Regulation of IL-2 Gene Expression and Nuclear Factor-90 Translocation in Vaccinia Virus-Infected Cells

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ABSTRACT

Nuclear factor-90 (NF-90) has been described as a regulatory subunit of a complex containing DNA-dependent protein kinase (DNA-PK), Ku, and NF-45, which are capable of binding the interleukin-2 (IL-2) enhancer region and stimulating IL-2 gene expression. Vaccinia virus (VV) infection of Jurkat cells induced a nuclear factor that bound specifically to the IL-2 promoter sequence and led to the expression of the IL-2 transcript. Induction of this IL-2 promoter binding factor occurred concomitantly with the induction of NF-90 and translocation of NF-90 to the nucleus. Electrophoretic mobility supershift analysis using specific anti-NF-90 serum suggested the presence of NF-90 in the IL-2 promoter binding complex. As NF-90 can bind to double-stranded RNA (dsRNA) and be phosphorylated by the dsRNA-dependent protein kinase, PKR, we investigated whether accumulation of dsRNA in VV-infected cells could regulate IL-2 gene expression. Infection of Jurkat cells with a VV mutant that produces free dsRNA led to similar levels of induced NF-90 within the cell, but the protein remained localized within the cytosol. This mutant did not lead to the accumulation of an IL-2 promoter binding complex or to the synthesis of IL-2 mRNA. Other VV mutants that produced excess dsRNA also inhibited protein binding to the IL-2 enhancer, suggesting that the presence of viral dsRNA has a role in retaining NF-90 in the cytosol and regulating IL-2 gene expression.

INTRODUCTION

INTERLEUKIN-2 (IL-2) IS THOUGHT TO PLAY A KEY ROLE IN T cell induction and proliferation.4,5 In activated T cells, transcriptional regulation of IL-2 involves the binding of specific transcription factors to the purine-rich enhancer element designated the antigen receptor response element-2 (ARRE-2).3 In vitro studies have demonstrated an antiviral role for many cytokines, including IL-2. Athymic mice, which lack mature functional T cells and typically die after infection with wild-type vaccinia virus (wtVV), rapidly resolve an infection with VV expressing IL-2.4,5 Many viruses or their gene products have been shown to have dramatic effects on cellular functions, including cytokine production.5

It has been suggested that nuclear factor-90 of activated T cells (NF-90) participates in numerous cellular functions, including cell cycle regulation, transcriptional activation, and translational control.6–8 Previous work suggests that cytoplasmic NF-90 translocates to the nucleus on T cell stimulation. Once in the nucleus, NF-90 combines with other nuclear components to form an ARRE-2 DNA-binding complex.9 This complex is thought to include NF-90, NF-45, the Ku autoantigen heterodimer (Ku80 and Ku70), and the large catalytic subunit of DNA-dependent protein kinase (DNA-PK).6 In stimulated human bronchial epithelial cells, this complex was found associated with an ARRE-2-like DNA sequence and subsequently led to the activation of IL-2 transcription.6,9 NF-90 may function as both a positive and a negative regulator of gene expression depending on the promoter context and interaction with NF-45.10 Binding of NF-90 to the ARRE-2 sequence has been suggested to occur through Ku, as a DNA-binding domain in NF-90 has not been identified.6,9

NF-90 appears to act similarly to a family of cytoplasmic ARRE-2 regulatory factors, including nuclear factor of activated T cells (NFATc1–NFATc4). The NFATc family of proteins remain cytoplasmic until dephosphorylated by calcineurin, a calmodulin-dependent phosphatase.11,12 On dephosphorylation, these proteins can translocate to the nucleus and bind directly to

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The cellular protein, PKR, which also contains this dsRNA-binding motif, functions in recognizing dsRNA produced during a viral infection. The ARRE-2 enhancer element and induce transcription. NFATc1 is required for the development of T cells and has been shown to contribute to the production of IL-2 and the development of Th2 responses. Translocation of NFATc1 can be inhibited by the addition of cyclosporin A, which is thought to act by inhibiting the activity of calcineurin. The role of NF-90 binding to dsRNA remains unknown. Binding to dsRNA appears to be specific in that this domain cannot bind to ssRNA or DNA. Recent evidence suggests that NF-90 is involved in the induction of the cellular antiviral interferon (IFN) response by transcriptional upregulation of several IFN response genes. Translocation of NF-90 from the cytoplasm to the nucleus is also inhibited by the addition of cyclosporin A, suggesting that the phosphorylation state of NF-90 may alter translocation. The functional domains of NF-90 include a motif previously characterized to bind dsRNA. This motif is conserved in many dsRNA-binding proteins, including the VV E3L protein. The ability of the VV E3L protein to bind dsRNA appears to aid the virus in evading the antiviral host defenses. The cellular protein, PKR, which also contains this dsRNA-binding motif, functions in recognizing dsRNA produced during a viral infection. On binding to dsRNA, PKR undergoes an autophosphorylation event leading to activation of the enzyme. Activated PKR proceeds to phosphorylate various substrates, including the translation initiation factor, eIF2α. The phosphorylation of eIF2α leads to a subsequent inhibition in initiation of translation. The VV E3L protein functions to bind to and sequester dsRNA produced during the infection, thereby blocking PKR recognition of the dsRNA activator. The role of NF-90 binding to dsRNA remains unknown. Binding to dsRNA appears to be specific in that this domain cannot bind to ssRNA or DNA. Recent evidence suggests that NF-90 is involved in the induction of the cellular antiviral interferon (IFN) response by transcriptional upregulation of several IFN response genes. Several homologs of NF-90 have been discovered, including the Xenopus CBTF protein, the mouse Spnr protein, and the human TCP80 and MPP4 proteins. The TCP80 protein has been shown to interact with β-glucosidase mRNA and inhibit translation. The MPP4 protein is a phosphoprotein induced during M-phase. The CBTF protein is a CCAAT box transcription factor that on translocation to the nucleus, activates expression of the hematopoietic regulatory factor, GATA-2. Evidence suggests that CBTF is negatively regulated and retained in the cytoplasm by binding RNA in an mRNP complex.

VV encodes a multitude of proteins that regulate the activity of various cytokines. Persistent infection of Jurkat cells has been shown to lead to the stimulation of lymphokine production. During an acute infection by VV, no increase in IL-2 activity could be detected, but after the establishment of a persistent infection, a dramatic increase in the synthesis of IL-2 and IL-6, but not IFN-γ, was observed. Furthermore, treatment of BSC-40 cells with cyclosporin A inhibits the replication of VV by an unknown mechanism. These results suggest that VV infection interplays with the IL-2 regulatory pathway and that certain components of this pathway may be required for VV replication.

In this study, we investigated VV regulation of ARRE-2 DNA-binding activity and alterations in NF-90 subcellular distribution during viral infection. VV infection of Jurkat cells led to an increase in nuclear factors with an affinity for the ARRE-2 DNA sequence. The presence of ARRE-2 DNA-binding nuclear factors coincided with the presence of NF-90 protein in the nucleus, and supershift analysis suggests the presence of NF-90 in the DNA-binding complex. Subsequently, during the VV infection, IL-2 mRNA levels were significantly increased. Infection of Jurkat cells with a VV mutant deleted of the E3L (VVΔE3L) gene did not lead to the formation of a nuclear ARRE-2 DNA-binding complex. Infection with VVΔE3L did lead to increased levels of NF-90 protein, although the protein remained in the cytoplasm. Infection of cells with VVΔE3L leads to the synthesis of excess free dsRNA. Infection of Jurkat cells with other VV mutants that alter the level of free dsRNA present in a cell demonstrated a correlation between the presence of dsRNA in the cytoplasm and a decrease in ARRE-2 DNA-binding factors present in the nucleus. These results suggest that NF-90 levels and subcellular localization are altered during infection with VV, and these alterations in the properties of NF-90 likely affect IL-2 gene expression.

MATERIALS AND METHODS

Cell lines and viruses

Jurkat cells were cultured in RPMI 1640 medium supplemented with 50 μg/ml gentamicin sulfate and 10% fetal bovine serum (FBS) (Hyclone, Ogden, UT). Vaccinia virus VC-2 (Copenhagen strain), designated in this paper as wtVV, was the parent strain for all viruses used in this study. VV constructs included virus deleted of the E3L gene (VVΔE3L), virus containing a deletion of the 7 C-terminal amino acids of E3L (VV(E3LΔ7C), and ts23 (a construct that produces excess dsRNA at the nonpermissive temperature). For all infections, 4 × 10⁶ cells were infected at an moi of 5. Prior to infection, the cells were pelleted, the supernatant was removed, and the cells were infected with virus in a total volume of 100 μl. After 1 h, fresh medium was added to the cell suspension and, unless otherwise indicated, incubated at 37°C until 6 h postinfection. As a control, cells were treated with 20 ng/ml phorbol myristate acetate (PMA) (Sigma, St. Louis, MO) and 2 μM ionomycin (Sigma) for 4 h prior to harvest. Cyclosporin A (Sigma) was used at 1 μg/ml 10 min prior to treatment or infection. Cytosine arabinoside (araC) (Sigma) treatment at a concentration of 40 μg/ml was begun 1 h prior to infection and maintained until cells were harvested.

Extract preparation

Nuclear extracts were prepared as described by Park et al. Briefly, pelleted cells were lysed in 50 μl lysis buffer (10 mM Tris, pH 7.4, 10 mM NaCl, 3 mM MgCl₂, 0.5 mM dithiothreitol, 2.5 mM EGTA, 5 μg aprotinin/ml, 5 μg antipain/ml, 100 μM benzamidine, 5 μg leupeptin/ml, 5 μg pepstatin/ml, 5 μg soybean trypsin inhibitor/ml, 0.05% Nonidet P-40—all reagents from Sigma) by incubation for 10 min at 4°C. Nuclear proteins were pelleted at 300g for 10 min and washed once in the same buffer. The supernatants were removed and centrifuged at 10,000g for 10 min, and the resulting supernatant was stored at −80°C (cytoplastic extract). The nuclear pellets were lysed in 50 μl nuclear lysis buffer (0.42 M NaCl, 1.5 mM MgCl₂, 20 mM HEPES, pH 7.5, 25% glycerol, 0.2 mM EDTA, 0.5 dithiothreitol, and the above protease inhibitors) by incubation for 30 min at 4°C, with occasional shaking. Nuclear debris was pe-
leted at 10,000g for 10 min, and the supernatant was dialyzed (in 20 mM HEPES, pH 7.5, 100 mM KCl, 0.2 mM EDTA, 20% glycerol, 1 mM PMSF, 0.5 mM dithiothreitol) and stored at −80°C (nuclear extract).

**Electrophoretic mobility shift assay (EMSA)**

EMSA assays were done according to Kao et al. EMSA reactions (25 μl volume) included 25 mM HEPES, pH 7.5, 0.1 mM EDTA, 10% glycerol, 50 mM KCl, 0.05 mM dithiothreitol, 1 ng labeled probe, 1 μg poly dI:dC, and 10 μg nuclear extract. Reactions were incubated at 25°C for 40 min, and DNA-protein complexes were separated on a 4% nondenaturing polyacrylamide gel and analyzed by autoradiography. Oligonucleotide probes were end-labeled using polynucleotide kinase and γ32P-ATP. The wtARRE-2 oligonucleotide had the sequence 5′-gatcGGAGGAAAAACTGTTTCATACAGAAGGCGT-3′ (corresponding to positions −255 to −285 of the human IL-2 enhancer). Mutant oligonucleotides were as follows: mutant 1: 5′-gatcGGAGACCTAACTTTTAATACAGAAGGCGT-3′; mutant 2: 5′-gatcGGAGACCTAACTGTGTTTCATACAGAAGGCGT-3′. Mutant 1 disrupts both the characterized NFATc binding motif and a weak AP-1 binding site. Mutant 2 was designed to disrupt only the NFATc binding site. Supershift analysis was performed by adding 1 μl specific NF-90 antiserum to the EMSA reaction prior to separation on the nondenaturing polyacrylamide gel.

**Western blot analysis**

Western blot analysis was performed as previously described using proteins separated by SDS-PAGE and transferred to nitrocellulose using CAPS transfer buffer (20 mM CAPS, pH 11.0, 20% [v/v] methanol). Nonspecific sites were blocked with BLOTTO, and the membrane was incubated overnight at 4°C with anti-NF-90 antiserum (provided by Peter Kao). After thorough washing, the membrane was incubated with secondary goat antirabbit IgG horseradish peroxidase (HRP) conjugate (Sigma), washed again, and developed using 0.1 mg 4-iodophenol per ml, 0.4 mg sodium luminal per ml, and 0.005% (v/v) H2O2. Immune complexes were visualized using autoradiography.

**IL-2 mRNA levels**

Quantification of IL-2 mRNA was done following the procedure described by Fan et al. Total RNA was isolated fol-
lowing guanidinium-isothiocyanate lysis of cells. The RNA was converted to cDNA using oligo dT primers, MMLV reverse transcriptase (RT) (GIBCO-BRL, Gaithersburg, MD) and 1 mM dNTP and incubation at 42°C for 15 min and 99°C for 5 min. The resulting cDNA was amplified in a PCR reaction containing 10 mM Tris, 2 mM MgCl\textsubscript{2}, 0.2 mM dNTP, 0.2 μM 5' and 3' oligonucleotide primers, and 1 U Taq DNA polymerase for 35 cycles of denaturation at 95°C for 1 min and annealing-extension at 60°C for 1 min. PCR products were analyzed by electrophoresis on a 1.8% TBE-agarose gel and visualized by staining with ethidium bromide. The sequences of the IL-2 primers were 5' - GAAATGGAATTAATATACGATCC - 3' and 5' - GTTGTTCACACCGCTTTGATTC - 3'. Control primers for β-actin were 5' - ATGGATGATGATATCGCCGCC - 3' and 5' - CTAGAAGCATTGGCCGATGATGGGGGCC - 3'.

IL-2 protein levels

Measurements of IL-2 protein synthesis and secretion were performed by ELISA using cell culture supernatants. Experimental procedures were performed according to the manufacturer’s protocols (Cytimmune Sciences, College Park, MD). Culture supernatants from infected cells were harvested at 0, 24, 48, and 72 h postinfection/treatment. Control cells were treated with PMA and ionomycin as described.

RESULTS

Previous studies have demonstrated a role of NF-90 in binding to the purine-rich ARRE-2 enhancer element found on the IL-2 gene.\(^{(9)}\) Binding to the ARRE-2 element does not involve direct DNA interaction by NF-90 but instead requires complex formation among NF-90, NF-45, DNA-PK, and Ku.\(^{(6)}\) This complex can then bind the enhancer element and induce IL-2 transcription. On T cell stimulation or treatment with PMA and ionomycin, NF-90 translocates from the cytoplasm to the nucleus, allowing complex formation to occur. NF-90 contains various motifs, including two that mediate binding to dsRNA, a bipartite nuclear localization signal, several epitopes with potential M-phase specific phosphorylation or phosphorylation by cyclin-dependent kinases, and an arginine and glutamine rich region identified as an RG2 motif found in many RNA binding proteins.\(^{(8,9,16)}\) The role of these motifs in regulating NF-90 activity has not been well studied. Several mutants in the VV system accumulate different levels of free dsRNA during infection,\(^{(28,29)}\) allowing the role of dsRNA in the function of NF-90 to be investigated.

As shown in Figure 1, treatment of cells with PMA-ionomycin led to the formation of a DNA complex not present in mock-treated cells (lanes B and A, respectively). Infection of Jurkat cells with wtVV led to the formation of a similar DNA complex with the same mobility shift as that observed with PMA/ionomycin (Fig. 1, lane C). A shifted band with a faster mobility was observed in all cellular nuclear extracts, including mock-infected extracts (Fig. 1). This nonspecific band has been observed by other investigators.\(^{(9,33,34)}\)

In order to test the specificity of ARRE-2 DNA complex formation observed during wtVV infection, competition EMSA was performed in the presence of excess wt and mutant probe. Two mutant probes were added to the EMSA reaction at a 5-fold excess over the radiolabeled wt probe. The sequence of these probes is described in Materials and Methods. As shown in Figure 2, excess unlabeled wt probe significantly competed binding of the VV-inducible complex to the labeled probe at a 5-fold excess and eliminated complex formation at a 50-fold excess (lanes A, B, and C). Unlabeled DNA did not effectively compete for the formation of the constitutive complex (Fig. 2, compare lanes A and B). Both mutant probes were unable to significantly reduce complex formation to the labeled probe even at a 50-fold excess (Fig. 2, lanes D, E, and F, G).

The VV E3L protein binds to dsRNA synthesized by the virus during replication. Binding of E3L to dsRNA sequesters and masks the dsRNA, preventing recognition of the dsRNA by host defense proteins, including PKR.\(^{(19,28)}\) To investigate how dsRNA may alter ARRE-2 DNA complex formation, a VV mutant deleted of the E3L gene (VVΔE3L) was used. As shown in Figure 1, infection of Jurkat cells with VVΔE3L inhibited formation of the ARRE-2 DNA complex observed with wtVV (compare lane D to lane C). These results suggest that free dsRNA present in the cytoplasm inhibited the formation of the ARRE-2 DNA complex within the nucleus.

To determine when the ARRE-2 DNA complex formation occurred during VV infection, nuclear extracts were prepared at 3 and 6 h postinfection and assayed by EMSA. Figure 3 shows that as early as 6 h after viral infection, the shifted ARRE-2 complex could be detected (lanes B and C). Again no complex formation could be detected in cells infected with VVΔE3L (Fig. 3, lanes D and E).

Several nuclear factors have been described as ARRE-2 binding elements. If NF-90 was the factor responsible for ARRE-2 binding activity observed during wtVV infection, NF-90 should have been present in the nucleus at the times during infection when ARRE-2 binding activity was detected. Previous research suggests that the presence of certain cytoplasmic RNAs leads to localization and retention of NF-90 in the cytoplasm.\(^{(7,24,35)}\) As dsRNA synthesized during a VV infection is localized to the cytoplasm and as NF-90 contains two dsRNA-binding motifs, the absence of AARE-2 binding activity observed during infection by VVΔE3L could be explained by dsRNA retention of NF-90 in the cytoplasm. If this were the case, NF-90 should remain localized to the cytoplasm during a VVΔE3L infection. To test for these possibilities, Western blot analysis was performed on nuclear and cytoplasmic extracts from VV-infected Jurkat cells. As shown in Figure 4, the appearance of NF-90 in the nucleus during a wtVV infection coincides with the ARRE-2 DNA binding activity observed in Figure 3 (lanes A and B). Previous reports state that NF-90 is constitutively produced in the cytoplasm.\(^{(9,15)}\) However, NF-90 is could not be detected at either 3 or 6 h in the cytoplasm of wtVV-infected Jurkat cells (Fig. 4, lanes E and F). These results suggest that VV infection leads to an increased level of NF-90, which then translocates and accumulates in the nucleus. During infection with VVΔE3L, NF-90 could not be detected in the nucleus (Fig. 4, lanes C and D). This also is in agreement with the lack of ARRE-2 DNA binding activity observed in Figure 3. Infection with VVΔE3L led to a similar induction in NF-90 levels as wtVV, but the protein accumulated in the cytoplasm of the infected cell (Fig. 4, lanes G and H). This supports the concept that the presence of free dsRNA blocks translocation of NF-90 from the cytoplasm to the nucleus.
In order to determine if nuclear NF-90 induced during wtVV infection was involved in the formation of the ARRE-2 DNA binding complex observed, EMSA supershift analysis was performed using specific anti-NF-90 serum. ARRE-2 DNA binding reactions were incubated with anti-NF-90 serum, followed by separation of the complex on a nondenaturing polyacrylamide gel. As shown in Figure 5, the ARRE-2 DNA complex induced using wtVV extracts was supershifted by the addition of anti-NF-90 serum.

### FIG. 3. Time course of vaccinia virus induction of an ARRE-2 DNA binding complex. Jurkat cells were mock treated (lane A), infected with wtVV (lanes B and C), or infected with VVΔE3L (lanes D and E). Nuclear extracts were prepared at 3 h postinfection (lanes B and D) or 6 h postinfection (lanes A, C, and E). Extracts were assayed by EMSA using a radiolabeled ARRE-2 DNA probe. Protein-DNA complexes were separated by gel electrophoresis and visualized by autoradiography. Inducible ARRE-2 complex formation is indicated by the arrow.

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### FIG. 4. Vaccinia virus induction of an ARRE-2 DNA binding complex occurred concomitantly with NF-90 presence in the nucleus. Jurkat cells were infected with wtVV (lanes A, B, E, and F) or with VVΔE3L (lanes C, D, G, and H). Nuclear (lanes A, B, C, D) and cytoplasmic (lanes E, F, G, H) extracts were prepared at 3 h postinfection (lanes A, C, E, and G) or 6 h postinfection (lanes B, D, F, and H). Proteins were separated by SDS-PAGE and assayed by Western blot analysis using anti-NF-90 antiserum. Proteins corresponding to NF-90 are indicated.

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of anti-NF-90 antiserum (compare lanes B and D). Addition of NF-90 antiserum to mock-infected extracts did not alter the mobility of the lower shifted band, further supporting the nonspecific nature of this complex (Fig. 5, lanes A and C). These results further support the presence of NF-90 in wtVV nuclear extracts and that NF-90 was part of the protein complex bound to the ARRE-2 DNA probe.

To further confirm the role of dsRNA in inhibiting ARRE-2 DNA complex formation, other VV mutants were used ts23 is a VV mutant that produces excess dsRNA at the restrictive temperature of 39°C. Infection with ts23 at the permissive temperature of 31°C produces an infection similar to wtVV infection. When Jurkat cells infected with this virus were assayed by EMSA as in the previous assays, ARRE-2 DNA complex formation was observed only at the permissive temperature when excess dsRNA was not present (Fig. 6, lane C). This shift was similar to that observed with PMA/ionomycin treatment or during infection with wtVV (Fig. 6, lanes B and E, respectively). Infection with ts23 at the restrictive temperature did not result in the presence of a similar shifted product (Fig. 6, lane D).

Another mutant of VV, VVE3LΔ7C, contains a 7-amino acid deletion from the C-terminus of the E3L gene product. E3LΔ7C protein is still able to bind dsRNA, but with an affinity 100-fold lower than full-length E3L. Jurkat cells infected with this virus still produced a shifted product on EMSA, but at a 5-fold reduction compared with wtVV (Fig. 6, compare lanes G and E). These data fit with a model in which the smaller amount of free dsRNA produced by VVΔE3L compared with VVΔÆ3L partially inhibits NF-90 translocation to the nucleus.

AraC is an effective inhibitor of poxvirus replication. Treatment of VV-infected cells with araC blocks viral DNA replication as well as transcription at late times postinfection. Because dsRNA present during VV infection requires mRNA formed during late transcription, treatment of VV-infected cells with araC blocks the formation of dsRNA. Treatment of VV-infected cells with araC inhibited the ability of nuclear extracts to form an ARRE-2 DNA complex (Fig. 6, lane H). These results suggest that the ARRE-2 binding factor induced during VV infection required progression of the viral life cycle through DNA replication and likely late transcription. As our previous data support a role of NF-90 in the ARRE-2 DNA complex, the level of NF-90 present in the nucleus might be expected to be reduced on araC treatment of VV-infected cells. As shown in Figure 7, the amount of NF-90 present in the nucleus of araC-treated, VV-infected cells was significantly reduced as compared with wtVV-infected, untreated cells (compare lanes C and B). Again, these results support a role of NF-90 in the formation of the ARRE-2 DNA complex after wtVV infection.

Our data, along with that of others, indicate that PMA/ionomycin treatment of cells leads to a strong induction of ARRE-2 DNA binding activity. Because VVΔÆ3L does not lead to induction of ARRE-2 DNA binding activity, experiments were performed to determine if infection with VVΔÆ3L would block PMA/ionomycin activity. As shown in Figure 8B, PMA/ionomycin treatment of Jurkat cells induced nuclear ARRE-2 DNA binding activity (lane B). Infection of cells with VVΔÆ3L inhibited the activity of PMA/ionomycin treatment where no ARRE-2 DNA complex formation could be detected (Fig. 8B, lane C). This suggests that free dsRNA can block PMA/ionomycin as well as viral induction of an ARRE-2 DNA binding complex. Both treatment of Jurkat cells with PMA/ionomycin and infection with wtVV caused increased levels of NF-90 in the nucleus (Fig. 8A, compare lane A with lanes B and C). Infection with VVΔÆ3L did not increase nuclear NF-90 levels, and infection with VVΔÆ3L inhibited the induction of nuclear NF-90 levels observed on PMA/ionomycin treatment (Fig. 8A, lanes D and E, respectively). These results are in agreement with VVΔÆ3Ls inhibiting the PMA/ionomycin ARRE-2 DNA complex by blocking NF-90 translocation to the nucleus.

With wtVV infection of Jurkat cells, the presence of an ARRE-2 DNA binding complex in the nucleus would suggest that IL-2 transcription should be present. To test for this, RNA was isolated from cells infected with wtVV, and the level of IL-2 mRNA was determined by RT-PCR. As shown in Figure 9, IL-2 transcription was significantly higher in cells infected with wtVV compared with mock-treated cells (compare lanes

**FIG. 5.** Supershift analysis of the vaccinia virus-induced ARRE-2 DNA complex. Jurkat cells were mock treated (lanes A and C) or infected with wtVV (lanes B and D). Nuclear extracts were prepared and assayed by EMSA using a radiolabeled ARRE-2 DNA probe. Prior to separation on a nondenaturating gel, specific anti-NF-90 serum was added to the EMSA binding reaction (lanes C and D). Protein-DNA complexes were separated by gel electrophoresis and visualized by autoradiography. Inducible ARRE-2 complex formation is indicated by the arrow. A supershifted complex.
scription, this result was somewhat unexpected. However, as wtVV is known to inhibit host protein synthesis soon after infection, the lack of IL-2 protein synthesis may be due to this block in host translation.

In fact, we detected, concomitant with early viral protein synthesis, an inhibition in host translation in cells infected with either wtVV or VV

**DISCUSSION**

IL-2 is thought to play a key role in immunoregulation. *In vitro*, IL-2 has been demonstrated to be crucial for T cell induction and proliferation. The transcription factors, NFATc1–NFATc4, play a pivotal role in the transcription of cytokine genes and other genes critical for the immune response. Corthesy and Kao isolated and cloned a different nuclear factor, NF-90, that also induced IL-2 transcription under conditions of T cell stimulation. For
NFATc, nuclear translocation appears to involve a dephosphorylation event catalyzed by calcineurin.\textsuperscript{(11,12)} For NF-90, the mechanism of translocation remains unclear. The homolog of NF-90, MPP4, is present in the nucleus in an unphosphorylated state during mitosis, whereas the cytoplasmic form is present during other stages of the cell cycle in a phosphorylated state.\textsuperscript{(18)} This may suggest a similar means of regulating NF-90 nuclear transport to that observed for NFATc. Data presented in this paper suggest that infection of T cells with wtVV led to the formation of a nuclear DNA binding complex capable of binding to the IL-2 enhancer region. This is in agreement with previous data where persistently VV-infected cells induced IL-2 secretion.\textsuperscript{(26)}

Multiple functional motifs have been described for NF-90. A previous report from our laboratory and other reports demonstrated that NF-90 was capable of finding dsRNA and that NF-90 could act as an \textit{in vitro} substrate for the dsRNA-dependent protein kinase, PKR.\textsuperscript{(15–17,42)} The data presented here suggest that VV infection of Jurkat cells led to the induction or accumulation of NF-90 protein levels. For wtVV infection, NF-90 was induced and translocated to the nucleus of infected cells. The migration of NF-90 to the nucleus occurred concomitantly with formation of an ARRE-2 DNA binding complex and subsequent IL-2 transcription. The presence of NF-90 in the shifted EMSA complex was supported by supershift analysis using NF-90 antiserum. Thus, or results suggest that NF-90 was likely in-
involved in the induction of IL-2 transcription during VV infection. Formation of the ARRE-2 DNA binding complex during VV infection required viral DNA synthesis or late transcription or both, as the addition of araC blocked both the shifted EMSA complex and the presence of NF-90 in the nucleus.

Infection of Jurkat cells with a VV mutant (VVΔE3L), which produced free cytoplasmic dsRNA during infection, caused accumulation of NF-90 protein levels similar to that of wtVV, but the protein remained localized to the cytoplasm. Subsequent formation of an ARRE-2 DNA binding complex and IL-2 transcription was not observed on infection with this and other phenotypically similar viruses, again supporting the concept that NF-90 was the IL-2 regulatory factor induced during VV infection. Because NFATc1 is known to be involved in the production of IL-2, we measured the levels of NFATc1 in the nucleus following VV infection. Both wtVV and VVΔE3L led to only a minor increase in NFATc1 in the nucleus (data not shown). The fact that both viruses caused the same level of NFATc1 in the nucleus suggests that NFATc1 was not sufficient for the induction differences in IL-2 transcription observed between wtVV and VVΔE3L. Treatment of cells with PMA/ionomycin has been shown to induce ARRE-2 DNA complex formation and IL-2 transcription. Our data supported these effects as well as demonstrating an increase in nuclear NF-90 levels following PMA/ionomycin treatment. In PMA/ionomycin-treated cells that were infected with VVΔE3L, ARRE-2 DNA complex formation and the induction of nuclear NF-90 were inhibited. These results suggest that cytoplasmic dsRNA may inhibit PMA/ionomycin-induced ARRE-2 complex formation by blocking transport of NF-90 to the nucleus.

Previous results also suggest that the presence of cytoplasmic RNA leads to retention of NF-90 in the cytoplasm. In Xenopus, the CCAAT box transcription factor, CBTF, is likely equivalent to the mammalian NF-90 protein. This protein translocates to the nucleus prior to activation of zygotic GATA-2 transcription at gastrulation. Prior to the midblastula transition, CBTF is associated with translationally quiescent mRNP complexes. Binding to RNA, by means of the conserved dsRNA-binding motif, acts as a cytoplasmic anchor for CBTF. Nuclear translocation of CBTF at midblastula likely occurs after degradation of the maternal mRNA. The cytoplasmic RNA

![Image](image_url)
association of NF-90 is also demonstrated in the TCP80 regulation of β-glucosidase translation. TCP80 is 96% identical to NF-90 and functions in binding to β-glucosidase mRNA and inhibiting translation. Therefore, it is likely that the cytoplasmic form of TCP80 remains bound to mRNA until translocation to the nucleus. In the data presented in this paper, during VV replication, the E3L protein acted to sequester and mask dsRNA synthesized during viral infection. As the dsRNA synthesized during wtVV infection was requested by the E3L protein, the induced NF-90 was capable of migration to the nucleus. For VV constructs that synthesize free dsRNA, it was likely that the induced NF-90 bound to the free dsRNA and remained localized to the cytoplasm. These data together support cytoplasmic regulation of NF-90 activity by association with RNA, likely dsRNA (this paper), or highly structured ssRNA. How VV led to the induction and subsequent translocation of NF-90 to the nucleus is unknown, but it is clear that the presence of dsRNA blocked this event. One possible mechanism may be related to the synthesis of E3L. As both E3L and NF-90 contain the conserved dsRNA-binding motif, it is likely that they compete for binding to RNA. If NF-90 is constitutively anchored in the cytoplasm until release from some structural RNA, the excess amount of E3L synthesized during wtVV replication may compete for RNA associated with NF-90, leading to the subsequent release and translocation of NF-90 to the nucleus.

As mentioned earlier, the phosphorylation state of NF-90 likely regulates nuclear translocation and function. Kao et al. suggest that the phosphorylation state of NF-90 alters association of NF-90 with the ARRE-2 enhancer element. Likewise, the homolog MPP4 was isolated as a dephosphorylated nuclear protein during mitotic M-phase. Therefore, in addition to a cytoplasmic RNA anchor, NF-90 nuclear translocation may be dependent on a dephosphorylation event. A likely candidate for the kinase regulating NF-90 phosphorylation is PKR. NF-90 shares homology (amino acids 166–235) with the putative PKR binding domain present on eIF2α, in agreement with NF-90 acting as an in vivo substrate for PKR. Endogenous cellular activators of PKR have been identified that may lead to localized activation of PKR or the highly structured RNA anchor may permit cobinding of NF-90 and PKR, leading to activation of PKR and a specific phosphorylation of NF-90. Work by Sauders et al. demonstrated an increase in NF-90-induced gene expression in fibroblast cells lacking PKR. In the VV system, the presence of E3L may lead to a similar result with the inhibition of PKR activity by blocking PKR recognition of the activator molecule. The inhibition of PKR activity may lead to the subsequent dephosphorylation of NF-90, leading to nuclear translocation. Cyclosporin A, which is thought to act by inhibiting the phosphatase activity of calcineurin, has been shown to block NF-90 transcripional activity. Because PKR is activated in the presence of dsRNA, NF-90 may be phosphorylated by PKR during an infection with VVΔE3L, inhibiting nuclear translocation. These results likely suggest that PKR does regulate the phosphorylation state of NF-90 and that the translocation of NF-90 to the nucleus depends on subsequent dephosphorylation of the protein. The data presented here suggest that wtVV infection induced NF-90 levels and led to subsequent nuclear translocation and the formation of an ARRE-2 DNA binding complex. Research by Damaso and Moussatché suggests that VV replication is inhibited by treatment with cyclosporin A. Cyclosporin A treatment blocks NF-90 ARRE-2 complex formation, and one possible mechanism may be that VV replication requires NF-90 activity in the nucleus of infected cells. VV replication occurs solely within the cytoplasm of infected cells. Interestingly, the E3L protein has been the only viral protein identified in both the cytoplasm and the nucleus of infected cells. VV may require NF-90 translocation to the nucleus to regulate the transcription of specific cellular genes or to allow release of cytoplasmic RNAs present in NF-90-RNP complexes. Alternatively, VV infection of T cells may induce a host defense mechanism in an attempt to release IL-2, leading to increased T cell response. NF-90 has been shown to regulate the transcription of IFN response genes, supporting an antiviral role for NF-90. The VV E3L protein is required for efficient VV replication. If E3L leads to the induction of NF-90 activity, the block in host protein synthesis observed during wtVV infection may be a means by which the virus prevents the synthesis and release of the IL-2 signal induced by NF-90. Notably, the synthesis of IL-2 during VV replication does lead to inhibitory effects. Work byFlexner et al. demonstrated that a recombinant VV construct expressing IL-2 caused an attenuated phenotype of the virus that was a direct result of the antiviral mechanisms induced by IL-2.

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