were grown as monolayers in MEM supplemented with 5% fetal calf serum and 50 μg of gentamicin sulfate per ml. COS-1 cells were grown as monolayers in Dulbecco's MEM (DMEM) supplemented with 5% fetal calf serum and 50 μg of gentamicin sulfate per ml. Vaccinia virus (WR strain) was propagated in HeLa cells (22).

Preparation of Cytoplasmic Extracts. For preparing extracts from vaccinia-infected L cells, suspensions of cells were infected with vaccinia virus (WR strain) at a multiplicity of 10 plaque-forming units per cell. Cytosine arabinoside was added to the growth medium at a final concentration of 40

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Abbreviations: dsRNA, double-stranded RNA; eIF-2, eukaryotic protein synthesis initiation factor 2; IFN, interferon; ORF, open reading frame; DTT, dithiothreitol.

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μg/ml to increase expression of p25 (20). At 5 h postinfection, cells were pelleted by centrifugation at 1000 × g. The cell pellet was suspended in Nonidet P-40 lysis buffer [20 mM Hepes, pH 7.5/120 mM KCl/5 mM $MgCl_2$/1 mM dithiothreitol (DTT)/10% (vol/vol) glycerol/0.5% Nonidet P-40; 100 μl of Nonidet P-40 lysis buffer per 10^7 cells] and incubated on ice for 30 min. The resuspended cells were sonicated twice for 10 sec each (Bransonic 52). Nuclei and cell debris were pelleted by centrifugation at 30,000 × g for 10 min at 4°C. The cytoplasmic extracts were collected and stored at −80°C. For preparing extracts from COS cells, monolayers were washed with cold isotonic buffer (35 mM Tris·HCl, pH 6.8/146 mM NaCl/11 mM glucose) and scraped into the buffer. Cells were then pelleted by centrifugation at 1000 × g and resuspended in Nonidet P-40 lysis buffer (100 μl per 10^7 cells) and briefly sonicated. Nuclei were removed by centrifugation at 30,000 × g for 10 min at 4°C.

Poly(rI)·poly(rC)-Agarose Binding. Poly(rI)·poly(rC)-agarose was washed three times in buffer A (150 mM KCl/20 mM Hepes, pH 7.5/10% glycerol/5 mM MgOAc/1 mM DTT/1 mM benzamidine). Cytoplasmic extracts or *in vitro* translation mixtures were added to the washed poly(rI)·poly(rC)-agarose and incubated at 4°C with occasional mixing for 1 h. The agarose was then washed four times in buffer A. dsRNA-binding proteins were eluted from the agarose by adding an equal volume of 2× SDS/PAGE sample buffer and boiling for 3 min. Proteins were separated by SDS/PAGE.

N-Terminal Sequencing. Protein was digested with chymotrypsin by the Cleveland mapping method (23). Briefly, proteins were resolved by SDS/PAGE and visualized with Coomassie R-250. The p25 band was excised from the gel and equilibrated in 125 mM Tris·HCl, pH 6.8/0.1% SDS/1 mM EDTA/1 mM DTT for 1 h at room temperature. The gel piece was load into a well on a second gel. RBII (0.125 M Tris·HCl, pH 6.8/0.1% SDS/1 mM EDTA) containing 20% glycerol was loaded over the gel piece and overlaid with 2.75 μg of chymotrypsin in RBII containing 10% glycerol. Electrophoresis was performed at 30 mA until the proteins reached the stacking gel/running gel interface. Then electrophoresis was stopped for 45 min, allowing for chymotrypsin digestion. Electrophoresis was then resumed at 30 mA. Peptides were transferred to a poly(vinylidene difluoride) membrane (Bio-Rad) in 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) buffer (20 mM CAPS/0.5 mM DTT), and stained with 0.025% Coomassie R-250. The membrane was destained in 50% methanol, followed by extensive washing in water. Bound peptides were cut out and N-terminally sequenced with a Porton Instruments automated protein sequencer system.

Construction of Vectors Containing the *E3L* Gene. The *E3L* gene of vaccinia virus (WR strain) was amplified by PCR from plasmid containing the *Hin*dIII E fragment of vaccinia virus (WR strain) (kindly provided by Bernie Moss) (24). Two primers: 5′-TCGCGAATTCATGGTCTAAGATC-TATATT-3′ and 5′-AGGCCTGCAGTCAGAATCTAAT-GATTAC-3′, which correspond to 5′ and 3′ sequences of the *E3L* ORF were used to amplify the gene. The amplified *E3L* gene was cloned into the *Eco*RI and *Pst* I sites of pBS-KSII+ (Stratagene) vector. The pBluescript plasmid containing the *E3L* gene was designated pBS-E3L.

pMT2/Va− vector (kindly provided by Randy Kaufman) (25) was modified as follows: pMT2/Va− was digested with *Eco*RI and blunt ended with Klenow fragment of DNA polymerase I. Calf intestinal alkaline phosphatase was used to remove the 5′-terminal phosphate group of the modified pMT2/Va− vector. The *E3L* gene was removed from pBS-E3L by *Eco*RI and *Bam*HI digestion. The *E3L* gene was then blunt ended by DNA polymerase I Klenow fragment-catalyzed filling and subcloned into the modified pMT2/Va− vector. The pMT2/Va− vector containing the correct orientation of *E3L* gene was designated pMT-E3L.

***In Vitro* Transcription and Translation.** Five micrograms of pBS-E3L DNA was linearized with *Sac* I, and T3 RNA polymerase (Promega) was used according to the manufacturer's specifications to transcribe RNA. After completion of the transcription reaction, RNA samples were treated with DNase and extracted with phenol/chloroform and chloroform. RNA samples were then precipitated with ethanol and resuspended in 20 μl of diethylpyrocarbonate-treated water. Aliquots of RNA (10 μl) were used in *in vitro* translation reactions (Promega). The translation reaction mixture contained 35 μl of nuclease-treated rabbit reticulocyte lysate, 20 units of RNasin, 20 μM amino acid mixture minus methionine and 50 μCi of [^{35}S]methionine (1186 Ci/mmol; 1 Ci = 37 GBq). The reactions were carried out at 30°C for 1.5 h. After completion of the translation reaction, 20 μl of the reaction mixture was used for poly(rI)·poly(rC)-agarose binding or radioimmunoprecipitation.

Transfection and Radiolabeling of COS Cells. Two micrograms of pMT-E3L or pMT2/Va− plasmid were transfected into COS cells by the DEAE-dextran method with chloroquine treatment (26). Briefly, subconfluent monolayers of COS cells seeded in 60-mm tissue culture plates were rinsed with phosphate-buffered saline (PBS). DNA in 0.4 ml of PBS containing 0.3 mg of DEAE-dextran was added to the cells in a 60-mm plate and incubated at 37°C for 30 min with occasional rocking. Four milliliters of medium containing 80 μM chloroquine was then added. After 3 h at 37°C, the medium was aspirated and replaced with 2 ml of medium containing 10% dimethyl sulfoxide (DMSO). Then, 2.5 min later, medium containing DMSO was replaced with 7 ml of growth medium. The cultures were incubated at 37°C for 48 h.

To radiolabel newly synthesized proteins, medium was aspirated off the COS cells and 1.5 ml of DMEM without methionine was added. After 30 min of incubation at 37°C, the medium was removed and 150 μl of DMEM lacking nonradioactive methionine and containing 1% dialyzed fetal calf serum and 30 μCi of [^{35}S]methionine per ml was added. The cells were incubated for 30 min at 37°C and cytoplasmic extracts were prepared. Ten microliters of extract was used for each immune precipitation and poly(rI)·poly(rC)-agarose binding. Six microliters of extracts were used in kinase reactions.

Radioimmune Precipitation. Seven microliters of normal rabbit serum or p25-specific antiserum was incubated with 20 μl of [^{35}S]methionine-labeled *in vitro* translation mixture or 10 μl of extract from [^{35}S]methionine-labeled COS cell extract for 2 h on ice. Seventy microliters of washed, fixed, *Staphylococcus aureus* cells (Boehringer) was then added and incubation was continued on ice for 1 h. The *S. aureus* cells were collected by centrifugation and washed three times in RIPA buffer (10 mM Tris·HCl, pH 7.4/1% deoxycholate/1% Nonidet P-40/0.15 M NaCl/1 mM benzamidine/1 mM DTT) and once in 0.1 M Tris·HCl (pH 7.4). Bound proteins were eluted by boiling with 2× SDS/PAGE sample buffer for 3 min. Proteins were resolved by SDS/PAGE and visualized by autoradiography.

Antibody Adsorption. Thirty microliters of normal rabbit serum or anti-p25 antiserum was incubated with 150 μl of buffer A washed *S. aureus* cells for 1 h on ice. The antibody-adsorbed bacterial cells were washed twice with buffer A and then incubated for 2 h at 4°C with 15 μl of extracts from pMT-E3L transfected COS cells. The bacterial cells were removed by centrifugation and the supernatant solution was incubated with fresh antibody-adsorbed *S. aureus* cells at 4°C overnight. The bacterial cells were removed by centrifugation and 3 μl of the supernatant solutions was used in each of the kinase assay reactions.

P1/eIF-2 Kinase Inhibitory Activity. Extracts prepared from COS cells transfected with pMT-E3L or pMT2/Va− plasmid were added to the kinase reaction mixture {20 mM Hepes, pH 7.5/120 mM KCl/5 mM MgOAc/1 mM DTT/100 μM [γ-^{32}P]ATP (1 Ci/mmol)} containing extract prepared from IFN-treated, uninfected L cells (5 × 10^5 cells) as a source of kinase and the indicated concentrations of reovirus dsRNA in a final vol of 25 μl. The kinase reaction was carried out at 30°C for 15 min and was stopped by addition of an equal vol of 2× SDS/PAGE sample buffer. The reaction samples were boiled for 3 min and proteins were resolved by SDS/PAGE and visualized by autoradiography.

RESULTS

Identification of the Gene That Encodes the dsRNA-Binding Protein p25. To identify the gene that encodes p25, N-terminal sequencing of p25 was performed. Cytoplasmic extracts of vaccinia (WR strain)-infected L cells were incubated with poly(rI)·poly(rC)-agarose. Proteins bound to the matrix were eluted with SDS/PAGE sample buffer and resolved by SDS/PAGE. The proteins were then electroblotted onto a poly(vinylidene difluoride) membrane and visualized by Coomassie R-250 staining. The band corresponding to p25 was excised and subjected to N-terminal sequencing. Initial sequencing of p25 indicated that the protein was N-terminally blocked. p25 was then partially digested with chymotrypsin and the products were resolved by SDS/PAGE, blotted, and stained with Coomassie blue. One of the fragments resulting from chymotrypsin digestion was cut out from the membrane and sequenced. The N-terminal sequence of the fragment was determined to be Xaa-Ser-Asp-Asp-Ile-Pro-Thr-Arg-Trp-Phe-Met (Xaa, undetermined). This amino acid sequence was found to closely match the predicted amino acid sequence of vaccinia virus (Copenhagan strain) ORF *E3L* (27) (Fig. 1).

Expression of *E3L in Vitro* and in COS Cells. To confirm that the *E3L* ORF encodes p25, the gene was cloned by PCR and expressed *in vivo* in COS cells and *in vitro* in rabbit reticulocyte lysates. pBluescript vector was used to express *E3L* gene products in a rabbit reticulocyte lysate. The *E3L* gene of vaccinia virus (WR strain) was amplified by PCR and inserted into the multiple cloning site of pBS-KSII+. The resulting plasmid was designated pBS-E3L (Fig. 2*A*). RNA was transcribed from linearized pBS-E3L *in vitro* with bacteriophage T3 RNA polymerase and was translated *in vitro* in a rabbit reticulocyte lysate. Three primary translation products of M_r 40,000, M_r 25,000, and M_r 20,000 were detected (data not shown). Both the M_r 25,000 protein and the smaller M_r 20,000 protein bound to poly(rI)·poly(rC)-agarose (Fig. 3, lane 1). All three translation products were precipitated by anti-p25 antiserum but not by normal rabbit serum (lanes 4 and 3, respectively). No products were detected when translation reaction mixtures lacking RNA were adsorbed to poly(rI)·poly(rC)-agarose or precipitated with anti-p25 antiserum (data not shown). These results suggest that the *E3L* gene encodes the dsRNA binding protein p25, since the *in vitro* translated *E3L* gene products could react with p25-specific antiserum and functioned as dsRNA-binding proteins.

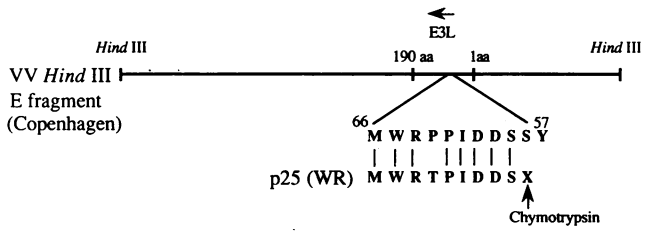

FIG. 1. Comparison of amino acid sequence of a chymotryptic fragment of p25 with the predicted amino acid sequence of vaccinia virus (vv) (Copenhagen strain) ORF *E3L*. p25 was digested with chymotrypsin as described and one of the isolated fragments was subjected to N-terminal sequencing. Amino acids (aa) are designated by standard single-letter abbreviations.

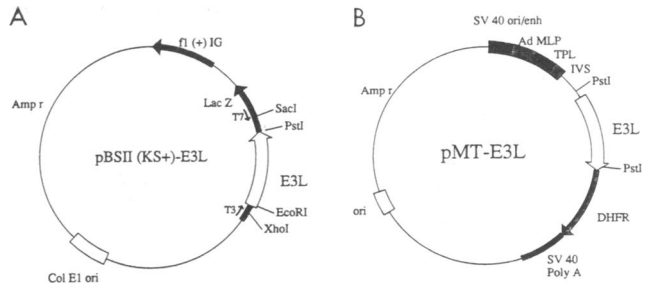

FIG. 2. Plasmid vectors containing the *E3L* gene. (*A*) The *E3L* gene of vaccinia virus (WR strain) was inserted downstream of the bacteriophage T3 promoter region of pBS-KSII+ (Stratagene) vector. (*B*) The *E3L* gene was removed from pBS-E3L and subcloned into the modified pMT2/Va− vector as described. pMT2/Va− vector was kindly provided by R. J. Kaufman (18). SV40 ori/enh, simian virus 40 origin and enhancer element; AdMLP, adenovirus major late promotor; TPL, adenovirus tripartite leader sequence; IVS, intervening sequence; DHFR, dihydrofolate reductase coding region; SV40 poly(A), SV40 early polyadenylylation signal. Amp r, ampicillin-resistance gene.

The *E3L* gene was subcloned from pBS-E3L into the eukaryotic expression vector pMT2/Va− and the resulting plasmid [pMT-E3L (Fig. 2*B*)] was transfected into COS cells. The M_r 25,000 and M_r 20,000 polypeptides were expressed at high levels in COS cells transfected with pMT-E3L (Fig. 4*A*, lane 3) but not in COS cells transfected with the parental plasmid pMT/Va− (lane 2) or in untransfected COS cells (lane 1). Both proteins could be immunoprecipitated with p25-specific antiserum (Fig. 4*B*, lane 3) and both bound to poly(rI)·poly(rC)-agarose (Fig. 4*C*, lane 3). These results confirm that the *E3L* gene encodes the dsRNA-binding protein p25.

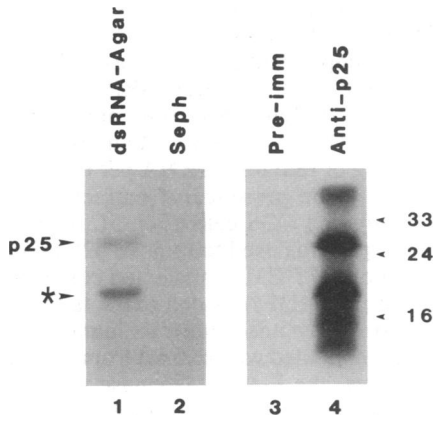

FIG. 3. *In vitro* synthesis of *E3L* gene products. Five micrograms of pBS-E3L vector DNA was linearized and T3 RNA polymerase was used to transcribe RNA. The *in vitro* transcribed pBS-E3L RNA was used to program *in vitro* translation reaction mixtures containing nuclease-treated rabbit reticulocyte lysate and [^{35}S]methionine. dsRNA-binding proteins were isolated by adsorption to and elution from poly(rI)·poly(rC)-agarose (lane 1) as described. As a control, extract was adsorbed to and eluted from underivatized Sepharose (lane 2). p25-related products were isolated by immunoprecipitation with p25-specific antiserum (lane 4) as described. As a control, immune precipitation reactions were performed with preimmune serum (lane 3). Proteins were resolved by SDS/PAGE and were detected by autoradiography. The electrophoretic mobilities of molecular weight standards are indicated on the right (×10^{-3}). p25, M_r 25,000 vaccinia virus-encoded dsRNA-binding protein. *, M_r 20,000 p25-related protein.

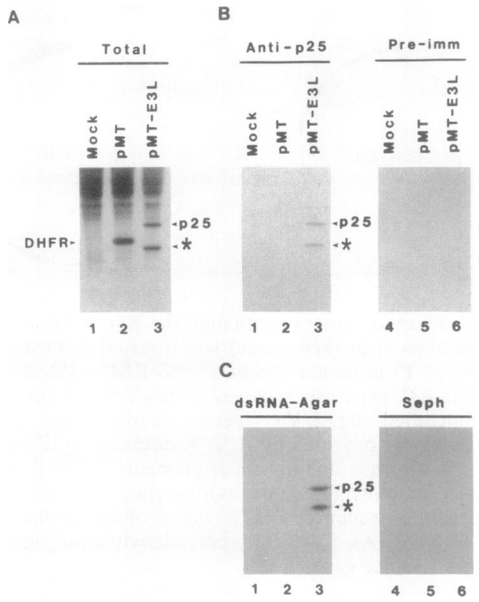

FIG. 4. Synthesis of *E3L* gene products in transfected COS cells. (*A*) COS cells were either mock transfected (lane 1) or transfected with pMT2/Va− (lane 2) or pMT-E3L (lane 3). Nonidet P-40 lysates of [^{35}S]methionine-labeled cells were prepared as described and mixed with 2× SDS/PAGE sample buffer. (*B*) Extracts from [^{35}S]methionine-labeled transfected COS cells were immunoprecipitated with p25-specific antiserum (lanes 1–3) or normal rabbit serum (lanes 4–6) as described. (*C*) Extracts from [^{35}S]methionine-labeled transfected COS cells were added to poly(rI)·poly(rC)-agarose (lanes 1–3) or to Sepharose (lanes 4–6). dsRNA binding proteins were isolated as described. Proteins were separated by SDS/PAGE and visualized by autoradiography. DHFR, dihydrofolate reductase; p25, M_r 25,000 vaccinia virus-encoded dsRNA-binding protein; *, M_r 20,000 p25-related protein.

P_1/eIF-2α Kinase Inhibitory Activity. Since p25 has been shown to be involved in inhibition of the P_1/eIF-2α kinase (20), the *E3L* gene products were tested for their kinase inhibitory activity. As shown in Fig. 5 *A* and *B*, the IFN-induced dsRNA-dependent protein kinase was activated at a concentration of 0.1 μg of reovirus dsRNA per ml in extracts prepared from COS cells that were mock transfected or transfected with pMT2/Va− (Fig. 5 *A* and B, lanes 2). On the other hand, 10 μg of reovirus dsRNA per ml was required to activate kinase in the presence of extracts prepared from pMT-E3L-transfected COS cells (Fig. 5*C*, lane 5).

Antiserum to p25 was used to remove p25-related proteins from an extract of pMT-E3L-transfected cos cells. As shown in Fig. 6, when pMT-E3L/cos cell extracts from which p25 had been removed by adsorption to immobilized anti-p25 antiserum were incubated with extract from IFN-treated cells as a kinase source, the P_1/eIF-2α kinase was activated at a dsRNA concentration of 0.1 μg/ml (lane 7). On the other hand, for COS cell extracts that had been preincubated with normal rabbit serum, 1 μg of dsRNA per ml was required to activate the kinase (lane 4). These results demonstrate that the vaccinia virus E3L gene, which encodes the dsRNA-binding protein p25, encodes an inhibitor of the dsRNA-dependent P_1/eIF-2α kinase.

DISCUSSION

In this paper, we demonstrate that the vaccinia virus (WR strain) *E3L* gene encodes both the dsRNA-binding protein p25 and specific inhibitory activity for the dsRNA-dependent protein kinase. The *E3L* gene products, when expressed in COS cells or in rabbit reticulocyte lysates *in vitro*, bound to poly(rI)·poly(rC)-agarose and reacted with p25-specific antiserum. Furthermore, when expressed in COS cells, the *E3L* gene products acted as an inhibitor of the P_1/eIF-2α kinase. These results demonstrate that the products of the *E3L* gene are necessary and sufficient for potent inhibitory activity for the dsRNA-dependent protein kinase.

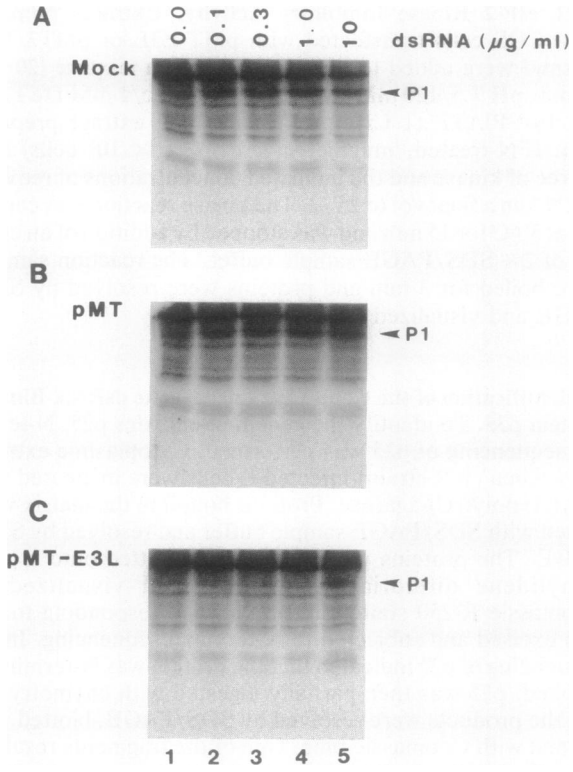

FIG. 5. Inhibition of P_1/eIF-2α kinase activity by extracts from pMT-E3L transfected COS cells. COS cells were either mock transfected (*A*) or transfected with pMT2/Va− (*B*) or pMT-E3L (*C*). Nonidet P-40 extracts of transfected COS cells were prepared as described and mixed with extracts from IFN-treated L cells as a source of kinase. The assay for kinase activity was performed as described. Phosphorylated proteins were separated by SDS/PAGE and visualized by autoradiography. The kinase reaction mixtures contained the following concentrations of reovirus dsRNA: lanes 1, no dsRNA; lanes 2, 0.1 μg/ml; lanes 3, 0.3 μg/ml; lanes 4, 1.0 μg/ml; lanes 5, 10 μg/ml. P_1, M_r 67,000 subunit of dsRNA-dependent protein kinase.

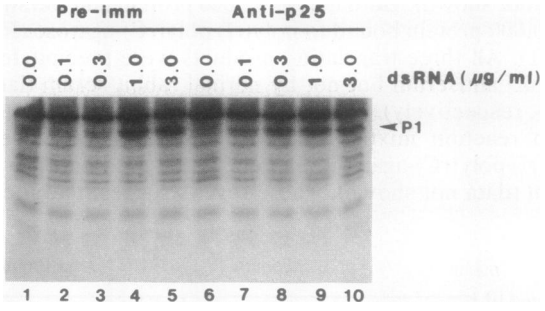

FIG. 6. Adsorption of extracts from pMT-E3L-transfected COS cells with immobilized p25-specific antiserum removes kinase inhibitory activity. Nonidet P-40 extracts from pMT-E3L-transfected COS cells were incubated with *S. aureus* cells that had been preincubated with either anti-p25 antiserum (lanes 6–10) or normal rabbit serum (lanes 1–5). Adsorbed proteins were removed by centrifugation and the supernatant solutions were used in kinase assays as described. The kinase reaction mixture contained the following concentrations of reovirus dsRNA: lanes 1 and 6, no dsRNA; lanes 2 and 7, 0.1 μg/ml; lanes 3 and 8, 0.3 μg/ml; lanes 4 and 9, 1.0 μg/ml; lanes 5 and 10, 3.0 μg/ml. P_1, M_r 67,000 subunit of dsRNA-dependent protein kinase.

The mechanism by which the *E3L* gene products prevent activation of the P_1/eIF-2α kinase is probably similar to the mechanism used by the reovirus σ3 protein and histone proteins (5, 8), both dsRNA-binding proteins. Histones and σ3 protein bind to the available dsRNA, preventing it from binding to and activating the kinase. Both function in a stoichiometric manner (ref. 5; F. Imani, J. O. Langland, and B.L.J., unpublished data). The vaccinia virus inhibitor of the dsRNA-dependent kinase has been suggested to interact in a stoichiometric manner with activator (19), consistent with the dsRNA-binding properties of the *E3L* gene products.

Two E3L-encoded polypeptides of M_r 25,000 and M_r 20,000 were detected in extracts of COS cells transfected with pMT-E3L. A third polypeptide of M_r 40,000 was detected in reticulocyte lysates programmed with E3L RNA transcribed *in vitro* from pBS-E3L. The M_r 40,000 polypeptide was not detected in reticulocyte lysates programmed with RNA transcribed from vaccinia virus cores (20), suggesting that this polypeptide is an artifact of transcribing *in vitro* in the bacterial system. The M_r 25,000 polypeptide is presumably the translation product of the entire *E3L* ORF. The M_r 20,000 polypeptide appears to be due to alternative initiation of translation (H. Yuwen and B. Moss, personal communication). Minor amounts of a similarly sized p25 related polypeptide have been detected in vaccinia virus-infected cells (20). Amounts similar to those reported in this paper were detected in reticulocyte lysates programmed with RNA transcribed *in vitro* from vaccinia virus cores (20). Since only minor and variable amounts of p20 were detected in extracts of vaccinia virus-infected cells that had potent kinase inhibitory activity, we believe that p25 is the major physiologically relevant product of *E3L*.

The role of p25 in the vaccinia virus life cycle remains unclear. Replication of vaccinia virus is resistant to IFN treatment in various cell lines (17). Vaccinia virus produces several inhibitors of the IFN-induced enzymes that are involved in the IFN-induced antiviral state. An ATPase and a phosphatase produced during vaccinia infection have been reported to degrade ATP and dephosphorylate 2′,5′-adenosine, thereby blocking the 2′,5′-adenosine synthetase pathway (28). In addition, it also has been reported that the *ts22* gene product of vaccinia virus prevents accumulation of 2′,5′-adenosine at permissive temperature (29). The other IFN-induced enzyme pathway, the P_1/eIF-2α kinase pathway, is inhibited by the *E3L* gene product p25, as described previously (20) and in this paper. The products of the vaccinia virus *K3L* gene also appear to have kinase inhibitory activity (32). The *K3L* gene products share homologous sequences with eIF-2α (30). Deletion mutants of *K3L* are IFN sensitive, suggesting that this gene is necessary for IFN resistance (30).

Comparison of the deduced amino acid sequence for ORF *E3L* to the protein sequence data base revealed a region near the C terminus of p25 (amino acids 139–182) with close homology to a region in the amino quarter of the dsRNA-dependent kinase (amino acids 32–75; Fig. 7). These regions shared 25 of 31 residues, either identical or highly conserved. A basic subregion also showed similarity to histone proteins and RNase III (amino acids 164–177; data not shown) (31). Since all four proteins bind to dsRNA (4, 5, 20, 31), this region may be involved in binding the ORF *E3L* gene products to dsRNA. Mutagenesis of the gene for the human dsRNA-dependent kinase suggests that the ability to bind to dsRNA maps to the N-terminal region of the enzyme, including the region of homology with *E3L* (S. J. McCormack, D. C. Thomis, and C. E. Samuel, personal communication).

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 32  NSGPPHDRRFTFQVIIDGREFPEGEGRSKKEAKNAAAKLAVEIL  75   p68
      ..    .  . .... .   . ..  ... ......  .
      .. .  .  . .... .  ..........  ......  .
139  SVGPSNSPTFYACVDIDGRVFDKADGKSKRDAKNNAAKLAVDKL  182  p25
```

FIG. 7. Comparison of predicted amino acid sequence of IFN-induced, dsRNA-activated P_1/eIF-2α kinase with predicted amino acid sequence of vaccinia virus (Copenhagen strain) ORF *E3L*. Residues 32–75 of the IFN-induced, dsRNA-activated P_1/eIF-2α kinase (p68) are compared with residues 139–182 of the *E3L* ORF (p25). Identical amino acids are denoted by double dots; conserved amino acids are denoted by single dots. Amino acids are designated by standard single-letter abbreviations.

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