

Nuclear Factor-90 of Activated T-Cells: A Double-Stranded RNA-Binding Protein and Substrate for the Double-Stranded RNA-Dependent Protein Kinase, PKR

J. O. Langland,[§] P. N. Kao,^{||} and B. L. Jacobs^{*,§,⊥}

Department of Microbiology and Division of Molecular and Cellular Biology, Arizona State University, Tempe, Arizona 85287-2701, and Division of Pulmonary and Critical Care, Stanford University Medical School, Stanford, California 94305

Received October 9, 1998; Revised Manuscript Received December 21, 1998

ABSTRACT: NFAT transcription factors play a central role in initiating T-cell activation through the induction of immediate-early T-cell specific genes including interleukin-2 (IL-2). NFAT transcription factors bind to a sequence in the IL-2 enhancer known as the antigen receptor response element 2 (ARRE-2). Multiple proteins exhibiting ARRE-2 binding activity have been isolated, including a heterodimer from stimulated T-cell nuclear extracts consisting of $M_r = 90\,000$ (NF90) and $M_r = 45\,000$ (NF45) subunits. The subunits of this heterodimer have been cloned, and NF90 was found to encode a protein containing two domains that are predicted to form motifs capable of binding to double-stranded RNA. Using in vitro translated polypeptides, we have demonstrated that NF90 specifically binds to double-stranded RNA. Furthermore, NF90 was phosphorylated in a double-stranded RNA-dependent manner likely by the interferon-induced, double-stranded RNA-dependent protein kinase, PKR. The NF90 protein was found to be expressed not only in T-cells, but also in nonimmune HeLa cells. In HeLa cells, the protein was almost exclusively localized to the ribosome salt wash fraction of cell lysates.

The transcription factors, nuclear factors of activated T-cells (NFATs),¹ appear to be essential for early T-cell gene activation (1–6). The transcriptional activation of interleukin 2 (IL-2), the first lymphokine secreted following T-cell activation (7), likely involves NFATs as well as other transcription factors including AP-1, NF- κ B, and Oct-1 (8, 9). Activation of the IL-2 gene after antigen recognition is a critical event for T-cell proliferation and effector function. In addition to binding to the IL-2 promoter, NFATs bind regulatory sites of several other cytokine genes, including tumor necrosis factor- α , IL-4, IL-3, and granulocyte-macrophage colony stimulating factor (10–16).

Various attempts have been made to identify the proteins involved in NFAT-DNA binding activity. The induction of IL-2 appears to be mediated by a transcriptional enhancer located between 52 and 319 bp upstream of the IL-2 gene (65). A sequence from this enhancer region, termed ARRE-2, is capable of activating linked promoters in response to antigen binding. A factor that was capable of interacting with ARRE-2 DNA sequences was defined by McCaffrey et al. as a DNA-binding protein ($M_r = 90\,000$ – $140\,000$) that was constitutively expressed in T-cells (17). This protein is present in resting T-cells in a nonnuclear compartment and

appears in nuclear extracts only after T-cell activation. Flanagan et al. also isolated a cytoplasmic component of NFAT that translocated to the nucleus upon T-cell stimulation, however, this protein combined with a nuclear component to form an ARRE-2 DNA-binding complex (18). Recently, distinct classes of NFAT proteins (NFAT1, NFATc, NFAT3, and NFAT4) have been defined as playing key roles in the transcription of cytokine genes and other genes during the immune response (19–24). These proteins define a novel family of transcription factors containing a conserved DNA-binding domain (19, 20, 22, 24). All four NFAT family proteins are cytosolic components capable of translocating to the nucleus and binding cooperatively with Fos and Jun family proteins at the ARRE-2 site of the IL-2 promoter (4, 20, 25, 26). Most of the research regarding NFAT activity has focused on the role of NFAT1. Within minutes of T-cell stimulation, NFAT1 is dephosphorylated, translocates from the cytoplasm into the nucleus, and binds to ARRE-2 DNA sequences (21, 27, 28). This effect is dependent on calcium mobilization and on activity associated with the serine-threonine phosphatase, calcineurin (1, 17, 21, 28–30).

The diversity of factors involved in transcriptional activation through binding to ARRE-2 DNA enhancer elements may involve other classes of proteins as well. Cortes and Kao purified to homogeneity a heterodimeric protein exhibiting ARRE-2 binding activity (31). This protein complex exhibited activities similar to those demonstrated for NFAT transcriptional factors including ARRE-2 DNA binding specificity, T-cell specific activation, enhancement of ARRE-2 DNA-binding activity upon T-cell stimulation, transcriptional activation of heterologous reporter genes, and sensitivity of

* To whom correspondence should be directed. E-mail: bjacobs@asu.edu. Phone: 1-602-965-4684. Fax: 1-602-965-0098.

[§] Department of Microbiology, Arizona State University.

^{||} Stanford University Medical School.

[⊥] Division of Molecular and Cellular Biology, Arizona State University.

¹ Abbreviations: NFAT, nuclear factor of activated T-cells; IL-2, interleukin-2; ARRE-2, antigen receptor response element 2; PKR, double-stranded RNA-dependent protein kinase; dsRNA, double-stranded RNA; eIF-2, eukaryotic translation initiation factor-2.

DNA-binding activity and transcriptional activation to the immunosuppressant drugs cyclosporin A and FK506 (31, 32). Both subunits of the heterodimeric complex have been cloned and found to consist of subunits of $M_r = 45\,000$ (NF45) and $M_r = 90\,000$ (NF90). The phosphorylation state of this ARRE-2 binding complex appears to be involved in regulating DNA-binding since the addition of a phosphatase to nuclear extracts inhibits DNA-binding activity (31). Upon examination of the NF90 clone, no discernible DNA-binding domain was identified. However, two regions of NF90 with similarity to known motifs previously demonstrated as being involved in double-stranded (ds) RNA-binding were identified (32).

This evidence may suggest that the phosphorylation state and possibly the presence of dsRNA may influence NF90/NF45 ARRE-2 binding and transcriptional activity. Several investigators have previously observed a $M_r = 90\,000$ protein that could be phosphorylated by the interferon-induced, dsRNA-dependent protein kinase, PKR (33–35). PKR has classically been defined as regulating translation through the phosphorylation of eIF-2 α (36–38). More recent evidence suggests that PKR is also involved in the regulation of transcription through NF- κ B activation (39–41) and in the control of apoptosis (42–44) and cell cycle regulation (45, 46). Rice et al., in particular, identified a 90 kDa protein that could bind to adenovirus VAI RNA and authentic dsRNA, but not single-stranded RNA (35). This protein was localized to the ribosome salt wash fraction of rabbit reticulocyte lysates and could act as a substrate for PKR (35). This reticulocyte lysate protein appears to have biochemical characteristics like those expected for NF90, including a phosphoprotein of $M_r = 90\,000$ that is capable of binding dsRNA.

Our investigation focuses on the characterization of the dsRNA-binding properties of NF90, the identification of NF90 in nonimmune HeLa cells, and the phosphorylation of NF90 by PKR. The HeLa cell NF90 protein was demonstrated to be a 90 kDa, dsRNA-binding protein localized primarily to the ribosome salt wash fraction of cell lysates. Furthermore, we have identified the HeLa cell NF90 and purified Jurkat cell NF90 as substrates for PKR.

While this work was in progress, Liao et al. purified a protein from human 293 cells, which, from partial peptide sequence, appears to be NF90. This protein was able to bind dsRNA and highly structured ssRNAs including the adenovirus VA RNAs (68).

EXPERIMENTAL PROCEDURES

Cells. HeLa cells were grown in suspension at 37 °C using glass round-bottom flasks in Eagle's suspension minimal essential media (Gibco) supplemented with 50 μ g of gentamicin/mL, 100 μ g of glucose/mL, 292 μ g of L-glutamine/mL, 110 μ g of sodium pyruvate/mL, 0.1 mM nonessential amino acids, and 5% fetal bovine serum (HyClone Laboratories). Cells, when at a density of 1×10^6 cells/mL, were treated with recombinant human IFN- α /D (kindly provided by Hoffmann-La Roche Inc.) at 100 IU/mL. Cells were harvested 18 h later by centrifugation, washed in isotonic buffer and extracts prepared.

Extract Preparation. Ribosome salt wash (RSW) fractions were prepared as previously described (47). Briefly, the cell

pellet was suspended in 2.5 volumes of hypotonic buffer and disrupted by Dounce homogenization (40 strokes). The suspension was brought to isotonic, and standard S-100 extracts were prepared. The ribosomal pellet was brought to 1.0 M KCl and repelleted by ultracentrifugation. The RSW fraction was dialyzed against buffer A (20 mM Hepes (pH 7.5), 5 mM MgOAc, 10% (v/v) glycerol, 50 mM KCl, 1 mM dithiothreitol, and 1 mM benzamidine).

For subcellular fractionation (29), 1×10^7 cells were lysed in 100 μ L of lysis buffer (0.05% Nonidet P-40, 10 mM Tris (pH 7.4), 10 mM NaCl, 3 mM MgCl₂, 0.5 mM dithiothreitol, 2.5 mM EGTA, 5 μ g aprotinin/mL, 5 μ g antipain/mL, 100 μ M benzamidine, 5 μ g leupeptin/mL, 5 μ g pepstatin/mL, 5 μ g soybean trypsin-chymotrypsin inhibitor/mL, and 1 mM PMSF) for 10 min on ice. The cell lysates were centrifuged at 300g for 10 min to separate nuclei from cytoplasmic fractions. Nuclear pellets were washed once with 1 mL of the same buffer and resuspended in 100 μ L of nuclear extraction buffer (20 mM Hepes (pH 7.9), 0.42 M NaCl, 1.5 mM MgCl₂, 25% v/v glycerol, 0.2 mM EDTA, 0.5 mM dithiothreitol, and the above protease inhibitors). Resuspended nuclei were incubated on ice for 30 min with occasional shaking followed by centrifugation at 10000g for 10 min. The supernatant was removed and stored at –80 °C until use. The cytoplasmic fraction was separated into cytosolic and RSW fractions as described above.

In Vitro Transcription and Translation. The plasmid pBSII-NF90.3 was used for in vitro synthesis of full-length NF90 protein. Coupled transcription/translation was performed using T7 RNA polymerase (Promega-TNT Coupled Reticulocyte Lysate System) in the presence of [³⁵S]methionine. The reactions were done according to manufacturer's specifications at 30 °C for 1 h.

Poly(rI).Poly(rC)-Sepharose Affinity Chromatography. DsRNA-binding assays were performed using HeLa cells extracts and in vitro translated products as previously described (48). Briefly 10–100 μ L of RSW extract or 20 μ L of in vitro translated products were incubated with 50 μ L of poly(rI).poly(rC)-Sepharose resin for 1 h at 4 °C. After incubation, the resin was collected by centrifugation (250g) and washed four times in buffer A containing 100 mM KCl. Bound proteins were eluted in SDS-sample buffer and separated by SDS-PAGE. To determine the specificity of dsRNA-binding, samples were preincubated with 100 μ g (5-fold excess) of soluble nucleic acid (dsRNA:poly(rI).poly(rC), ssRNA:poly(rA), dsDNA:calf thymus DNA, ssDNA:single-stranded calf thymus DNA (Sigma)) for 5 min on ice prior to the addition of dsRNA-agarose. The specificity of binding was also confirmed by washing the resin in buffer A (two times) containing higher concentrations of KCl (200 mM, 400 mM, and 800 mM) followed by two washes in buffer A with 100 mM KCl. For the purification of PKR activity, the resin was washed two times in buffer A containing 1.2 M KCl and twice in buffer A containing 100 mM KCl.

Immunoanalysis. Western blot analysis was performed (49) using proteins separated by SDS-PAGE which were transferred to nitrocellulose using CAPS transfer buffer (20 mM CAPS (pH 11.0), 14 mM B-mercaptoethanol, 20% (v/v) methanol) for 45 min at room temperature. Nonspecific sites were blocked with BLOTTO and the membrane was incubated overnight at 4 °C with anti-NF90 serum. After

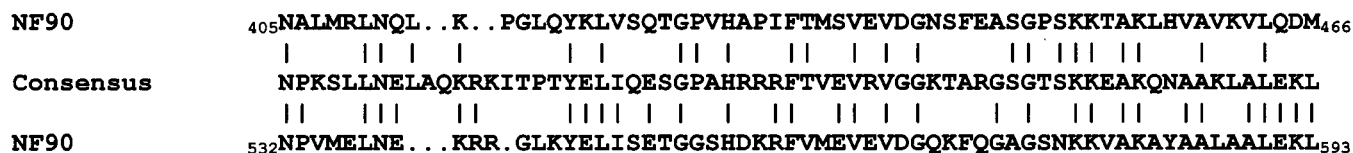


FIGURE 1: Identification of putative dsRNA-binding domains in NF90. The NF90 amino acid sequence revealed two regions of homology to a consensus dsRNA-binding domain (53). Regions of homology correspond to amino acids 405–466 and 532–593 in NF90.

thorough washing, the membrane was incubated with secondary goat anti-rabbit IgG horseradish peroxidase conjugate (Sigma), washed again, and developed using 0.1 mg of 4-iodophenol/mL, 0.4 mg of Na-luminal/mL, and 0.005% v/v H₂O₂. Immune complexes were visualized by autoradiography.

Immunoprecipitation of radiolabeled proteins was performed as previously described (50). Phosphorylated proteins were incubated with anti-NF90 serum for 1 h at 4 °C followed by incubation with protein A-agarose. The resin was washed five times in wash buffer (0.1 M Tris (pH 7.5), 0.15 M NaCl, 0.65% Nonidet P-40), and bound proteins were eluted, separated by SDS-PAGE, and visualized by autoradiography.

Peptide Mapping Analysis. Peptide mapping was performed as described (51). Extract proteins were purified by dsRNA-agarose affinity purification or as an *E. coli* expressed protein. Purified proteins were electrophoresed by standard SDS-PAGE followed by brief staining in Coomassie Brilliant Blue R-250 and destaining in methanol/acetic acid. The desired protein band was excised and equilibrated in 125 mM Tris (pH 6.8), 0.1% SDS, 1 mM EDTA, 1 mM dithiothreitol. Each gel piece was placed into a well on a 15% SDS-polyacrylamide gel, and sample buffer containing 10% glycerol and *Staphylococcus* V-8 protease was loaded over the gel piece. The gel was electrophoresed at 30 mA until the dye front was just above the stacking gel/running gel interface. Electrophoresis was stopped, and protease digestion was allowed to occur for the next 30 min. Standard electrophoresis was then continued followed by analysis of the peptide fragments by Western blot analysis.

In Vitro Phosphorylation. For soluble phosphorylation assays (50), HeLa RSW fractions (2 μ L) were incubated with the indicated concentrations of poly(rI).poly(rC) and kinase reaction buffer (5 mM MgOAc, 5 mM MnCl₂, 10% (v/v) glycerol, 20 mM Hepes (pH 7.5), 100 mM KCl, 1 mM dithiothreitol, 1 mM benzamide, 100 μ M [γ -³²P]ATP (1 Ci/mmol)) for 10 min at 30 °C. The reaction was stopped by the addition of an equal volume of 2 X SDS-sample buffer. The proteins were resolved by SDS-PAGE and detected by autoradiography.

For phosphorylation of proteins bound to dsRNA-agarose (47), the washed resin was incubated with an equal volume of kinase reaction buffer for 10 min at 30 °C. To test for the phosphorylation of NF90, 2 μ g purified *Escherichia coli* expressed NF90 was added to the reaction buffer. The reaction was stopped by the addition of an equal volume of 2 X SDS-sample buffer. The proteins were resolved by SDS-PAGE and detected by autoradiography.

RESULTS

Identification of a Consensus dsRNA-Binding Motif in NF90. Several proteins have previously been identified to

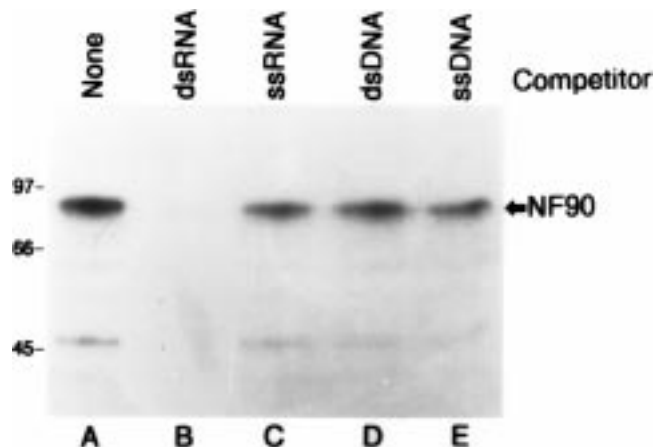


FIGURE 2: Specific affinity of NF90 for dsRNA. NF90 protein was expressed in vitro from pBSII-NF90.3. Cell free translation products incorporating [³⁵S]methionine were incubated with soluble competitor nucleic acids (see Material and Methods) followed by incubation with dsRNA-agarose. Bound proteins were eluted in SDS-sample buffer, separated by SDS-PAGE, and visualized by autoradiography. Molecular weight markers (kDa) and the position of NF90 are shown.

contain a conserved protein motif involved in binding to dsRNA (52, 53). NF90 contains two regions, from amino acids 405–466 and 532–593 that demonstrate significant similarity to the consensus dsRNA-binding motif (Figure 1).

Given the conservation of the dsRNA-binding motif in NF90, the ability of NF90 to bind to dsRNA was examined. NF90 protein from a Jurkat T-cell cDNA clone was synthesized from the plasmid pBSII-NF90.3 using T7 RNA polymerase in a coupled transcription-translation system. Radiolabeled products were incubated with dsRNA-agarose and washed thoroughly, and bound proteins were analyzed by SDS-PAGE and autoradiography. As shown in Figure 2, a protein having a $M_r = 90\,000$ bound to the resin (lane A). In vitro translation with the parental plasmid, pBSII, did not produce any products of a similar molecular weight (data not shown). To determine the specificity of this binding, competition experiments were performed. Incubation with a 5-fold excess of soluble dsRNA eliminated NF90 binding to the matrix (Figure 2, lane B). Preincubation with excess ssRNA, dsDNA, or ssDNA did not significantly reduce the level of NF90 bound to the dsRNA-linked resin (Figure 2, lanes C–E). In vitro synthesized NF90 was incubated with dsRNA-agarose and washed in buffer of varying salt concentrations. As shown in Figure 3, NF90 remained associated with the matrix to KCl concentrations of 400 mM (lane C). Binding was completely eliminated when the resin was washed in buffer containing 800 mM KCl (lane D). These results support the concept that NF90 binding to dsRNA is not due to nonspecific nucleic acid interactions or interactions with the agarose and that NF90 has a specific affinity for dsRNA.

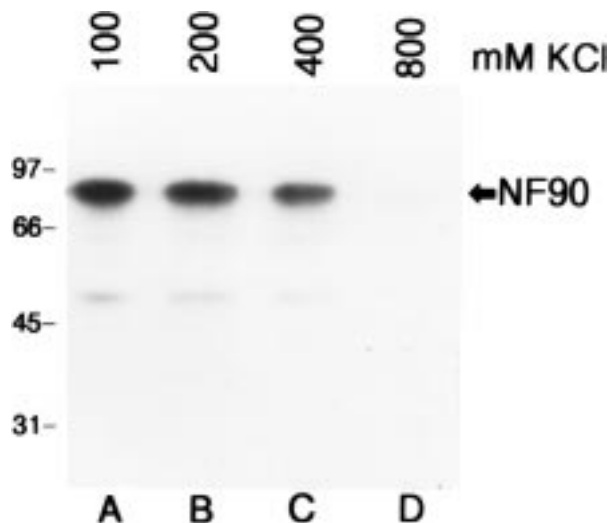


FIGURE 3: Properties of NF90 binding to dsRNA. In vitro translated, [35 S]methionine labeled NF90 protein was incubated with dsRNA-agarose. The resin was thoroughly washed in buffer A (see Materials and Methods) containing the indicated concentration of KCl. Bound proteins were eluted in SDS-sample buffer, separated by SDS-PAGE, and visualized by autoradiography. Molecular weight markers (kDa) and the position of NF90 are shown.

Analysis of a HeLa Cell Expressed Equivalent the T-Cell Expressed NF90 Protein. Previous studies have suggested that NF90 is not a T-cell specific product. A 90 kDa protein that binds to adenovirus VAI RNA has previously been identified in the ribosomal salt wash fraction of rabbit reticulocyte lysates (35). A partial peptide sequence suggests the presence of NF90 in human 293 cells (68). Furthermore, immunoreactive proteins to NF90 have been identified but uncharacterized in HeLa cells (32). Therefore, experiments were performed to determine if NF90 was expressed in HeLa cells and, if so, to characterize the biochemical and cellular properties associated with this protein.

Ribosome salt wash fractions were prepared from HeLa cells, followed by incubation with dsRNA-agarose. Bound proteins were eluted, separated by SDS-PAGE, and detected by Western blot analysis using anti-NF90 serum. As shown in Figure 4, a single immunoreactive protein having a $M_r = 90\,000$ was observed in the HeLa cell fraction (lane A). This protein bound specifically to dsRNA, and binding could be competed with the addition of a 5-fold excess of soluble dsRNA (Figure 4, lane B). No proteins were detected on the Western blot when probed with preimmune serum (data not shown). These results suggest that nonimmune HeLa cells express a ribosome-associated, dsRNA-binding protein having a $M_r = 90\,000$, which was an immunologically related protein to NF90. Densitometer comparison to a Western blot with known concentrations of *E. coli* expressed NF90 suggested that the amount of this protein present in HeLa cell ribosome salt wash fractions was approximately $1\,\mu\text{g}/10^7$ cells (data not shown).

To confirm that the HeLa p90 protein was analogous to the Jurkat cell NF90 protein, V-8 protease peptide mapping analysis was performed. *E. coli* expressed NF90 protein was obtained and compared to the HeLa p90 protein, which was affinity purified from ribosome salt wash fractions by binding to dsRNA-agarose. The peptide fragments were detected by Western blot analysis using anti-NF90 serum. As shown in Figure 5, at all V-8 protease concentrations used, similar

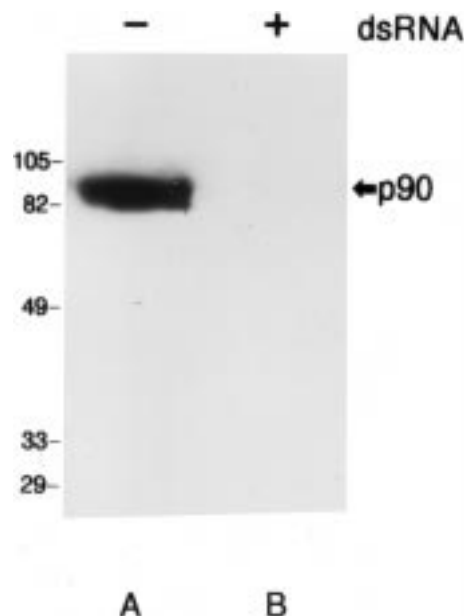


FIGURE 4: Identification of an immunologically related protein to NF90 expressed in HeLa cells. A HeLa cell ribosome salt wash fraction was prepared followed by incubation with (lane B) or without (lane A) soluble dsRNA. Samples were subsequently incubated with dsRNA-agarose and bound proteins eluted in SDS-sample buffer, separated by SDS-PAGE, and transferred to nitrocellulose. Chemiluminescent Western blot analysis was performed using specific polyclonal antiserum to NF90. Luminescent signals were visualized by autoradiography. Molecular weight markers (kDa) and the position of the 90 kDa (p90) protein are shown.

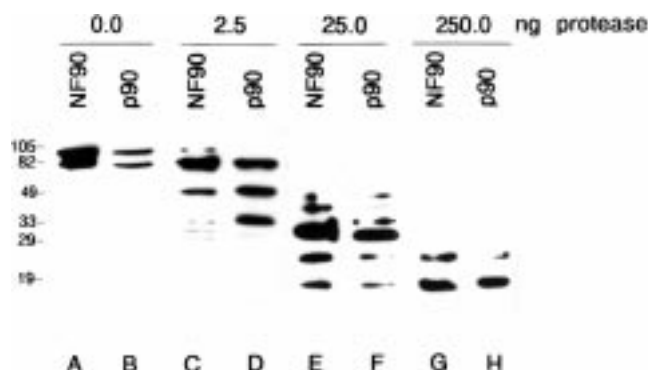


FIGURE 5: Peptide mapping comparison of NF90 and the HeLa cell p90 protein. The HeLa cell p90 protein was partially purified from ribosome salt fractions by binding to dsRNA-agarose. The eluted protein along with *E. coli* expressed and purified NF90 were separated by SDS-PAGE and the 90 kDa region of the gel excised. Gel pieces were incubated with the indicated amounts of *Staphylococcus* V-8 protease followed by separation on SDS-PAGE and transfer to nitrocellulose. Chemiluminescent Western blot analysis was performed using specific polyclonal antiserum to NF90. Luminescent signals were visualized by autoradiography. Molecular weight markers (kDa) are shown.

peptide mapping patterns were observed between the NF90 and p90 proteins. Minor differences in mapping patterns were detected (compare lanes C and D), possibly due to post-translational processing of the HeLa p90 protein, which did not occur with the *E. coli* expressed NF90 protein or due to protein differences between the HeLa cell and Jurkat cell proteins. These results support the presence of NF90 in HeLa cells.

Rice et al. identified a 90 kDa adenovirus VAI RNA-binding protein, which was ribosome associated (35). NFAT

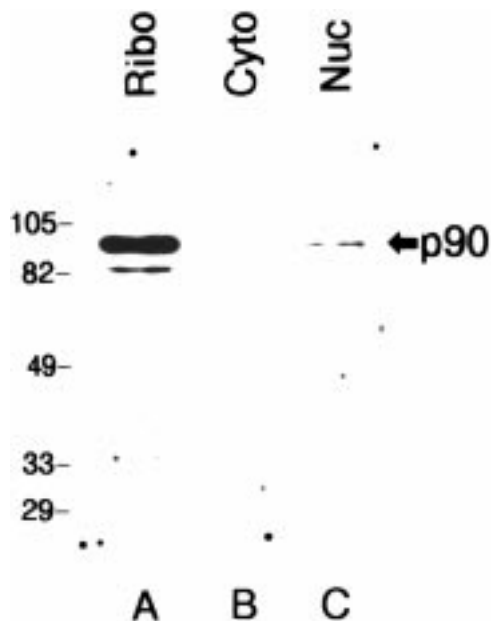


FIGURE 6: Subcellular distribution of the HeLa cell p90 protein. HeLa cell subcellular fractions were prepared as described in Materials and Methods. An equal volume of SDS-sample buffer was added to each of the subcellular fractions (ribosomal (lane A), cytosolic (lane B), and nuclear (lane C)) followed by separation of the proteins by SDS-PAGE and transfer to nitrocellulose. Chemiluminescent Western blot analysis was performed using specific polyclonal antiserum to NF90. Luminescent signals were visualized by autoradiography. Molecular weight markers (kDa) and the position of the 90 kDa (p90) protein are shown.

activation appears to require the translocation of a cytosolic component of the NFAT complex from the cytoplasm into the nucleus during antigen stimulation (4, 18, 20, 25, 26). To determine the cellular distribution of the HeLa cell NF90 protein, subcellular fractions were prepared. Protein fractions were separated by SDS-PAGE and analyzed by Western blot analysis using anti-NF90 serum. Figure 6 demonstrates that the HeLa cell NF90 protein was predominantly localized to the ribosome salt wash fraction (lane A). The NF90 protein could not be detected free in the cytosol (lane B), and a minor amount was present in the nuclear fraction (lane C). The quality of the subcellular fractionation was monitored by the presence of PKR, which has previously been shown to localize primarily to the ribosome salt wash fraction (47).

In Vitro Phosphorylation of NF90 Protein. The 90 kDa adenovirus VAI RNA binding protein has been reported to be phosphorylated by PKR (35). Other investigators have also reported the phosphorylation of a 90 kDa protein by PKR (33, 34). To assay for PKR phosphorylation of NF90, HeLa RSW fractions were tested for dsRNA-dependent phosphorylation of NF90. In Figure 7, RSW fractions were incubated in the presence or absence of dsRNA and [γ - 32 P]-ATP. Phosphorylated proteins were immune precipitated with anti-NF90 serum or preimmune serum. As shown in Figure 7, a HeLa cell p90 protein was specifically immune precipitated with anti-NF90 serum (lane D), and this protein could not be precipitated with preimmune serum (lane C). The phosphorylation of the 90 kDa immune precipitated protein was only observed in the presence of dsRNA (lane B). Activation of PKR occurs through an autophosphorylation event. We have previously demonstrated that the only detectable 68 kDa dsRNA-dependent phosphoprotein is PKR

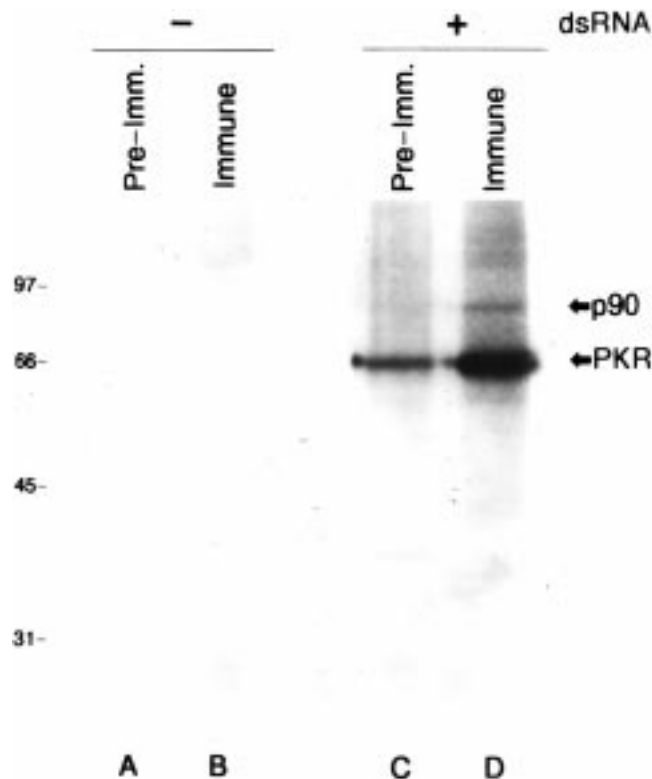


FIGURE 7: In vitro phosphorylation of the HeLa cell p90 protein. HeLa ribosome salt wash fractions were incubated with (lanes C and D) or without (lanes A and B) 3 μ g/mL dsRNA and [γ - 32 P]-ATP. Phosphorylated proteins were incubated with antiserum to NF90 (lanes B and D) or preimmune serum (lanes A and C). Immune complexes were precipitated with protein A-Sepharose and solubilized in SDS-sample buffer. Phosphoproteins were separated by SDS-PAGE and visualized by autoradiography. Molecular weight markers (kDa) and the positions of the 90 kDa (p90) protein and PKR are shown.

(47). A protein corresponding to PKR was also immune precipitated in extracts phosphorylated in the presence of dsRNA. It is likely PKR was coprecipitated with the p90 protein due to the association of both proteins with dsRNA during the phosphorylation reaction. A small amount of phosphorylated PKR was also detected in the preimmune precipitate, likely due to the large amount of radiolabeled PKR present in the assay (lane C).

To confirm the dsRNA-dependent phosphorylation of the HeLa p90 protein and thereby support the phosphorylation of p90 by PKR, kinase reactions were performed with varying levels of dsRNA. HeLa RSW fractions were incubated with increasing amounts of dsRNA and [γ - 32 P]-ATP. Phosphorylated products were immune precipitated with anti-NF90 serum, and precipitated proteins were analyzed by SDS-PAGE and autoradiography. Maximal phosphorylation of p90 occurred concomitantly with the autophosphorylation of PKR (Figure 8, lane E). The autophosphorylation of PKR is not only dependent on the presence of dsRNA but is inhibited at high concentrations of dsRNA (30–100 μ g/mL) (54, 55). The phosphorylation of the HeLa p90 protein was markedly inhibited at high concentrations of dsRNA (lanes G and H), supporting the phosphorylation of p90 by PKR.

The HeLa p90 protein appeared to be analogous to the Jurkat NF90 protein. Since *E. coli* expressed NF90 protein was available (32), we tested if purified NF90 could act as

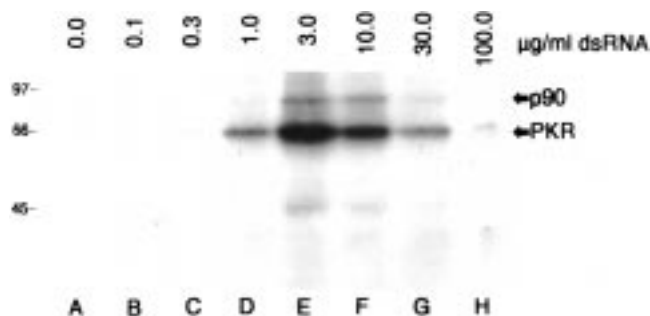


FIGURE 8: DsRNA-dependent phosphorylation of the HeLa cell p90 protein. HeLa ribosome salt wash fractions were incubated with increasing concentrations of dsRNA (as indicated) and [γ - 32 P]ATP. Phosphorylated proteins were incubated with antiserum to NF90 and immune complexes precipitated with protein A-Sepharose. Precipitated phosphoproteins were solubilized in SDS-sample buffer, separated by SDS-PAGE and visualized by autoradiography. Molecular weight markers (kDa) and the positions of the 90 kDa (p90) protein and PKR are shown.

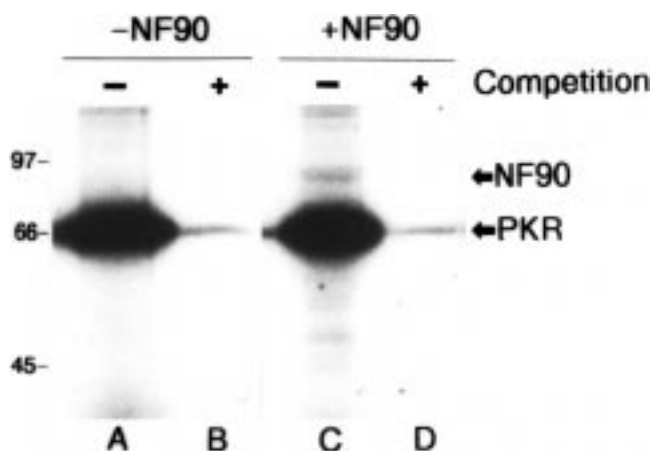


FIGURE 9: Phosphorylation of NF90 by PKR. PKR was affinity purified from HeLa ribosome salt wash fractions by binding to dsRNA-agarose and washing in buffer A (see Materials and Methods) containing 1.2 M KCl. Prior to incubation with the dsRNA-agarose, the fractions were incubated with (lanes B and D) or without (lanes A and C) soluble dsRNA. The washed matrix was incubated with [γ - 32 P]ATP and with (lanes C and D) or without (lanes A and B) *E. coli* expressed and purified NF90 protein (2 μ g). Phosphoproteins were solubilized in SDS-sample buffer, separated by SDS-PAGE, and visualized by autoradiography. Molecular weight markers (kDa) and the positions of the NF90 protein and PKR are shown.

a substrate for purified PKR. Previously, when bacterial expressed substrates of PKR have been used for in vitro analysis, high levels (microgram amounts) of the substrate protein have been used (66, 67, unpublished observations). This is likely due to poor renaturation of the bacterially expressed protein. For this assay, PKR was purified from HeLa RSW fractions by affinity purification on dsRNA-agarose. Washing the matrix in 1.2 M KCl results in PKR being the only kinase bound to the resin (48). Bound PKR was incubated in the presence or absence of 2 μ g of *E. coli* expressed NF90 and [γ - 32 P]ATP. Phosphorylated products were analyzed by SDS-PAGE and autoradiography. As shown in Figure 9, a protein corresponding to NF90 was phosphorylated by PKR (compare lanes A and C). When the binding of PKR to the matrix was competed by the addition of soluble dsRNA, the phosphorylation of NF90 was no longer observed (lane D), supporting the phosphorylation of NF90 by PKR.

DISCUSSION

The NF45/NF90 heteroduplex appears to be a novel class of ARRE-2 DNA-binding factors and transcriptional activators (31, 32). The NF90 sequence analysis reveals similarity in two regions of the protein that are homologous to previously described dsRNA-binding motifs (32). Using in vitro synthesized NF90 protein, we have shown that NF90 was capable of specific binding to dsRNA. These data are in support of work done concurrently by Liao et al. where 293 cell isolated NF90 was shown to bind dsRNA (68). Bass et al. (69) previously isolated the 4F.1 protein from *Xenopus*, which binds specifically to dsRNA and is likely the *Xenopus* analogue to the mammalian NF90 protein. Our results, demonstrating binding to synthetic dsRNA, along with the results of Liao et al. and Bass et al., suggest that NF90 is a sequence nonspecific dsRNA binding protein. The role of the dsRNA interaction of NF90 remains unclear, but given the regulatory function of ARRE-2 DNA binding proteins, dsRNA may function to regulate NF90 activity. This is not a novel activity associated with dsRNA, since previous studies have demonstrated dsRNA regulatory effects related to NF- κ B activation (39–41). Activation of NF- κ B occurs through the phosphorylation of I- κ B on the I- κ B/NF- κ B heteroduplex, leading to the release of NF- κ B and translocation to the nucleus. The presence of dsRNA likely leads to the phosphorylation of I- κ B through the activation of PKR (39–41). We have demonstrated NF90 as an additional substrate for PKR. Phosphorylation of ARRE-2 binding proteins, including NFAT1 and likely NF90, appears to be an essential regulatory mechanism (1, 17, 21, 28–30). Cortesy and Kao suggest that the phosphorylation of NF90/NF45 heteroduplex is necessary for ARRE-2 binding and/or transcriptional activity (31). Other investigators have identified 90 kDa proteins that display biochemical characteristics similar to those of NF90 (33–35). First, Rice et al. identified a 90 kDa protein that could bind to adenovirus VAI RNA and authentic dsRNA, but not single-stranded RNA (35). This protein was localized to the ribosome salt wash fraction of rabbit reticulocyte lysates and could act as a substrate for PKR. Second, antiserum to PKR has been described to co-immunoprecipitate a 90 kDa protein (34). This immune precipitation may be due to cross-reactive antiserum or due to both PKR and the 90 kDa protein associating with dsRNA. In vivo evidence suggests that this 90 kDa protein can be phosphorylated by PKR during herpes simplex virus I infection (33).

The mechanism of activation and nuclear translocation of NF90 and NF45 have not been determined. Activation can be induced in the presence of PMA and ionomycin and partially inhibited by the addition of cyclosporin A (1, 17, 21, 27–30, 56, 57). With NFAT1, it is believed that cyclosporin A acts by binding to intracellular immunophilin receptors and inhibiting the activity of the phosphatase, calcineurin (56, 57). These results may suggest that ARRE-2 DNA-protein interaction may be regulated by the phosphorylation state of regulatory subunit(s).

NFAT1 protein is expressed in several classes of immune system cells consistent with the detection of nuclear ARRE-2 DNA-binding activity in activated T-cell, B-cells, mast cells, and natural killer cells (58–64). However, the ARRE-2 DNA binding class of proteins does not appear to be localized

solely to immune cells. NFATc mRNA is expressed at high levels in skeletal muscle and in activated lymphoid cells, NFAT4 mRNA is predominantly expressed in the thymus and NFAT3 mRNA is most strongly expressed outside the immune system (20, 22, 24). Given that these four proteins differ in their patterns of expression in cells and tissues and in their representation in resting and activated cells, it may suggest that each protein may serve specific functions. This hypothesis is supported through distinct ARRE-2 DNA binding activity associated with NF90/NF45 heteroduplex formation (31, 32). Our results suggest the presence of NF90 not only in T-cells but also in nonimmune cells. Antiserum to NF90 has previously been used to identify the presence of an antigenically related protein in HeLa cell nuclear fractions (32). These authors also tested cytoplasmic fractions (10000g supernatant) for this protein but observed a large amount of background binding of the antiserum, making evaluation of the amount of NF90 impossible. Our Western blots to detect HeLa cell NF90 utilized nuclear, cytosolic (100000g supernatant) and ribosome-salt wash (100000g pellet) fractions. Results indicated that NF90 in HeLa cells was primarily localized to the ribosome salt wash fraction of the cell lysate, with a small amount detectable in the nuclear fraction. In another nonimmune cell line, 293 cells, NF90 was found to be approximately equally distributed between the nuclear and the cytoplasmic 100000g pellet fraction (68). These results may suggest that NF90 is regulated by a similar subcellular localization mechanism as that observed with other ARRE-2 binding proteins.

PKR is primarily localized to the ribosome salt wash fraction of cell lysates (47). This colocalization of PKR and NF90 may provide a unique mechanism of regulating ARRE-2 DNA binding activity. Activation of PKR in the presence of dsRNA may lead not only to the regulation of translation through the phosphorylation of eIF-2 α and the activation of NF- κ B leading to transcriptional activation but also to the regulation of NF90 activity. At this point it is unclear if the phosphorylation of NF90 by PKR leads to increased or decreased ARRE-2 DNA binding and subsequent transcriptional activity.

In summary, we have characterized NF90 as a dsRNA-binding protein that can act as a substrate for PKR. It is likely that dsRNA and/or phosphorylation of NF90 regulates subsequent ARRE-2 DNA binding and transcriptional activity possibly by modulating movement from the ribosome or translocation to the nucleus. NF90 was identified in both T-cells and nonimmune cells, although the transcriptional role of NF90 in nonimmune cells is unclear. Future investigations will focus on deciphering the regulatory potential of dsRNA related to ARRE-2 binding and transcriptional activity.

REFERENCES

- Rao, A. (1994) *Immunol. Today* 15, 274–281.
- Crabtree, G. R., and Clipstone, N. A. (1994) *Annu. Rev. Biochem.* 63, 1045–1083.
- Jain, J., Loh, C., and Rao, A. (1995) *Curr. Opin. Immunol.* 7, 333–342.
- Luo, C., Burgeon, E., and Rao, A. (1996) *J. Exp. Med.* 184, 141–147.
- Schreiber, S. L., and Crabtree, G. R. (1992) *Immunol. Today* 13, 136–142.
- Serfling, E., Avois, A., and Neumann, M. (1995) *Biochim. Biophys. Acta* 1263, 181–200.
- Crabtree, G. R. (1989) *Science* 243, 355–361.
- Shibuya, H., and Taniguchi, T. (1989) *Nucl. Acids Res.* 17, 9173–9185.
- Riegel, J. S., Cortes, B., Flanagan, W. M., and Crabtree, G. R. (1992) in *Chemical Immunology* (Kishimoto T., ed) Vol. 51, pp 266–298, Karger, Basel.
- Cockerill, P. N., Shannon, F., Bert, A. G., Ryan, G. R., and Vadas, M. A. (1993) *Proc. Natl. Acad. Sci. U.S.A.* 90, 2466–2470.
- Masuda, E. S., Tokumitsu, H., Tsuboi, A., Shlomai, J., Hung, P., Arai, K. I., and Arai, N. (1993) *Mol. Cell. Biol.* 13, 7399–7407.
- Chuvpilo, S., Schomberg, C., Gerwig, R., Heinfli, A., Reeves, R., Grummt, F., and Serfling, E. (1993) *Nucl. Acids Res.* 21, 5694–5704.
- Szabo, S. J., Gold, J. S., Murphy, T. L., and Murphy, K. M. (1993) *Mol. Cell. Biol.* 13, 4793–4805.
- Rooney, J. W., Hodge, M. R., McCaffrey, P. G., Rao, A., and Glimcher, L. H. (1994) *EMBO J.* 13, 625–633.
- Lee, H. J., Masuda, E. S., Arai, N., Arai, K., and Yokota, T. (1995) *J. Biol. Chem.* 270, 17541–17550.
- Goldfield, A. E., McCaffrey, P. G., Strominger, J. L., and Rao, A. (1993) *J. Exp. Med.* 178, 1365–1379.
- McCaffrey, P. G., Perrino, B. A., Soderling, T. R., and Rao, A. (1993) *J. Biol. Chem.* 268, 3747–3752.
- Flanagan, W. M., Cortes, B., Bram, R. J., and Crabtree, G. R. (1991) *Nature* 352, 803–807.
- Ho, S., Thomas, D. J., Timmerman, L. A., Li, X., Franke, U., and Crabtree, G. R. (1995) *J. Biol. Chem.* 270, 19898–19907.
- Hoey, T., Sun, Y.-L., Williamson, K., and Xu, X. (1995) *Immunity* 2, 461–472.
- Luo, C., Burgeon, E., Carew, J. A., McCaffrey, P. G., Badalian, T. M., Lane, W. S., Hogan, P. G., and Rao, A. (1996) *Mol. Cell. Biol.* 16, 3955–3966.
- Masuda, E. S., Naito, Y., Tokumitsu, H., Campbell, D., Saito, F., Hannum, C., Arai, K.-I., and Arai, N. (1995) *Mol. Cell. Biol.* 15, 2697–2706.
- McCaffrey, P. G., Luo, C., Kerppola, T. K., Jain, S., Badalian, T. M., Ho, A. H., Burgeon, E. B., Lane, W. S., Lambert, J. N., Curran, T., Verdine, G. L., Rao, A., and Hogan, P. G. (1993) *Science* 262, 750–754.
- Northrop, J. P., Ho, S. N., Chen, L., Thomas, D. J., Timmerman, L. A., Nolan, G. P., Admon, A., and Crabtree, G. R. (1994) *Nature* 369, 497–502.
- Jain, J., Burgeon, E., Badalian, T. M., Hogan, P. G., and Rao, A. (1995) *J. Biol. Chem.* 270, 4138–4145.
- Nolan, G. P. (1994) *Cell* 77, 795–798.
- Loh, C., Shaw, K. T.-Y., Carew, J., Viola, J. P. B., Luo, C., Perrino, B. A., and Rao, A. (1996) *J. Biol. Chem.* 271, 10884–10891.
- Loh, C., Carew, J. A., Kim, J., Hogan, P. G., and Rao, A. (1996) *Mol. Cell. Biol.* 16, 3945–3954.
- Park, J., Yaseen, N. R., Hogan, P. G., Rao, A., and Sharma, S. (1995) *J. Biol. Chem.* 270, 20653–20659.
- Luo, C., Shaw, K. T.-Y., Raghavan, A., Aramburu, J., Garcia-Cozar, F., Perrino, B. A., Hogan, P. G., and Rao, A. (1996) *Proc. Natl. Acad. Sci. U.S.A.* 93, 8907–8912.
- Cortes, B., and Kao, P. N. (1994) *J. Biol. Chem.* 269, 20682–20690.
- Kao, P. N., Chen, L., Brock, G., Ng, J., Kenny, J., Smith, A. J., and Cortes, B. (1994) *J. Biol. Chem.* 269, 20691–20699.
- Chou, J., Chen, J.-J., Gross, M., and Roizman, B. (1995) *Proc. Natl. Acad. Sci. U.S.A.* 92, 10516–10520.
- Thomis, D. C., Doohan, J. P., and Samuel, C. E. (1992) *Virology* 188, 33–46.
- Rice, A. P., Kostura, M., and Mathews, M. B. (1989) *J. Biol. Chem.* 264, 20632–20637.
- Farrell, P. J., Balkow, K., Hunt, T., Jackson, R. J., and Trachsel, H. (1977) *Cell* 11, 187–200.
- Levin, D., and London, I. M. (1978) *Proc. Natl. Acad. Sci. U.S.A.* 75, 1121–1125.

38. Samuel, C. E. (1979) *Proc. Natl. Acad. Sci. U.S.A.* 264, 600–604.
39. Kumar, A., Haque, J., Lacoste, J., Hiscott, J., and Williams, B. R. (1994) *J. Biol. Chem.* 269, 6288–6292.
40. Maran, A., Maitra, R. K., Kumar, A., Dong, B., Xiao, W., Li, G., Williams, B. R., Torrence, P. F., and Silverman, R. H. (1994) *Science* 265, 789–792.
41. Offermann, M. K., Zimring, J., Mellits, K. H., Hagan, M. K., Shaw, R., Medford, R. M., Mathews, M. B., Goodbourne, S., and Jagus, R. (1995) *Eur. J. Biochem.* 232, 28–36.
42. Lee, S. B., and Esteban, M. (1994) *Virology* 199, 491–496.
43. Kibler, K. V., Shors, T., Perkins, K. B., Zeman, C. C., Banaszak, M. P., Biesterfeldt, J., Langland, J. O., and Jacobs, B. L. (1997) *J. Virology* 71, 1992–2003.
44. Lee, S. B., Rodriguez, D., Rodriguez, J. R. and Esteban, M. (1997) *Virology* 231, 81–88.
45. Chong, K. L., Schappert, K., Meurs, E., Feng, F., Donahue, T. F., Friesen, J. D., Hovanessian, A. G., and Williams, B. R. G. (1992) *EMBO J.* 11, 1553–1562.
46. Meurs, E., Galabru, G. N., Barber, G. N., Katze, M. G., and Hovanessian, A. G. (1993) *Proc. Natl. Acad. Sci. U.S.A.* 90, 232–236.
47. Langland, J. O., and Jacobs, B. L. (1992) *J. Biol. Chem.* 267, 10729–10736.
48. Langland, J. O., Pettiford, S. M., and Jacobs, B. L. (1995) *Protein Expression Purification* 6, 25–32.
49. Chu, N. M., Janckila, A. J., Wallace, J. H., and Yam, L. T. (1989) *J. Histochem. Cytochem.* 37, 257–263.
50. Chang, H.-W., Watson, J. C., and Jacobs, B. L. (1992) *Proc. Natl. Acad. Sci. U.S.A.* 89, 4825–4829.
51. Cleveland, D. W., Fischer, S. G., Kirschner, M. W., and Laemmli, U.K. (1977) *J. Biol. Chem.* 252, 1102–1106.
52. St. Johnston, D., Brown, N. H., Gall, J. G., and Jantsch, M. (1992) *Proc. Natl. Acad. Sci. U.S.A.* 89, 10979–10983.
53. Gibson, T. J., and Thompson, J. D. (1994) *Nucl. Acids Res.* 22, 2552–2556.
54. Samuel, C. E. (1993) *J. Biol. Chem.* 268, 7603–7606.
55. Hovanessian, A. G. (1989) *J. Interferon Res.* 9, 641–647.
56. Liu, J., Farmer, J. D., Lane, W. S., Friedman, J., Weissman, I., and Schreiber, S. L. (1991) *Cell* 66, 807–815.
57. Liu, J., Albers, M. W., Wandless, T. J., Luan, S., Alberg, D. G., Belshaw, P. J., Cohen, P., MacKintosh, C., Klee, C. B., and Schreiber, S. L. (1992) *Biochemistry* 31, 3896–3901.
58. Aramburu, J. L., Azzoni, L., Rao, A., and Perussia, B. (1995) *J. Exp. Med.* 182, 801–810.
59. Brabletz, T., Pietrowski, I., and Serfling, E. (1991) *Nucl. Acids Res.* 19, 61–67.
60. Choi, M. S. K., Brines, R. D., Holman, M. J., and Klaus, G. G. B. (1994) *Immunity* 1, 179–187.
61. Hutchinson, L. E., and McCloskey, M. A. (1995) *J. Biol. Chem.* 270, 16333–16338.
62. Prieschl, E. E., Gouilleux-Gruart, V., Walker, C., Harrer, N. E., and Baumruker, T. (1995) *J. Immunol.* 154, 6112–6119.
63. Venkataraman, L., Francis, D. A., Wang, Z., Liu, J., Rothstein, T. L., and Sen, R. (1994) *Immunity* 1, 189–196.
64. Yaseen, N. R., Maizel, A. L., Wang, F., and Sharma, S. (1993) *J. Biol. Chem.* 268, 14285–14293.
65. Shaw, J. P., Utz, P. J., Durand, D. B., Toole, J. J., Emmel, E. A. and Crabtree, G. R. (1988) *Science* 241, 202–205.
66. Langland, J. O., Langland, L. A., Browning, K. S., and Roth, D. A. (1996) *J. Biol. Chem.* 271, 4539–4544.
67. Judware, R., and Petryshyn, R. (1991) *Mol. Cell. Biol.* 11, 3259–3267.
68. Liao, H. J., Kobayashi, R., and Mathews, M. B. (1998) *Proc. Natl. Acad. Sci. U.S.A.* 95, 8514–8519.
69. Bass, B. L., Hurst, S. R., and Singer, J. D. (1994) *Curr. Biol.* 4, 301–314.

BI982410U