Characterization of Two Evolutionarily Conserved, Alternatively Spliced Nuclear Phosphoproteins, NFAR-1 and -2, That Function in mRNA Processing and Interact with the Double-stranded RNA-dependent Protein Kinase, PKR*

Laura R. Saunders, Darren J. Perkins, Siddharth Balachandran, Rebecca Michaels, Ryan Ford, Akila Mayeda‡§, and Glen N. Barber¶

From the Department of Microbiology and Immunology and Sylvester Comprehensive Cancer Center and ‡Department of Biochemistry and Molecular Biology, University of Miami School of Medicine, Miami, Florida 33136

We report here the isolation and characterization of two proteins, NFAR-1 and -2, which were isolated through their ability to interact with the dsRNA-dependent protein kinase, PKR. The NFAR proteins, of 90 and 110 kDa, are derived from a single gene through alternative splicing and are evolutionarily conserved nuclear phosphoproteins that interact with double-stranded RNA. Both NFAR-1 and -2 are phosphorylated by PKR, reciprocally co-immunoprecipitate with PKR, and colocalize with the kinase in a diffuse nuclear pattern within the cell. Transfection studies indicate that the NFARs regulate gene expression at the level of transcription, probably during the processing of pre-mRNAs, an activity that was increased in fibroblasts lacking PKR. Subsequent functional analyses indicated that amino acids important for NFAR's activity were localized to the C terminus of the protein, a region that was found to specifically interact with FUS and SMN, proteins also known as regulators of RNA processing. Accordingly, both NFARs were found to associate with both pre-mRNAs and spliced mRNAs in post-transcriptional studies, similar to the known splicing factor ASF/SF-2. Collectively, our data indicate that the NFARs may facilitate double-stranded RNA-regulated gene expression at the level of post-transcription and possibly contribute to host defense-related mechanisms in the cell.

The interferons (IFNs)[1] are pleotropic cytokines, considered important in host defense, that manifest their biological properties by inducing a number of responsive genes (1). One key IFN-inducible gene is the serine/threonine, dsRNA-dependent protein kinase, PKR, with a molecular mass of 68 kDa in human cells (2). PKR is a member of a previously described family of evolutionary conserved dsRNA binding molecules that includes *Escherichia coli* RNase III, *Drosophila* staufen, and *Xenopus* 4F.1 (3, 4). Interaction with dsRNA structures of greater than 35 bp causes PKR to autophosphorylate and subsequently to catalyze the phosphorylation of substrate targets, the most well characterized being the α subunit of eukaryotic initiation factor, eIF2α (5–7). Phosphorylated eIF2α effectively sequesters eIF2B, a rate-limiting component of translation, and causes a dramatic inhibition of protein synthesis in the cell (8, 9).

The majority of studies relating to PKR's function have focused on the kinase's participation in IFN's retaliatory response to virus infection. For example, PKR can be activated by a number of virus specific RNAs (10–12). PKR-deficient mice are extremely sensitive to lethal pulmonary disease following infection with vesicular stomatitis virus or influenza virus, further emphasizing the importance of PKR in host defense (13). Since activation of PKR is highly detrimental to the virus life cycle, numerous viruses have evolved mechanisms to neutralize the function of this kinase. For example, vaccinia virus encodes two proteins capable of inhibiting PKR. The first of these products, referred to as E3L, contains a dsRNA binding domain similar to those found in PKR (14–16). E3L, which is primarily detected in the nucleus, competes for dsRNA activators and may even bind to and inhibit the kinase through dsRNA bridging (17). A second vaccinia protein, K3L, shares homology to the known PKR substrate eIF2α and is thought to function by competitively sequestering the kinase (15, 18, 19). In addition, adenovirus may utilize VAI RNA to suppress PKR, while hepatitis C has been reported to encode PKR-interacting proteins referred to as NS5a and E2 (20–23).

Recent data have also revealed that PKR almost certainly plays a role in signaling events in the cell, including dsRNA-induced apoptosis. For example, recombinant vaccinia viruses overexpressing wild-type PKR have been shown to induce the rapid apoptosis of HeLa cells (16, 24, 25). Murine fibroblasts deficient for PKR activity have also been reported to be more resistant to dsRNA and tumor necrosis factor-α-induced apoptosis (26, 27). Our laboratory has recently demonstrated that cells inducibly overexpressing functional PKR, but not a dominant-negative PKR variant, are sensitive to dsRNA-mediated and virus-induced cell death (28). PKR-dependent apoptosis was accompanied by the induction of Fas, a member of the tumor necrosis factor receptor family, and was mediated

---

[1] The abbreviations used are: IFN, interferon; dsRNA, double-stranded RNA; bp, base pair(s); eIF2α, eukaryotic initiation factor 2α; PMSF, phenylmethylsulfonyl fluoride; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-PCR; MEF, mouse embryonic fibroblast; snRNP, small nuclear ribonucleoprotein.

This paper is available on line at http://www.jbc.org

through the FADD death-signaling pathway (28). Although the mechanisms of PKR-induced apoptosis remain to be fully clarified, it has been reported that PKR may play a role in the induction of NF-κB, a key regulator of cell survival, in response to viral infection and dsRNA. For example, murine fibroblasts lacking PKR exhibit diminished responses to dsRNA signaling as indicated by reduced activity of NF-κB and IRF-1 (29). Although presently controversial, this regulation may involve PKR influencing the activity of the IκB kinase (IKK) complex leading to the autophosphorylation of IKKβ and degradation of IκB (30, 31). In addition to these roles, PKR may also be involved in signaling pathways involving Ras, interleukin-3, and growth factors such as platelet-derived growth factor (32). Collectively, these data strongly suggest that substrates of PKR other than eIF2α probably exist in the cell, although their identification and characterization have thus far remained elusive.

Accordingly, to identify possible regulators and/or substrates of PKR, we used the yeast two-hybrid system to isolate proteins that associate with this kinase. Here we report the isolation and characterization of two evolutionarily conserved, dsRNA-binding nuclear proteins, NFAR-1 and -2 (for nuclear factors associated with dsRNA). This work confirms that the NFAR gene on chromosome 19 gives rise to two alternatively spliced variants and correctly identifies the two NFAR proteins. Aside from demonstrating that the NFARs associate with PKR in yeast, we show that both NFAR-1 and -2 share homology with eIF2α, the known PKR cytoplasmic substrate, and are themselves substrates for PKR. In transfected cells, both NFARs were found to regulate the transcription of co-transfected reporter genes, probably at the level of mRNA elongation, an effect that was increased in PKR null fibroblasts. Transfection studies further indicated that NFAR-2 rather than NFAR-1 appeared to have more potent regulatory activity and that the C-terminal region unique to NFAR-2 exhibited constitutive activity and interacted with SMN and FUS, two proteins know to play a role in mRNA processing. Finally, we report that NFAR can associate with active spliceosomes. Our data indicate that the NFARs are evolutionary conserved mediators of gene expression that may function as proximal targets for PKR and may play a role in regulating gene expression in response to dsRNA-regulated signaling events in the cell.

EXPERIMENTAL PROCEDURES

Plasmids—Construction of PKR K296R in pET11a (Novagen, CA) has been previously described (33). To fuse the GAL4 DNA binding domain in frame with PKR, the *Eco*RI site in pGBT9 (CLONTECH) was mutagenized to an *Nde*I site to create pGBT10. Full-length PKR K296R was digested with *Nde*I and *Bam*HI and then inserted into similarly treated pGBT10, generating pGBT DII.

The full-length cDNAs of NFAR-1 and NFAR-2 were generated by subcloning C terminus *Pst*I-*Bgl*II NFAR fragments from yeast two-hybrid pGAD10 vectors into pGAD424 (CLONTECH). The N terminus of NFAR was amplified by PCR from cDNAs isolated by screening a Jurkat T-cell library and was joined to the C terminus of NFAR at an endogenous *Pst*I site. An *Nde*I site was engineered into the N terminus of NFAR during PCR. For bacterial expression, *Nde*I-*Bgl*II-digested NFAR-1 and -2 from pGAD424 were subcloned into pET14b digested with *Nde*I and *Bam*HI (Novagen). For mammalian cell expression, NFAR cDNAs from pGAD were digested with *Eco*RV, followed by treatment with Klenow fragment and dNTPs to blunt the N terminus. NFAR was then subsequently digested with *Bgl*II and subcloned into *Eco*RV-*Bam*HI-digested pcDNA3.1 (Invitrogen, CA). To fuse the C terminus of NFAR-2 to the Gal4 binding domain, amino acids 666–894 of NFAR-2 were amplified by PCR using NFAR-2 as a template and subcloned into the *Eco*RI and *Pst*I sites of pGBT9.

NFAR-2 deletion mutations M5 (Δ7–173), M7 (Δ174–232), M8 (Δ238–384), M10 (Δ1–369), M11 (Δ1–588), and NFAR-1 M10 (Δ1–369) were created in pET14b and pcDNA3.1 vectors using the Stratagene QuickChange site-directed mutagenesis kit. NFAR-2 M12 (Δ586–894) and NFAR M13 (Δ1–369, 586–894) were created by digesting the con-

structs with *Hin*dIII, which cuts at an endogenous site in NFAR after the dsRNA binding domains, and in the MCS of pcDNA3.1 and then religating the vector to remove the C terminus of NFAR-2. The NFAR M9 variant (amino acids 1–417) was created by cloning the NFAR *Nde*I-*Pst*I fragment into pGAD424 and subsequently into pET11 and pcDNA3.1. All NFAR mutants were confirmed by DNA sequencing.

Yeast Strains and Two-hybrid Screen—PKR DII and NFAR (amino acids 666–894) in pGBT9 were used to transfect the yeast strains *Saccharomyces cerevisiae* Y190 and PJ694a, respectively, as described (CLONTECH).

Histidine-tagged Fusion Protein Expression and Purification—PKR was placed into pET14b such that the amino terminus of the kinase was fused in frame with 6× histidine residues (Novagen). The resultant plasmid (pET14bPKR) was used to transfect *E. coli* BL21(DE3)pLysS (Novagen), and PKR was purified over nickel columns as previously described (34). His-tagged proteins were further purified on a gel filtration column, Superose 12 (Amersham Pharmacia Biotech), examined by Coomassie Blue-stained SDS-polyacrylamide gels or immunoblot using anti-PKR monoclonal antibody (35).

NFAR cDNAs in pET11a were expressed in *E. coli* BL21(DE3)pLysS. Bacteria were lysed in 5 mM imidazole, 0.5 mM NaCl, 20 mM Tris-HCl (pH 7.9), 0.2 mM PMSF, and 100 μg/ml aprotinin. Inclusion bodies were resuspended in the above lysis buffer containing 6 M urea and applied to a nickel affinity column (Novagen). The column was washed with the above lysis buffer containing 6 M urea, followed by washes containing 60 mM NaCl. The proteins were eluted with 1 M imidazole, 0.5 M NaCl, 20 mM Tris (pH 7.5), 0.2 mM PMSF, 100 μg/ml aprotinin, and 6 M urea and dialyzed overnight (20 mM Tris-HCl, pH 8.0, 50 mM NaCl, 0.1 mM EDTA, 0.2 mM PMSF, 100 μg/ml aprotinin, 10% glycerol) to remove the denaturing reagent. Purified His-tagged NFAR proteins were examined by Coomassie Blue-stained SDS-polyacrylamide gels and by immunoblot using a monoclonal antibody to the His tag (R & D Systems).

For expression of ^{35}S-labeled NFAR *in vitro*, the TNT coupled transcription/translation system (Promega) was used to synthesize proteins from the pcDNA3.1 NFAR vectors using T7 polymerase.

dsRNA Binding Assays—The dsRNA binding activity of NFAR was measured by incubating purified His-tagged NFAR proteins, ^{35}S-labeled NFAR proteins, or lysate from HeLa cells with poly(I-C)-agarose beads. The HeLa cells were lysed in binding buffer (20 mM Tris-HCl, pH 7.5, 50 mM KCl, 1% Nonidet P-40, 1 mM EDTA, 1 mM dithiothreitol, 100 mM PMSF, and 1 μg/ml of aprotinin). The lysates were adjusted to 200, 500, and 1000 mM NaCl concentrations and incubated with 100 μl of poly(I-C)-agarose slurry (Amersham Pharmacia Biotech). Beads were incubated at 4 °C for 3 h and then washed five times with 1 ml of the binding buffer at the indicated NaCl concentrations. After the final wash, the beads were resuspended in protein loading dye, boiled, and loaded onto polyacrylamide gels. Proteins were transferred to nitrocellulose membranes and probed with anti-NFAR antiserum or with anti-histidine monoclonal antibodies.

Confocal Microscopy—HeLa cells grown on coverslips were fixed at room temperature in freshly prepared 2% paraformaldehyde in 1× PBS, washed with 1× PBS, and permeabilized for 30 min on ice with 1% Triton X-100 in 1× PBS and 0.1 M glycine. Antibodies used were anti-PKR (33), anti-SMN (Transduction Laboratories), and hybridoma supernatant of anti-FUS monoclonal antibody (gift from David Ron) followed by anti-mouse secondary antibody conjugated with Texas Red (Molecular Probes, Inc., Eugene, OR). Cells were double-labeled with anti-NFAR rabbit polyclonal serum and then rabbit secondary antibody conjugated with fluorescein isothiocyanate (Molecular Probes).

Kinase Assays—For *in vitro* assays, purified PKR was incubated with approximately stoichiometric amounts of His-tagged NFAR-1 or -2 in equal volumes of KCl buffer (20 mM Tris-HCl, pH 7.4, 25 mM KCl, 1 mM dithiothreitol, 0.1 mM PMSF, 1 μg/ml aprotinin, and 5% glycerol) in the presence or absence of dsRNA as described (28).

Northern Blot Analysis—Adult and fetal human multiple tissue Northern blots (CLONTECH) were hybridized with radiolabeled cDNA representing NFAR-1. Blots were washed and autoradiographed prior to being reprobed with radiolabeled actin cDNA.

Co-immunoprecipitation Assays—For *in vitro* analysis, equal amounts of His-tagged PKR and NFAR-1 were incubated in binding buffer (0.2% Nonidet P-40, 80 mM NaCl, 1 mM EDTA, 1 μg/ml aprotinin, 0.2 mM PMSF, 1 mM dithiothreitol, 20 mM Tris-HCl, pH 7.5) on ice for 10 min. To immunoprecipitate PKR, anti-PKR monoclonal antibody or preimmune murine IgG was added to the mix and rotated at 4 °C for 2 h. Protein G-agarose was added for 1 h. The proteins were washed four times in cold incubation buffer and boiled in protein disruption buffer, as above. Proteins were resolved on SDS-polyacrylamide gels and analyzed by immunoblot using anti-NFAR rabbit serum. To immu-

noprecipitate NFAR, rabbit antiserum raised to NFAR-1 or control preimmune rabbit serum was added to the proteins as above. After washing, the proteins were electrophoresed as described and immunoblotted using anti-PKR monoclonal antibody.

For *in vivo* co-immunoprecipitation analysis, HeLa cells (3×10^6/ incubation) were lysed in radioimmune precipitation buffer (1% deoxycholate, 0.1% SDS, 1% Nonidet P-40, 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.2 mM NaF, 0.2 mM PMSF, 0.1 mM dithiothreitol, 1 μg/ml aprotinin, 1 μg/ml leupeptin) and precleared with preimmune rabbit serum or normal mouse IgG and protein G for 1 h. Anti-PKR, anti-NFAR, anti-FUS, anti-SMN, or preimmune antibodies were added to precleared cell extracts and incubated for 1.5 h. Protein G-agarose was added to the mix, and after 3 h the cells were washed with 1 ml of radioimmune precipitation buffer five times. For RNase treatment, 20 units of RNase T1 (Roche Molecular Biochemicals) was added during the third wash and incubated at 4 °C for 15 min. The proteins were examined by immunoblot using anti-PKR, anti-NFAR, anti-FUS, or anti-SMN antibodies.

Transient Transfection Assays—COS-1 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. 6×10^4 cells were plated out in 12-well dishes for luciferase assays, and 0.7 μg of the pcDNA3.1 vector and 0.3 μg of the pGL3 promoter vector were co-transfected using the LipofectAMINE Plus reagents (Life Technologies, Inc.). Cells were lysed 48 h post-transfection in 40 μl of lysis buffer (20 mM Tris-HCl, pH 7.5, 50 mM KCl, 100 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, and 0.2 mM PMSF). Luciferase assays were read on a luminometer (TD 20/20; Promega). All assays were performed independently, a minimum of three times, in duplicate. Mice lacking PKR (strain 129/terSv) were courtesy of Dr. John Bell of Ontario University (36).

RT-PCR—COS-1 cells were transfected as above, except that 1×10^5 cells were seeded into six-well dishes and transfected with 1 μg of the pcDNA3.1 vector and 0.4 μg of the reporter vector. Total RNA was isolated using TRIzol (Life Technologies) according to the manufacturer's protocol. After quantitation, the samples were treated with DNase I (Life Technologies). RT-PCR was performed using Stratagene's ProSTAR Ultra HF RT-PCR system. First strand cDNA synthesis was performed at 37 °C for 30 min. As a control for equal mRNA concentrations, β-actin was reverse transcribed using the following primers: 5′-TGAC-GGGGTCACCCACACTGTGCCCATCTA-3′ and 5′-CTAGAAGCATTT-GCGGTGGACGATGGAGGG-3′. Luciferase mRNA was reversed transcribed using 5′-CAGCGAGACGGAGTATCTTGACGG-3′ and 5′-GAAGGAATCTCTCCCCTCGCGGTG-3′.

PCR products were visualized on 1% agarose gels and quantitated using the Quantity One program (Bio-Rad).

Nuclear Run-on Assay—NFAR-1 or NFAR-2 was co-transfected with pGL3 promoter (Promega) into 100 mm dishes of 1×10^7 COS-1 cells. At 36 h post-transfection, the cells were washed three times in ice-cold $1\times$ SSC, collected, and resuspended in 100 μl of Nonidet P-40 lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl$_2$, 0.1 mM EDTA, 0.5% Nonidet P-40) by pipetting 10 times. Cells were incubated on ice for 5 min, centrifuged, and washed twice in 1 ml of the above buffer and then resuspended in 100 μl of freezing buffer (50 mM Tris-HCl, pH 8.3, 40% glycerol, 5 mM MgCl$_2$, 0.1 mM EDTA) and frozen in liquid nitrogen. The nuclei were thawed on ice and mixed with 100 μl of reaction buffer (10 mM Tris-HCl, pH 8.0; 5 mM MgCl$_2$; 300 mM KCl; 0.5 mM each ATP, CTP, and GTP; and 100 μCi of [α-^{32}P]UTP, 800 Ci/mmol). Following a 20-min incubation at 30 °C, 10 mg/ml DNase I was added, and the nuclei were incubated at 42 °C for 30 min. Labeled RNAs were isolated using TRIzol (Life Technologies). cDNAs representing the N and C terminus of luciferase as well as full-length GAPDH were denatured in NaOH for 30 min at room temperature, followed by neutralization with 6$\times$ SSC on ice. The cDNAs were applied under low vacuum to Hybond N (Amersham Pharmacia Biotech) using a slot blot apparatus. The dried membrane was UV-cross-linked and incubated at 70 °C with prewarmed Rapid-Hyb buffer (Amersham Pharmacia Biotech) for 30 min. Labeled RNAs were denatured at 100 °C for 5 min before being incubated with the blot overnight at 70 °C. The blot was washed for 20 min in 2$\times$ SSC with 0.1% SDS at room temperature, followed by two washes in 0.1$\times$ SSC with 0.1% SDS at 70 °C. The blot was exposed to film for 2–3 weeks.

Immunoprecipitation of Spliceosomes—Immunoprecipitations of *in vitro* splicing reactions using NFAR-specific antiserum and other control antibodies were performed as previously described (37). Anti-heterogeneous nuclear ribonucleoprotein A1 monoclonal antibody (9H10) was a generous gift from Dr. G. Dreyfuss.

RESULTS

Isolation and Identification of NFAR-1 and -2—To identify novel proteins that interact with PKR, we used the yeast two-hybrid system and screened for proteins that associated with the full-length kinase (38). Wild-type PKR is toxic to yeast, since it becomes activated *in vivo* and phosphorylates the yeast eIF2α homologue, SUI2, causing a block in translation (39, 40). Therefore, a catalytically inactive PKR variant with an amino acid substitution in catalytic domain II (K296R) was fused to the Gal4 DNA binding domain and used as bait to screen a Jurkat T-cell cDNA library. Eight positive clones from 4×10^6 transformants screened were found to be identical at the N terminus of the putative open reading frames but to differ in their carboxyl ends, indicating the existence of two related gene products, which we refer to as NFAR-1 and -2. Further isolation of cDNA clones retrieved from hybridization screening of T-cell cDNA libraries and the complete sequencing of the entire NFAR gene from PAC clones confirmed the existence of two related cDNAs that are generated through alternative splicing from a single gene on chromosome 19 (Fig. 1, *A* and *B*; Ref. 41). The two NFAR cDNAs represented open reading frames encoding proteins containing 702 (NFAR-1) and 894 (NFAR-2) amino acids, respectively. The coding region of both alternatively spliced variants begins in exon 2 and encodes through exon 17, at which point NFAR-1 continues to encode one additional exon, exon 18, while NFAR-2 splices to exon 19 and encodes three additional exons not contained in NFAR-1 (Fig. 1*B*).

Data base searches revealed that amino acids 401–468 and 524–591 of both NFAR-1 and -2 represent potential dsRNA binding motifs, similar to those found in PKR (4, 42). Thus, NFAR-1 and -2 are almost certainly members of the dsRNA binding family of proteins that includes the *Drosophila* maternal effect protein staufen and the vaccinia virus protein E3L (4). Data bank comparative analysis further revealed that NFAR-1 and -2 are ~75% homologous at the amino acid level with the *Xenopus* dsRNA-binding proteins 4F.1 and 4F.2, whose function is unknown (3). Significantly, both NFAR proteins were also found to share homology from amino acids 166–235 with the known PKR substrate eIF2α (Fig. 1*C*; 23% identity, 49% conservation over 69 amino acids). It is notable that this homologous region of eIF2α contains serine 51, the single target site for PKR phosphorylation (9). More extensive regions of homology exist from residue 222 to 235 and include a conserved VIRV box. This area also entirely overlaps with the vaccinia virus-encoded PKR inhibitor, K3L, an eIF2α homologue (15, 18). It has been reported that K3L binds 400-fold more efficiently than eIF2α to PKR and suppresses the function of the kinase (19).

The sequences of the NFAR cDNA clones were homologous to two partially overlapping clones in the data banks, referred to as MPP-4 (43). However, the two MPP-4 clones are partial representations of the NFAR gene with the coding regions of these genes only extending to amino acids 404 and 611 of NFAR-1. Our sequence was also similar (85% identity at the amino acid level) to a protein referred to as NF-90 (44). However, careful comparison of the NFAR-2 cDNA sequence indicates that 2 bp have been inserted into the NF-90 sequence (nucleotides 1798 and 1799 of the NF-90 coding region), producing a shift in the reading frame to generate a nonnatural hybrid protein. A recent isolate, DRBP76, with unknown function, appears to be 99% identical to NFAR-1 (45), while the human NFAR-2 gene does not appear to have been previously isolated.

In Vivo Expression and Tissue Distribution of NFAR-1 and -2—To start to characterize the expression profile of the NFARs, selected cell lines were examined by immunoblot anal-

A

```
NFAR 1  M R P M R I F V N D D R H V M A K H S S V Y P T Q E E L E A V Q N M V S H T E R A L K A V S D W I D  50
NFAR 2  M R P M R I F V N D D R H V M A K H S S V Y P T Q E E L E A V Q N M V S H T E R A L K A V S D W I D  50

NFAR 1  E Q E K G S S E Q A E S D N M D V P P E D D S K E G A G E Q K T E H M T R T L R G V M R V G L V A K  100
NFAR 2  E Q E K G S S E Q A E S D N M D V P P E D D S K E G A G E Q K T E H M T R T L R G V M R V G L V A K  100

NFAR 1  C L L L K G D L D L E L V L L C K E K P T T A L L D K V A D N L A I Q L A A V T E D K Y E I L Q S V  150
NFAR 2  C L L L K G D L D L E L V L L C K E K P T T A L L D K V A D N L A I Q L A A V T E D K Y E I L Q S V  150

NFAR 1  D D A A I V I K N T K E P P L S L T I H L T S P V V R E E M E K V L A G E T L S V N D P P D V L D R  200
NFAR 2  D D A A I V I K N T K E P P L S L T I H L T S P V V R E E M E K V L A G E T L S V N D P P D V L D R  200

NFAR 1  Q K C L A A L A S L R H A K W F Q A R A N G L K S C V I V I R V L R D L C T R V P T W G P L R G W P  250
NFAR 2  Q K C L A A L A S L R H A K W F Q A R A N G L K S C V I V I R V L R D L C T R V P T W G P L R G W P  250

NFAR 1  L E L L C E K S I G T A N R P M G A G E A L R R V L E C L A S G I V M P D G S G I Y D P C E K E A T  300
NFAR 2  L E L L C E K S I G T A N R P M G A G E A L R R V L E C L A S G I V M P D G S G I Y D P C E K E A T  300

NFAR 1  D A I G H L D R Q Q R E D I T Q S A Q H A L R L A A F G Q L H K V L G M D P L P S K M P K K P K N E  350
NFAR 2  D A I G H L D R Q Q R E D I T Q S A Q H A L R L A A F G Q L H K V L G M D P L P S K M P K K P K N E  350

NFAR 1  N P V D Y T V Q I P P S T T Y A I T P M K R P M E E D G E E K S P S K K K K K I Q K K E E K A E P P  400
NFAR 2  N P V D Y T V Q I P P S T T Y A I T P M K R P M E E D G E E K S P S K K K K K I Q K K E E K A E P P  400

NFAR 1  Q A M N A L M R L N Q L K P G L Q Y K L V S Q T G P V H A P I F T M S V E V D G N S F E A S G P S K  450
NFAR 2  Q A M N A L M R L N Q L K P G L Q Y K L V S Q T G P V H A P I F T M S V E V D G N S F E A S G P S K  450

NFAR 1  K T A K L H V A V K V L Q D M G L P T G A E G R D S S K G E D S A E E T E A K P A V V A P A P V V E  500
NFAR 2  K T A K L H V A V K V L Q D M G L P T G A E G R D S S K G E D S A E E T E A K P A V V A P A P V V E  500

NFAR 1  A V S T P S A A F P S D A T A E Q G P I L T K H G K N P V M E L N E K R R G L K Y E L I S E T G G S  550
NFAR 2  A V S T P S A A F P S D A T A E Q G P I L T K H G K N P V M E L N E K R R G L K Y E L I S E T G G S  550

NFAR 1  H D K R F V M E V E V D G Q K F Q G A G S N K K V A K A Y A A L A A L E K L F P D T P L A L D A N K  600
NFAR 2  H D K R F V M E V E V D G Q K F Q G A G S N K K V A K A Y A A L A A L E K L F P D T P L A L D A N K  600

NFAR 1  K K R A P V P V R G G P K F A A K P H N P G F G M G G P M H N E V P P P P N L R G R G R G G S I R G  650
NFAR 2  K K R A P V P V R G G P K F A A K P H N P G F G M G G P M H N E V P P P P N L R G R G R G G S I R G  650

NFAR 1  R G R G R G F G G A N H G G Y M N A G A G Y G S Y G Y G G N S A T A G Y S D F F T D C Y G Y H D F G  700
NFAR 2  R G R G R G F G G A N H G G Y M N A G A G Y G S Y G Y G G N S A T A G Y S Q F Y S N G G H S G N A S  700

NFAR 1  S S                                                                                             702
NFAR 2  G G G G G G G G G G S S S G Y G S Y Y Q G D N Y N S P V P P K H A G K K Q P H G G Q Q K P S Y G S G Y Q  750

NFAR 1                                                                                                  702
NFAR 2  S H Q G Q Q Q S Y N Q S P Y S N Y G P P Q G K Q K G Y N H G Q G S Y S Y S N S Y N S P G G G G G S D  800

NFAR 1                                                                                                  702
NFAR 2  Y N Y E S K F N Y S G S G G R S G G N S Y G S G G A S Y N P G S H G G Y G G G S G G G S S Y Q G K Q  850

NFAR 1                                                                                                  702
NFAR 2  G G Y S Q S N Y N S P G S G Q N Y S G P P S S Y Q S S Q G G Y G R N A D H S M N Y Q Y R  894
```
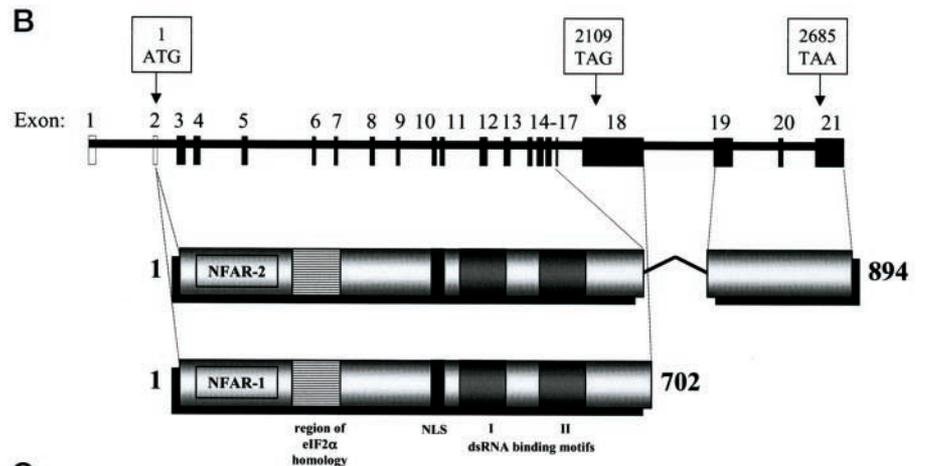
FIG. 1. **Structure and sequence of NFAR-1 and -2.** *A*, amino acid alignment of NFAR-1 and -2. The *shaded area* represents a region of homology to eIF2α. The *underlined sequence* indicates a bipartite nuclear localization signal. The *boxed regions* highlight the dsRNA binding domains. The *boxed* and *shaded* regions of NFAR-1 indicate additional amino acids encoded from exon 18 present in NFAR-1 and absent from NFAR-2. GenBank™ accession numbers for NFAR-1 and -2 cDNAs are AF167569 and AF167570, respectively. The NFAR genomic sequences are AF320227–AF320247. *B*, intron-exon organization of the NFAR gene. Both NFAR cDNAs begin coding at the end of exon 2 and continue through exon 17. Only NFAR-1 contains exon 18, which encodes a stop codon (TAG) at nucleotide 2109, and the 3′-untranslated region of NFAR-1. NFAR-2 splices from exon 17 to encode three additional exons not found in NFAR-1. The stop codon is found in exon 21 (TAA) at nucleotide 2685 and also encodes the 3′-untranslated region. *C*, amino acid alignment of NFAR (amino acids 166–235), eIF2α (*H*, human; *R*, rat; *D*, *Drosophila*), and the vaccinia virus-encoded PKR inhibitor K3L. Ultraconserved residues are *highlighted* in *red*, and conservative substitutions are in *blue*.

B

C

```
NFAR-1     HLTSPVVREEMEKVLAGETLSVNDPPDVLDRQKCLAALASLRHAKWFQARANGLKSCVIVIRVLRD    235
eIF2α(H)   PEVEDVVMVNVRSIAEMGAYVSLLEYNNIEGMILLSELSRRRIRSINKLIRIGRNECVVVIRVDKE    78
eIF2α(R)   PEVEDVVMVNVRSIAEMGAYVSLLEYNNIEGMILLSELSRRRIRSINKLIRIGRNECVVVIRVDKE    78
eIF2α(D)   PEIEDVVMVNVLSIAEMGAYVHLLEYNNIEGMILLSELSRRRIRSINKLIRVGKTEPVVVIRVDKE    77
K3L        PNAGDVIKGRVYEKDYAL-YIYLFDYPHSEAILAESVKMHMDRYVEYRDKLVGKTVKVKVIRVDYT    73
```

ysis using a novel rabbit antiserum raised in our laboratory to *E. coli* purified His-tagged NFAR-1. Fig. 2*A* confirmed that NFAR exists as two major species with approximate molecular

masses of 90 and 110 kDa in the cells examined, including Jurkat and HeLa cells. To determine if the isolated NFAR cDNA clones encoded similar sized proteins, His-tagged
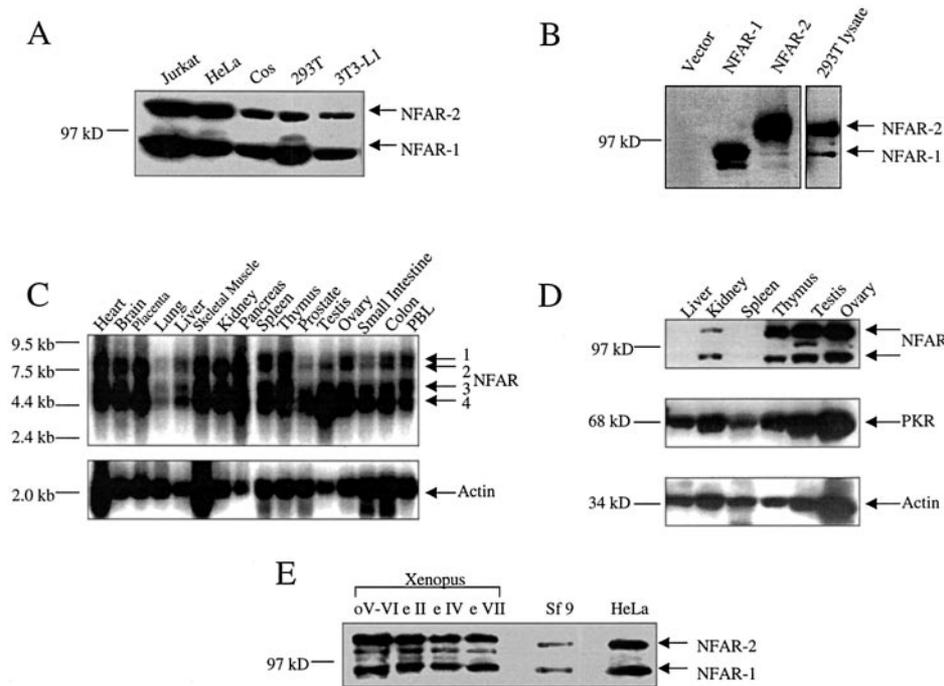
FIG. 2. **NFAR-1 and -2 cDNA expression and tissue distribution.** *A*, endogenous expression of NFAR-1 and -2 in cell lines. Equal amounts of whole cell extracts from Jurkat, HeLa, COS-7, 293T, and 3T3-L1 cells were electrophoresed on 8.5% polyacrylamide gels and immunoblotted with rabbit antiserum recognizing recombinant purified NFAR-1. *B*, *in vivo* expression of NFAR-1 and -2 cDNA. Putative full-length His-tagged cDNAs representing NFAR-1 and -2 in pcCDNA3.1 or vector alone were transfected into 293T cells. After 48 h, the cells were lysed, and equal amounts of cell extracts were precipitated using nickel beads. Precipitates were washed, electrophoresed on 8.5% polyacrylamide gels, and immunoblotted using antiserum to purified NFAR-1 (*left panel*). As a control, total lysate from untransfected 293Ts was electrophoresed and analyzed by immunoblotting with NFAR antiserum (*right panel*). *C*, mRNA analysis of the NFARs in human tissues. A Northern blot was incubated with an NFAR-specific ^{32}P-labeled probe, followed by a control actin probe, to show that NFAR is ubiquitously expressed. *D*, protein expression of NFAR and PKR in human tissues. Equal amounts of protein extract, taken from the above corresponding tissues, were electrophoresed on 8.5% polyacrylamide gels and immunoblotted with antiserum raised to recombinant NFAR-1 or PKR. Antibody to actin served as a further control. *E*, antiserum raised to human NFAR-1 cross-reacts with dsRNA-binding proteins in *Xenopus* oocytes and insect cells. *Xenopus laevis* oocytes or embryos at various stages of development were lysed and incubated with poly(I-C)-agarose beads. Precipitates were washed, electrophoresed on 8.5% polyacrylamide gels, and immunoblotted with antiserum raised to recombinant human NFAR-1. *Spodoptera frugiperda* (Sf9) insect cells were analyzed similarly.

NFAR-1 and -2 cDNAs were expressed transiently in mammalian cells (Fig. 2*B*). Immunoblot analysis of transfected 293T cell lysates precipitated with poly(I-C) and immunoblotted with anti-NFAR antiserum indicated that the recombinant NFAR-1 migrated identically to the 90-kDa protein observed in 293T cells probed with NFAR antiserum, while recombinant NFAR-2 migrated similarly to the 110-kDa product. Taken together, these observations strongly indicate that the cloned NFAR cDNAs are full-length representations of the major NFAR species of 90 and 110 kDa that exist in mammalian cells.

To extend our analysis of the expression profile of the NFAR genes, mRNA and protein were examined from various human tissues. Northern blot analysis indicated that NFAR mRNAs were transcribed in nearly all of the human tissue examined (Fig. 2*C*). Although our antiserum indicated the existence of only two major NFAR polypeptides, we found that a number of NFAR-related mRNA species were apparent (4–8 kb). Thus, a variety of spliced variants of the NFAR gene could exist in the cell, or our radiolabeled probe could cross-react with other dsRNA-binding proteins. To check the translational status of these transcripts, representative proteins from tissue analyzed by Northern blot were examined by immunoblot analysis using NFAR antiserum (Fig. 2*D*). In the majority of tissues examined, the two major species of NFAR, namely the 90- and 110-kDa proteins, were visible. Interestingly, lesser amounts of NFAR were seen in the liver and spleen and to a certain extent kidney, suggesting that the regulation of these proteins may be governed to some extent at the translational or post-translational level.

Since the NFARs showed extensive homology to *Xenopus* 4F.1 and 4F.2, we next examined whether antiserum raised to human NFAR could cross-react with *Xenopus* proteins. *Xenopus* oocytes and embryos at various stages of development were therefore lysed, precipitated with poly(I-C)-agarose, and immunoblotted using our human anti-NFAR serum. Fig. 2*E* shows that the antiserum detected two poly(I-C)-binding proteins with molecular masses of 95 and 115 kDa in all extracts examined. Furthermore, similar sized dsRNA-binding proteins were observed in insect Sf9 cells. These data strongly suggest that the proteins detected in *Xenopus* and insect cell extracts are homologues of NFAR-1 and -2 and that both alternatively spliced proteins have been conserved through evolution.

NFAR-1 and -2 Are dsRNA-binding Proteins—To demonstrate that the NFAR proteins are able to interact with dsRNA, poly(I-C)-agarose beads or agarose beads alone were used to precipitate endogenous NFAR-1 and -2 from extracts of HeLa cells. Complexes were then washed under various salt conditions, electrophoresed, and immunoblotted using NFAR antiserum. Fig. 3*A* shows that both NFAR-1 and -2 bound efficiently to poly(I-C)-agarose beads but not to agarose beads alone. Increasing the salt concentration to 1 M NaCl prevented the binding of the NFAR proteins to the beads. Furthermore, purified recombinant NFAR-1 and -2, expressed in *E. coli* (His-NFAR-1 and -2), could also bind to poly(I-C)-agarose beads but not to agarose beads alone.

To confirm that the dsRNA binding motifs were responsible for the interaction with dsRNA, a number of NFAR variants were generated using a rabbit reticulocyte, *in vitro* coupled
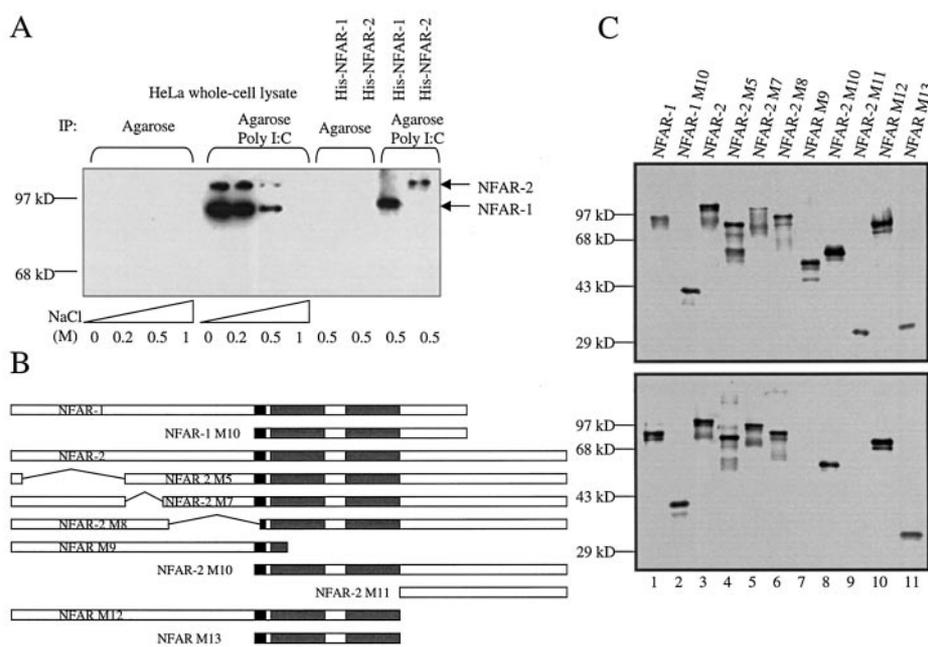
FIG. 3. **NFAR-1 and -2 preferentially bind dsRNA.** *A*, both NFAR-1 and -2 bind dsRNA. Equal amounts of whole HeLa cell extracts were incubated under various salt conditions (0–1 M NaCl) with poly(I-C)-agarose beads or agarose beads alone. Washed precipitates were electrophoresed on 8.5% polyacrylamide gels and immunoblotted with rabbit antiserum specific to NFAR-1. *E. coli* expressed recombinant His-tagged NFAR-1 and -2 were also incubated with poly(I-C)-agarose or with agarose alone and treated similarly. *B*, schematic diagram of NFAR variants used in dsRNA binding experiments. NFAR variants were generated with the following amino acids deleted: NFAR-2 M5 (Δ7–173), NFAR-2 M7 (Δ174–232), NFAR-2 M8 (Δ238–384), NFAR M9 (Δ418–702), NFAR-1 and -2 M10 (Δ1–369), NFAR-2 M11 (Δ1–588), NFAR-2 M12 (Δ586–894), and NFAR M13 (Δ1–369, 586–894). *C*, the dsRNA binding motifs of NFAR are necessary for the interaction with dsRNA. ^{35}S-Labeled NFAR proteins were generated using an *in vitro* coupled transcription and translation system. Equal amounts of protein were loaded on an 8.5% polyacrylamide gel and electrophoresed (*top panel*). Equal amounts of NFAR protein were incubated with poly(I-C)-agarose beads, and after extensive washing in 400 mM NaCl, the proteins were electrophoresed (*bottom panel*). *IP*, immunoprecipitate.

transcription and translation system (Fig. 3*B*). Equal amounts of the various His-tagged NFAR proteins (Fig. 3*C*, *top panel*) were incubated with poly(I-C)-agarose and washed with high salt buffer (Fig. 3*C*, *bottom panel*). Only the NFAR constructs containing both dsRNA binding motifs were able to bind poly(I-C)-agarose, while NFAR M9 and NFAR-2 M11, which lack both dsRNA binding domains, failed to bind dsRNA (Fig. 3, *B* and *C*). Thus, we confirm that the region of the NFARs that contain both dsRNA binding motifs is essential for the interaction with dsRNA.

PKR and NFAR Reciprocally Co-immunoprecipitate and Co-localize in the Nucleus of the Cell—To further analyze the association of the NFARs with PKR, we determined whether NFAR-1 and -2 could co-immunoprecipitate with PKR. *E. coli* expressed and affinity-purified His-tagged PKR and NFAR-1 or NFAR-2 were incubated *in vitro* and co-immunoprecipitated using an anti-PKR monoclonal or isotyped control antibody. Extracts were treated with a variety of RNases to eliminate potential dsRNA bridging between the proteins. Immunoblotting with the NFAR antiserum revealed that both NFAR-1 and NFAR-2 associated with PKR when both recombinant proteins were included in the immunoprecipitation reaction (Fig. 4*A*, *right panel*). Similarly, PKR was found to reciprocally co-immunoprecipitate with NFAR-1 and -2 when NFAR antiserum was used only when both recombinant proteins were included (Fig. 4*A*, *left panel*).

To demonstrate the interaction of the NFARs and PKR *in vivo*, untransfected HeLa cell extracts, treated with RNases, were incubated with NFAR antiserum. As shown in Fig. 4*B*, endogenous PKR co-immunoprecipitated with endogenous NFAR-1 and -2 from HeLa cells as confirmed using anti-PKR monoclonal antibodies in immunoblot analysis (Fig. 4*B*, *left panel*). PKR was not detected when preimmune rabbit serum was used in the immunoprecipitation procedure. Likewise,

both NFAR-1 and -2 co-immunoprecipitated with PKR when the anti-PKR monoclonal antibody was used in the precipitation procedure but not when isotyped control antibody was used (Fig. 4*B*, *right panel*). Taken together, our data strongly indicate that PKR associates with NFAR-1 and -2 not only *in vitro* but also *in vivo*.

PKR is predominantly found in the cytoplasm, where it is closely associated with ribosomes (46, 47). However, electron micrograph analysis and immunogold labeling has also indicated that a fraction of PKR resides in the nucleus (48, 49). Since the NFARs have a consensus bipartite nuclear localization signal (Fig. 1, *A* and *B*), we determined if PKR colocalizes with NFAR in the cell. We employed confocal microscopy double labeling studies using rabbit polyclonal and murine monoclonal antibodies directed to NFAR and PKR, respectively. HeLa cells incubated with both antibodies revealed that the distribution of NFAR is predominantly in the nucleus (Fig. 4*C*, *middle panel*). However, although the majority of PKR was found in the cytoplasm, a fraction ($\sim$20–25%) was diffusely distributed in the nucleus (Fig. 4*C*, *left panel*). Importantly, overlaying the images revealed that NFAR and PKR showed evidence of colocalization in the nucleus of the cell (Fig. 4*C*, *right panel*).

NFAR-1 and -2 Are Nuclear Phosphoproteins and Substrates for PKR—Sequence analysis revealed that the NFAR proteins exhibited homology with the known PKR substrate eIF2α (Fig. 1*C*). To determine if the NFAR proteins are indeed substrates for PKR, NFAR-1 and -2 were expressed as His-tagged proteins in *E. coli* and purified over nickel affinity columns to $\sim$90% purity as estimated by Coomassie Blue-stained gels (data not shown). PKR was also expressed as a His-tagged protein and underwent a similar purification protocol followed by a gel filtration step. Our data indicated that NFAR-1 or -2 alone showed no indication of autophosphorylation when incubated
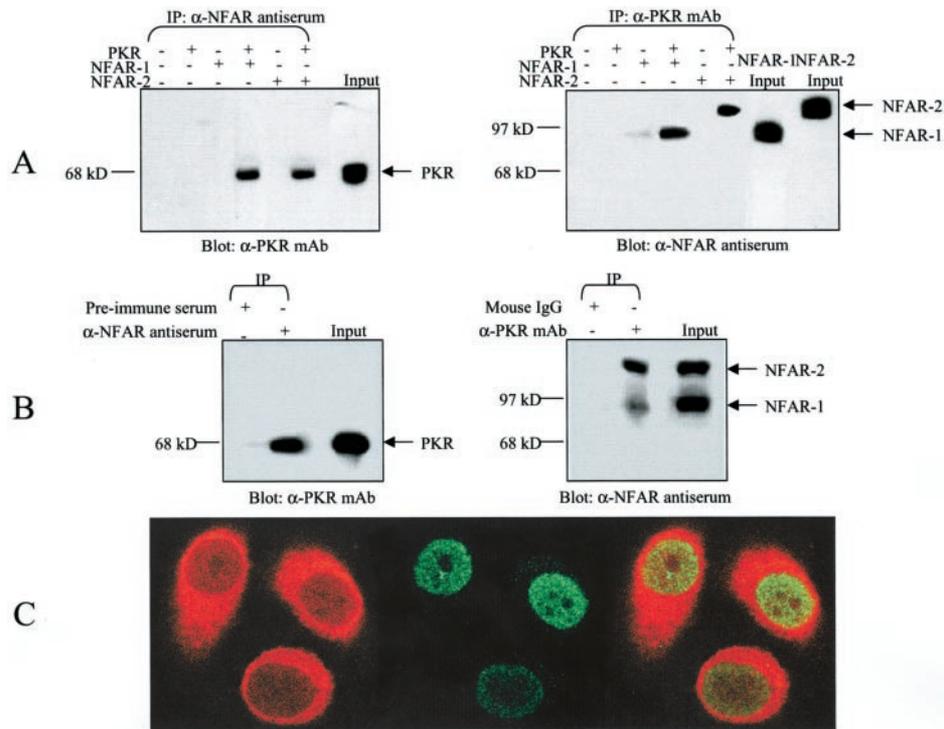
FIG. 4. **The NFARs co-immunoprecipitate with PKR *in vitro* and *in vivo* and colocalize in a diffuse nuclear pattern.** *A*, co-immunoprecipitation of NFAR-1 and -2 with PKR *in vitro*. *E. coli* expressing His-tagged NFAR-1 or -2 or PKR was lysed and purified over nickel beads, and purified NFAR-1 and PKR or NFAR-2 and PKR were co-incubated or incubated alone at 4 °C for 1 h. Anti-NFAR rabbit serum was then used to immunoprecipitate NFAR-1 and -2 (*left panel*). Precipitated extracts were washed, treated with RNase, electrophoresed on 8.5% polyacrylamide gels, and immunoblotted using an anti-PKR monoclonal antibody. Similarly, *E. coli* extracts containing NFAR-1 or -2 and PKR were coincubated and immunprecipitated using an anti-PKR monoclonal antibody (*right panel*). Precipitated extracts were washed, treated with RNase, electrophoresed, and immunoblotted using rabbit anti-NFAR serum. Inputs represent ~10% of the total amount of NFAR and PKR proteins used in the incubation reaction. *B*, co-immunoprecipitation of NFAR-1 and -2 *in vivo*. HeLa cells were lysed and incubated with anti-NFAR rabbit serum or with control preimmune rabbit serum. The immunoprecipitates were washed, treated with RNase, electrophoresed on 8.5% polyacrylamide gels, and immunoblotted using an anti-PKR monoclonal antibody (*left panel*). Reciprocally, HeLa extracts were incubated with an anti-PKR monoclonal antibody or isotype control antibody (*right panel*). Immunoprecipitates were then washed, treated with RNase, electrophoresed, and immunoblotted with anti-NFAR rabbit serum. Input values represent ~10% of the HeLa cell extract used in the assays. *C*, NFAR and PKR colocalize in a diffuse nuclear pattern. HeLa cells were fixed and double-labeled consecutively with anti-NFAR rabbit serum and anti-PKR monoclonal antibody. NFAR antibody was detected with fluorescein isothiocyanate-conjugated anti-rabbit IgG (*middle panel*) and PKR antibody with Texas Red-labeled anti-murine IgG (*left panel*). Merged filter analysis reveals co-localization of the NFARs and PKR in the nucleus (*yellow*; *right panel*). *IP*, immunoprecipitate.

in the presence of dsRNA and $[\gamma\text{-}^{32}P]$ATP (data not shown). In contrast, PKR efficiently autophosphorylated when incubated with dsRNA, as has been previously described (Fig. 5*A*, *left panel*). At high levels of dsRNA, PKR autophosphorylation is typically inhibited, arguably since intermolecular PKR-PKR interactions are prevented by each PKR molecule individually binding to dsRNA molecules (50–52). Significantly, PKR was clearly able to phosphorylate stoichiometric amounts of both NFAR-1 as well as NFAR-2 when incubated together (Fig. 5*A*, *middle* and *right panels*). At higher concentrations of dsRNA, phosphorylation of NFAR-1 and -2 by PKR was inhibited, similar to the effect seen with PKR alone.

To examine the role of the dsRNA binding domains in the interaction between PKR and NFAR, an NFAR variant lacking both dsRNA binding motifs (NFAR M9, amino acids 1–415; Fig. 3*C*) was next examined as a substrate for the kinase. Fig. 5*B* demonstrates that NFAR M9 that lacks the dsRNA binding domains was also able to serve as an efficient substrate for PKR. These data suggest that dsRNA is not essential for the association of the two proteins and emphasizes that PKR phosphorylates a site(s) in the N terminus of NFAR that includes the region with homology to eIF2α.

Attempts at comparing NFAR phosphorylation levels in PKR null and control mouse embryonic fibroblasts (MEFs) illustrated a high basal level of phosphorylation, probably due to several kinases regulating NFAR activity (data not shown). To

start to evaluate whether the NFAR proteins can be phosphorylated *in vivo* by PKR, control of 3T3 L1 cells inducibly overexpressing PKR were treated with poly(I-C) in the presence of $[^{32}P]$orthophosphate (28), followed by precipitation of NFAR and PKR using poly(I-C)-agarose beads. Fig. 5*C* confirmed that at least a proportion of the NFARs exist as phosphoproteins *in vivo*. Significantly, 3T3 L1 cells overexpressing the heterologous PKR (WT-PKR) severalfold higher than the endogenous murine PKR in the control cells (VEC) contained 3–4-fold higher levels of phosphorylated NFAR-1 and -2 (Fig. 5*C*, *right panel*). Immunoblotting equal amounts of the poly(I-C)-precipitated protein complexes using NFAR antiserum (Fig. 5*C*, *left panel*) confirmed the identity of the NFARs and ensured that the level of the NFAR proteins was equal in both the control and PKR overexpressing cells (demonstrating that the increase in NFAR phosphorylation is not a reflection of elevated NFAR protein levels). Taken together, these data imply that the NFARs exist as nuclear phosphoproteins and are conceivably substrates for PKR *in vivo*.

NFAR-1 and -2 Regulate Gene Expression at the Level of Transcription—To begin to examine a possible role of the NFARs in the regulation of gene expression, NFAR-1 and -2 were co-transfected into COS-1 cells with a luciferase reporter gene driven by the SV40 promoter. Fig. 6*A* indicates that luciferase production was augmented in the presence of NFAR-1 (~2-fold) and NFAR-2 (~3-fold), signifying that both
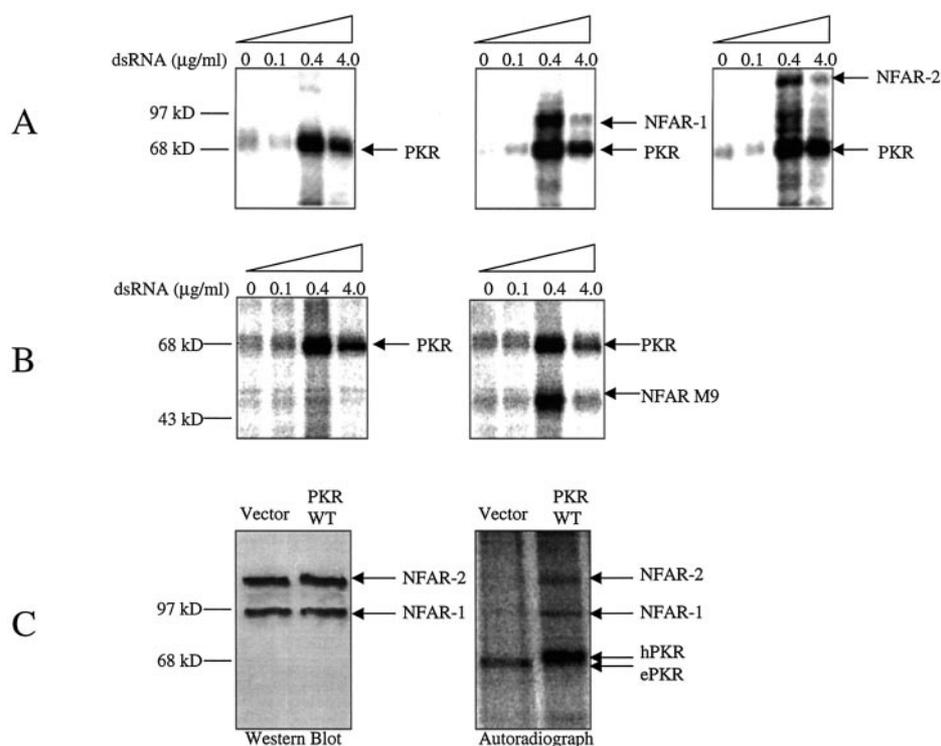
FIG. 5. **NFAR-1 and -2 are substrates for PKR *in vitro* and *in vivo*.** *A*, *in vitro* phosphorylation of NFAR-1 and -2 by PKR. His-tagged PKR and NFAR-1 and -2 were purified from *E. coli* using nickel beads. Equal amounts of NFAR-1 and -2 and PKR, as determined from Coomassie Blue-stained gels, were incubated in the presence of [γ-^{32}P]ATP and increasing amounts of dsRNA, as outlined. *Left panel*, PKR incubated alone; *middle panel*, PKR incubated with equal amounts of NFAR-1; *right panel*, PKR incubated with equal amounts of NFAR-2. NFAR-1 and -2 incubated alone with dsRNA gave no indication of autophosphorylation (data not shown). *B*, the dsRNA binding domains of NFAR are not essential for phosphorylation by PKR. His-tagged NFAR variant NFAR M9, lacking the dsRNA binding domains, was purified by nickel affinity columns and incubated with PKR in the presence of [γ-^{32}P]ATP and increasing amounts of dsRNA. *Left panel*, PKR incubated alone; *right panel*, PKR incubated with NFAR M9. *C*, *in vivo* phosphorylation of NFAR-1 and -2 by PKR. Murine 3T3 L1 cells inducibly overexpressing PKR or carrying vector alone were incubated with poly(I-C) in the presence of [^{32}P]orthophosphate for 4 h. Cells were lysed and extracts incubated with poly(I-C)-agarose beads. After washing, the precipitates were electrophoresed and autoradiographed. Markers refer to human PKR (*hPKR*) inducibly overexpressed only in the PKR WT cells, and the endogenous murine PKR (*ePKR*) expressed in both cell types (*right panel*). Proteins were confirmed as NFAR-1 and -2 by immunoblotting equal aliquots of the precipitated extracts with NFAR antiserum (*left panel*).

NFARs can indeed influence the regulation of gene expression. To further examine NFAR's function, several NFAR variants were similarly examined for their ability to modulate gene expression. All NFAR constructs were His-tagged, and immunoblots were carried out on transfected cells to ensure equal expression of the heterologous proteins (Fig. 6*B*). Deletion of various regions in the N terminus of NFAR-2, namely NFAR M5 (Δ7–173), NFAR M7 (Δ174–232), and NFAR M8 (Δ238–384), did not significantly impair the ability of NFAR-2 to stimulate luciferase synthesis (Figs. 3*B* and 6*A*). However, deletion of the first 369 N-terminal amino acids from either NFAR-1 or -2 (NFAR-1 and -2 M10) did appear to increase NFAR's capability to regulate gene expression, suggesting that the C terminus of the protein may function as a constitutively active form of the NFARs. This data was complemented by showing that the amino terminus region of the NFARs (NFAR M9), lacking the active C-terminal portion of the molecule, exhibited greatly reduced function (Figs. 3*B* and 6*A*). However, NFAR-2 M12, in which the protein terminates after the dsRNA binding domains, and NFAR-2 M11 and M13, which contain the dsRNA binding motifs or only the C terminus region, respectively, were also found to be impaired in their ability to manipulate luciferase synthesis (Figs. 3*B* and 6*A*). Thus, our data indicate that both the dsRNA binding motifs and C-terminal portion of the NFARs are required for optimal NFAR function.

To determine whether the regulation of gene expression was governed at the transcriptional or translational level, cells were co-transfected with NFAR-1 or -2 and a luciferase reporter gene driven by the SV40 promoter. Total RNA was isolated after 48 h, and RT-PCR measured luciferase mRNA levels as well as actin mRNA levels to ensure that approximately equal amounts of RNA were analyzed. Fig. 6*C* demonstrates increases (>2-fold) in the level of reporter gene transcripts in cells transfected with NFAR-1 or -2 but not vector alone, suggesting that the NFARs function to regulate transcription. Conceivably, the NFARs could function at the initiation, elongation, or post-transcriptional stage of mRNA production. However, further investigations using highly purified NFAR proteins in EMSAs indicated that the NFARs do not appear to bind directly to SV40 promoter sequences (data not shown). Furthermore, no transcription activation activity was apparent in co-transfection experiments with reporter genes under control of the Gal4 promoter and NFAR-1 or -2 fused to a Gal4 binding domain (data not shown). To examine if NFAR influences mRNA stability, we again co-transfected COS-1 cells with NFAR and the luciferase reporter construct, and 24 and 48 h after co-transfection the cells were treated with actinomycin D. Luciferase levels were then measured at several intervals up to 55 h, and NFAR did not appear to affect the stability of mRNA (data not shown). Collectively, these data strongly suggest but do not entirely validate that the mechanisms of NFAR action are downstream from the initiation of transcription.

To further elucidate at which step of transcription/post-transcription the NFARs function, nuclear run-on assays were performed. Accordingly, NFAR-1 and -2 were co-transfected in COS-1 cells along with an SV40 luciferase reporter construct,
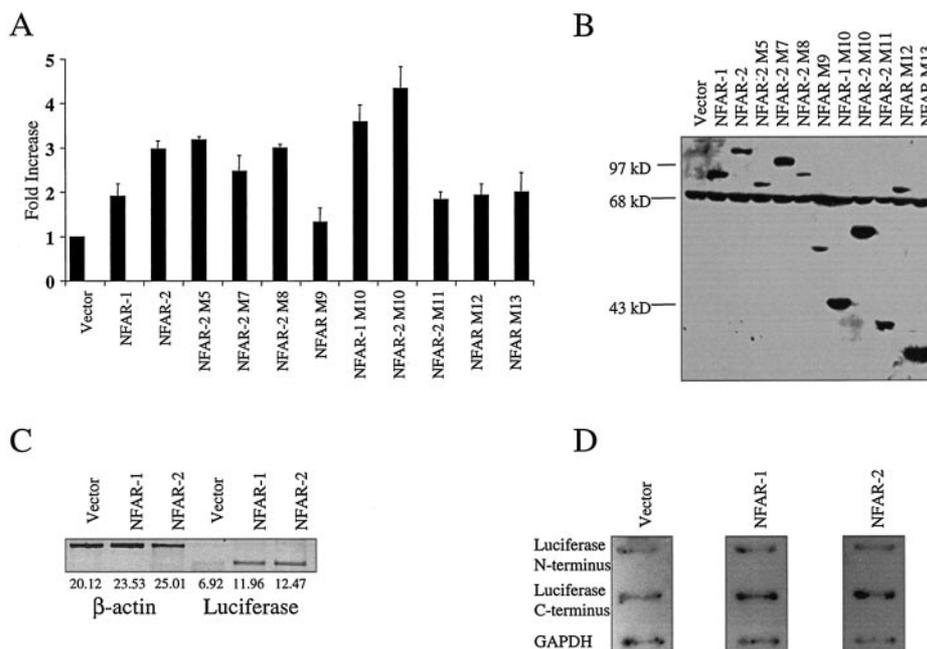
FIG. 6. **The NFARs function to regulate gene expression.** *A*, NFAR-1 and -2 stimulate the expression of co-transfected reporter genes under control of the SV40 promoter. Equal quantities of eukaryotic expression vectors (pcDNA3.1) carrying NFAR-1 or -2 or variants were co-transfected in COS-1 cells with a luciferase reporter gene placed downstream of the SV40 promoter (pGL3 promoter). After 48 h, the cells were lysed, and luciferase production was assayed. Experiments were done in duplicate three times. The mean values of the experiments are shown. *B*, immunoblot of NFAR variants transiently expressed in COS-1. NFAR-1 and -2 and NFAR variants (Fig. 3*B*) were transfected into COS-1 cells. After 48 h, NFAR protein expression was monitored by lysing the cells and immunoblotting with a histidine-specific antibody (His-G; Invitrogen). *C*, the NFARs are regulators of transcription in the cell. NFAR-1 and -2 were co-transfected in COS-1 cells with luciferase reporter genes under control of the SV40 promoter. After 48 h, the cells were lysed, and total RNA was extracted. mRNA was quantitated by RT-PCR using probes to actin as described under "Experimental Procedures." Luciferase mRNA was measured using primers specific to the luciferase gene. *D*, the NFARs enhance transcription elongation. A nuclear run-on assay was performed and showed that co-transfected NFAR-1 or -2 increased the density of elongating RNA polymerases at the 3′-end of luciferase ~1.5–2-fold over vector alone. The density of engaged RNA polymerases on GAPDH was measured to control for the amount of RNA on each slot blot.

and nuclei were isolated and labeled rNTPs were added to allow elongation of engaged polymerases. To equalize the amount of isolated RNA, GAPDH mRNA was measured along with luciferase. The ratio of GAPDH probe to the N terminus of luciferase showed that neither NFAR-1 nor NFAR-2 increased the rate of transcription over the vector control. However, a probe to the C terminus of the luciferase mRNA indicated that NFAR-1 and -2 did appear to moderately increase the rate of transcription ~1.4- and 2-fold, respectively, compared with vector-transfected (Fig. 6*D*). Collectively, our data would indicate that the NFARs function at the level of transcription elongation or in the processing of mRNAs.

Analysis of the Regulation of NFAR by PKR—Our yeast genetic studies, co-immunoprecipitation data, confocal analyses, and phosphorylation experiments indicate that PKR may associate with the NFARs in the cell. To examine if PKR influences NFAR's ability to regulate gene expression *in vivo*, immortalized control and PKR null MEFs were transfected with NFAR-1 or -2 and the SV40 promoter luciferase reporter construct. Immunoblot analysis was carried out on control and PKR null MEFs to demonstrate that endogenous levels of NFAR were equal in both cell types and that PKR is lacking in PKR$^{-/-}$ cells (Fig. 7*B*). Surprisingly, unlike transfected COS-1 cells, NFAR did not appear to strongly stimulate gene expression in the control immortalized MEFs, perhaps due to cell-specific factors being absent in the MEFs compared with COS-1 cells or due to the fact that COS-1 cells are transformed. However, in PKR null fibroblasts, NFAR-1 was found to stimulate gene expression ~4.0-fold, and NFAR-2 stimulated expression ~3.0-fold, compared with vector alone (Fig. 7*A*). Although the addition of IFNα/β and poly(I-C), which are known stimulators of PKR expression and activity, respectively, had no effect on

NFAR-regulated luciferase expression in the control MEFs, treatment of the PKR null MEFs with poly(I-C) reduced NFAR's ability to augment gene expression. This effect may be due to the NFARs interacting with other dsRNA binding proteins besides PKR or due to dsRNA binding to the NFARs and inducing a conformational change, which may mask functional domains in the C terminus. Collectively, our data indicate that PKR can modulate the ability of the NFARs to influence gene expression.

NFAR-2 Interacts with the mRNA-processing Proteins SMN and FUS—NFAR-1 and -2 appear to be analogous, alternatively spliced products that have been conserved through evolution, with the only difference between the two being an extra 192 amino acids at the C terminus of NFAR-2. This unique region may allow NFAR-2 to interact with partners not eligible for NFAR-1 and thus to endow extra properties that NFAR-1 lacks. To identify proteins that interact specifically with the C terminus of NFAR-2, amino acids 666–894 were fused in frame to the Gal4 binding domain and used to screen a Jurkat cDNA library in a yeast two-hybrid screen. Full-length cDNA clones were retrieved that encoded proteins able to specifically interact with the C terminus of NFAR-2 (Fig. 8*A*). One of the interacting clones was identified as SMN (survival of motor neurons), a protein that has been implicated in pre-mRNA splicing and in causing spinal muscular atrophy (53). Interestingly, neither full-length NFAR-1 nor 2 bound efficiently to SMN in yeast studies, typically due to the Gal4 binding domain or the amino terminus region of the NFARs masking the sites responsible for interaction. SMN has been reported to be localized in the cytoplasm and in nuclear bodies called gems, which are adjacent to snRNP-rich coiled bodies (54, 55). Confocal analysis on untransfected HeLa cells using anti-SMN and anti-

FIG. 7. **PKR can influence NFAR-mediated gene expression.** *A*, PKR appears to down-regulate NFAR's ability to stimulate gene expression in immortalized MEFS. Immortalized control and PKR null MEFs were co-transfected with vector, NFAR-1 or -2, and the pGL3 promoter vector, and luciferase production was measured. NFAR had a modest effect in control MEFs, while in MEFs lacking PKR, transiently transfected NFAR stimulated gene expression. *B*, immunoblot analysis on lysates from control and PKR null MEFs showed equal expression of NFAR-1 and -2.
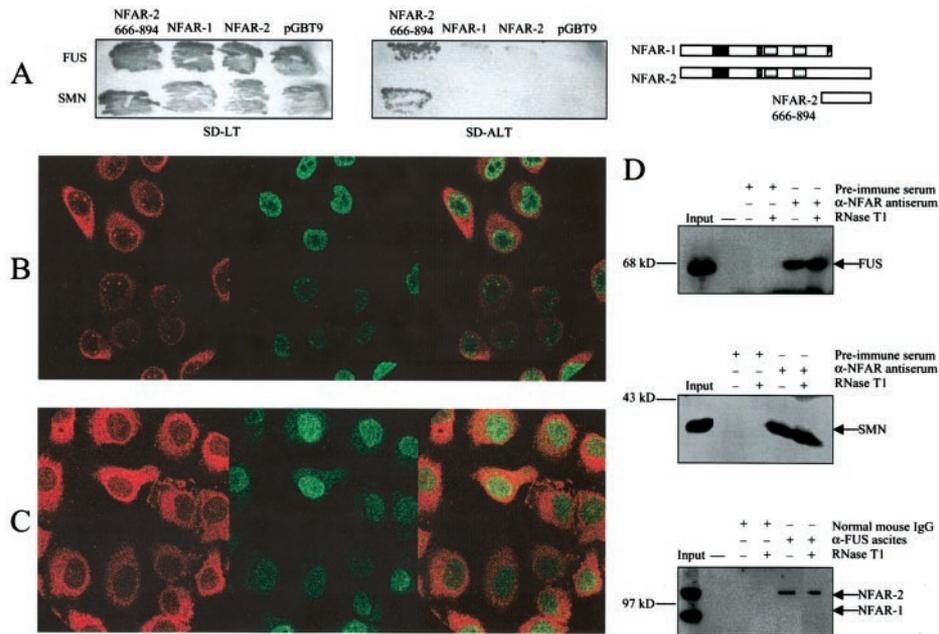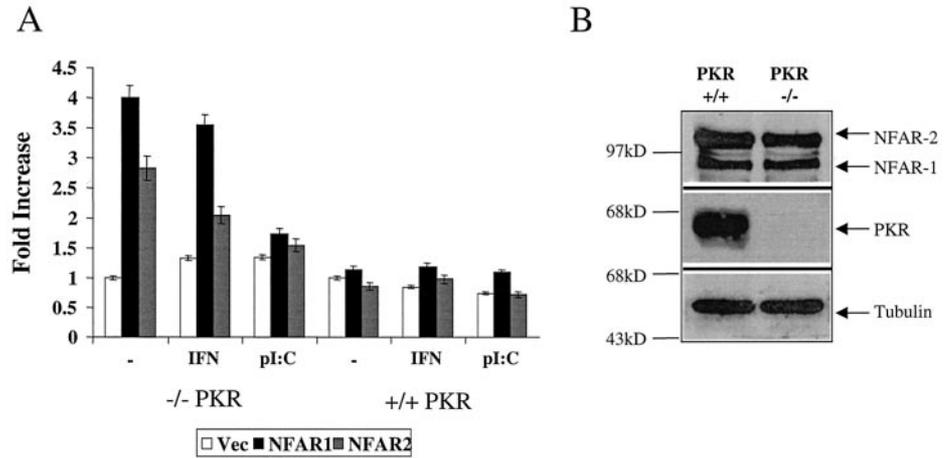

FIG. 8. **The C terminus of NFAR-2 specifically interacts with FUS and SMN.** *A*, the C terminus of NFAR-2 interacts with full-length FUS and SMN in a yeast two-hybrid assay. Amino acids 666–894 of NFAR-2 were fused to the Gal4 binding domain and shown to interact specifically with full-length FUS and SMN fused to the Gal4 activation domain. *B*, SMN localizes to nuclear gems. Confocal analysis of HeLa cells using anti-NFAR rabbit serum (*green*) and anti-SMN mouse monoclonal antibodies (*red*) indicates that SMN localizes to nuclear gems, sites enriched in splicing factors, and colocalizes with NFAR (*yellow*). *C*, FUS localizes to both the cytoplasm and nucleus of HeLa cells. Confocal analysis using anti-NFAR rabbit serum (*green*) and hybridoma supernatant of anti-FUS monoclonal antibodies (*red*) shows FUS and NFAR co-localized in a diffuse nuclear pattern (*yellow*). D, reciprocal co-immunoprecipitation of NFAR with FUS and SMN *in vivo*. HeLa cells were lysed and incubated with anti-rabbit NFAR serum or preimmune rabbit serum (*top* and *middle panels*) or normal mouse IgG or hybridoma supernatant of anti-FUS ascites fluid (*bottom panel*). The immunoprecipitates were washed and incubated with RNase T1, electrophoresed, and immunoblotted using antibodies to SMN or FUS (*top* and *middle panels*) or NFAR antiserum (*bottom panel*). Input represents ~10% of the HeLa cell extract used in the assay.

NFAR antiserum confirmed that SMN was predominantly localized to nuclear gems, although it also indicated that SMN showed evidence of colocalization with the NFARs in the nucleus (Fig. 8*B*).

A second protein found to specifically interact with the C terminus of NFAR-2 was FUS/TLS (t̲ransformed in l̲ip̲o̲s̲arcomas), whose N-terminal transactivation domain is fused to the DNA binding domain of CHOP in human myxoid and round cell liposarcomas (56, 57). The C terminus of FUS has conserved RNP consensus sequences as well as RGG repeats, both of which are implicated in RNA binding. Similar to SMN, FUS has been demonstrated to be involved in RNA splicing and/or shuttling of mRNAs as well as in homologous DNA pairing and recombination (58–61). As shown in Fig. 8*C*, the majority of FUS was localized to the cytoplasm, with a fraction found in a

diffuse pattern in the nucleus, where it was also found to colocalize with the NFARs.

To confirm an *in vivo* interaction between NFAR, FUS, and SMN, HeLa cell lysates were incubated with NFAR, FUS, SMN, or control antiserum. As Fig. 8*D* illustrates, NFAR antiserum co-immunoprecipitated both FUS and SMN (*top* and *middle panels*). This interaction was not prevented using treatment with RNase T1, indicating that the specificity of the interaction is not simply through RNA bridging. In a reciprocal assay, antibodies to FUS were able to specifically co-immunoprecipitate NFAR-2 but not NFAR-1 (Fig. 8*D*, *bottom panel*). This is in agreement with our yeast two-hybrid data that implicate the C terminus of NFAR-2 in the interaction with FUS and SMN. Thus, NFAR-2 but not NFAR-1 is able to interact with proteins such as FUS and SMN, which are known to form
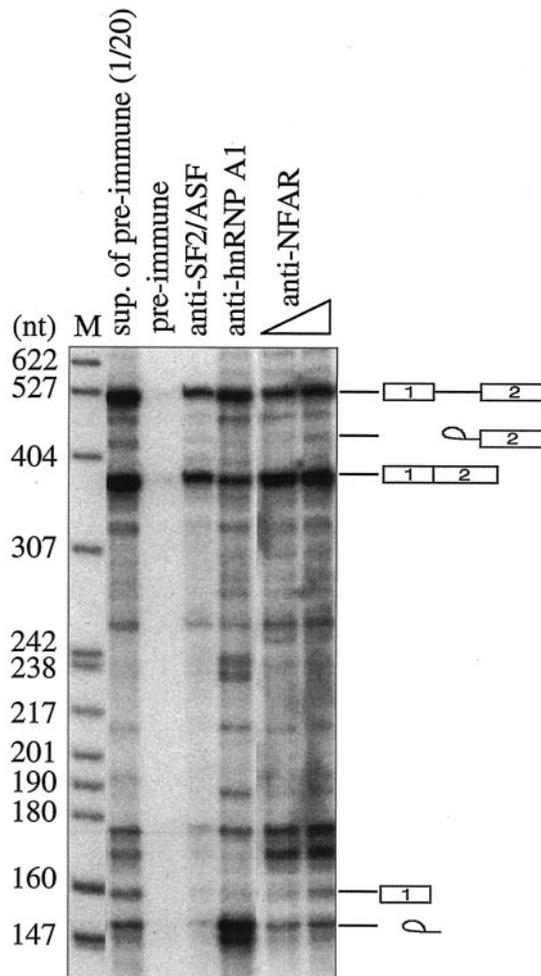
FIG. 9. **NFAR associates with the active spliceosome *in vitro*.** Labeled β-globin was incubated with splicing-competent HeLa nuclear cell extract and immunoprecipitated with NFAR-specific antiserum together with control antibodies. RNA moieties of the precipitated complexes were analyzed by denaturing polyacrylamide gel electrophoresis followed by autoradiograph.

large complexes with snRNP proteins and to be involved in the processing of pre-mRNAs. Aside from indicating divergent properties between the NFARs, these data further indicate the possibility that the NFARs are involved in mRNA processing.

Immunoprecipitation of NFAR with the Spliceosome—Interaction of the transcription machinery with protein complexes involved in capping, splicing, polyadenylation, and other steps of mRNA processing have been recently described (62). Given our above data and to further define NFAR's function, we began to examine a potential role for NFAR in mRNA splicing. We used a labeled β-globin pre-mRNA in an *in vitro* splicing reaction and performed an immunoprecipitation with our NFAR antiserum and several other control antibodies. Labeled RNA was recovered from each immunoprecipitated complex, electrophoresed by denaturing polyacrylamide gel electrophoresis, and then detected by autoradiography. As shown in Fig. 9, NFAR preferentially bound to complexes containing spliced mRNAs but was also associated with complexes containing pre-mRNAs as well as intermediate products. This was similar to an immunoprecipitation with an antibody against ASF/SF-2, which is a member of the essential SR protein family, but distinct from that using a control antibody against heterogeneous nuclear ribonucleoprotein A1, which is a regulator of alternative splicing (reviewed in Ref. 63). Collectively, our data suggest that the NFARs may associate with mRNA

processing complexes following the initiation of transcription in the cell.

DISCUSSION

Using PKR as bait in a yeast genetic screen, we have identified two evolutionary conserved proteins, NFAR-1 and -2, that are derived through alternative splicing from a single gene on chromosome 19. The NFARs are members of a previously described family of dsRNA-binding proteins that include PKR and vaccinia virus protein E3L (4, 7, 42, 64–66). Our data confirm that both NFAR-1 and -2 are able to interact with dsRNA and establish that the conserved motifs are indeed necessary and sufficient for these interactions (Fig. 3). In addition, using our antiserum to the human NFAR protein, we have identified dsRNA binding proteins that are potential homologs in both insect cells and *Xenopus* oocytes (Fig. 2*E*). *Xenopus* 4F.1 is 74% identical at the amino acid level to NFAR-1, and the partial clone 4F.2 is 61% identical at the amino acid level to NFAR-2 (3). Subsequent data bank searches also revealed the existence of a number of NFAR related clones. For example, NFAR-1 is similar to two partial clones called MPP4 and is probably identical to human DRBP76 (99% identical at the amino acid level), which was also reported to be phosphorylated by PKR *in vitro* (43, 45). A recent report further indicates the existence of a probable mouse homologue of NFAR-2, referred to as mILF3 (91% identical at the amino acid level) and a related human clone, NF-90, whose cDNA sequence is related to NFAR-2 but whose protein appears to be largely artificial, perhaps due to a PCR error introduced during the cloning of the cDNA at the C terminus (41, 44, 67, 68). Collectively, this report represents the first accurate identification and cloning of both alternatively spliced variants of NFAR and confirms that they are derived from a single human gene.

Our data indicate that the NFARs are almost exclusively localized to the nucleus of the cell, and while PKR is known to be distributed predominantly throughout the cytoplasm, this kinase has also been reported to reside in the nucleus (12, 48). Confocal analysis subsequently confirmed that the NFARs and PKR co-localize in a diffuse nuclear pattern within the cell, and co-immunoprecipitation data indicate that the NFARs and PKR could form inactive heterodimers (Fig. 4). It is plausible that dsRNA may facilitate PKR and NFAR interactions by acting as a bridge between two molecules in close proximity, a possibility that has been suggested to occur among other dsRNA binding family members (69). Indeed, data obtained from the yeast two-hybrid system do suggest that dsRNA may facilitate the interaction of the NFARs with PKR (data not shown). Thus, once bound to dsRNA, PKR may preferentially activate nearby targets that also contain dsRNA binding motifs, such as the NFARs.

We further demonstrate that both NFAR-1 and -2 are substrates for PKR (Fig. 5). The phosphorylation of NFAR-1 and -2 was enhanced in the presence of low amounts of dsRNA although inhibited at higher concentrations of dsRNA, speculatively since the dsRNA binding domains of PKR and NFAR may each accommodate an independent RNA molecule (50–52). This may prevent PKR from associating with target substrates such as the NFARs or inhibit the kinase's catalytic activity. Importantly, an NFAR variant lacking the dsRNA binding domains, NFAR M9, retained its ability to function as a substrate for PKR, indicating that the dsRNA binding domains of the NFARs are not necessary for the interaction with PKR (Fig. 5*B*). Presently, the residues in the amino region of NFAR-1 and -2 that are specifically targeted for phosphorylation by PKR remain to be resolved. Sequence comparisons have revealed a region of extensive homology between eIF2α, the

viral homologue K3L, and NFAR-1 and -2 (Fig. 1*C*). Although this homologous area includes a conserved VIRV box and the region of eIF2α containing serine 51 that is phosphorylated by PKR, this serine residue is not identically preserved in the NFAR proteins (9, 39). Furthermore, four serine and two threonine residues located within the eIF2α region of homology do not appear to be singularly targeted by PKR for phosphorylation (data not shown). It is possible that this region of homology may facilitate interactions between the NFARs and PKR, similar to the situation between PKR and K3L. Data bank analysis indicates that the NFARs may be targeted by a number of kinases in the cell, since consensus phosphorylation sites for both protein kinase C and casein kinase II are evident. Finally, a recent report also indicates that the partial NFAR clone NF-90 may also be a substrate for DNA-PK (70).

The family of dsRNA-binding proteins has diverse properties in the cell. For example, the adenosine deaminases (ADAR1–3), are interferon-inducible, RNA editing enzymes that catalyze the deamination of adenosine to inosine in dsRNA structures (71–73). In contrast, *Drosophila* staufen, which has five dsRNA binding motifs, has been shown to play a role in the localization of several mRNAs to specific sites of the developing oocyte and early embryo and in some cases to also activate translation of the specific mRNAs to which it binds (74). The dsRNA-binding proteins, NFAR-1 and -2, that we have identified appear to increase gene expression at the level of transcription elongation and may associate with both pre-mRNA and spliced mRNAs in the cell (Figs. 8 and 9). Through association with splicing proteins, like FUS and SMN, it is plausible that NFAR may be regulated by molecules such as PKR to influence the processing of target mRNAs during viral infection. Interestingly, studies with the NFAR homologues *Xenopus* 4F.1 and 2 indicate that these molecules were able to associate with RNA/DNA hybrids, also suggesting that they could be associated with a transcriptional elongation/mRNA processing complex (3). However, although the partial NFAR-1 homologue NF-90 was originally identified as binding to ARRE elements in the interleukin-2 promoter, we have been unable to demonstrate that recombinant NFAR proteins or endogenous NFAR bind to these same DNA elements or to the SV40 promoter, this difference perhaps being due to the NF-90 clone lacking an authentic C-terminal region (75, 76).

In addition to NFAR-1 and -2, other dsRNA-binding proteins have been reported to interact with PKR. For example, human SPNR, which has been proposed to have a role in controlling cell growth, has been shown to associate with PKR (69). Further, human TRBP, first isolated by its ability to associate with human immunodeficiency virus TAR RNA, has been documented as being a putative regulator of the kinase (77). In addition, human PACT and its murine homologue RAX have been reported to actually activate PKR and to perhaps play a role in stress response (78, 79). Importantly, none of these PKR-interacting proteins have been reported to be substrates for PKR, and to date eIF2α remains the only well documented substrate for this kinase. One possible model to explain the molecular relationship between PKR and NFAR suggests that in the presence of dsRNA, of either cellular or viral origin, PKR becomes activated and is then able to phosphorylate NFAR-1 and -2 to modulate their activity and function. In the case of the SR family of splicing factors, phosphorylation has indeed been shown to be important for regulating the ability of these factors to interact with polymerase II and to form functional splicing complexes (80). Interestingly, PKR has been implicated in influencing the splicing of the tumor necrosis factor-α mRNA, although the exact mechanism remains to be determined (81).

It also remains to be determined why there are two spliced variants of NFAR residing in the cell. NFAR-1 and -2 appear to be expressed concomitantly in nearly all cells and tissue analyzed (Fig. 2), arguing against one spliced form being preferentially translated in certain types of tissue in the absence of the other. It is not yet clear whether the NFARs function as dimers or regulate one another's activity, although preliminary data argue against NFAR-1 being able to interact with NFAR-2.[2] It is possible that the regulation of NFAR-2 may be governed by factors not associated with NFAR-1, since the former protein has an extra 192 amino acids at the extreme carboxyl-terminal end, which is rich in arginine and glycine residues, commonly found in RNA binding and splicing proteins (58). Indeed, yeast two-hybrid screening indicates that the C terminus of NFAR-2 can associate with proteins involved in RNA processing such as FUS and SMN (Fig. 8*A*). FUS has recently been demonstrated to interact with RNA polymerase II through its N terminus and to interact with members of the SR protein family of splicing factors through its C terminus. Evidence also indicates that FUS may serve as a docking protein that recruits splicing factors to RNA polymerase II, thus coupling gene transcription and splicing (82). In contrast, SMN has been reported to interact with SIP-1 (SMN-interacting protein), forming a large complex containing several U snRNP essential splicing proteins, suggesting a cytoplasmic role for this protein in snRNP biogenesis (83). A nuclear role for SMN in pre-mRNA splicing, probably in the regeneration or recycling of snRNPs and other splicing factors, has similarly been reported (54). That the NFARs were further found to associate with both pre-mRNAs and spliced mRNAs, *in vitro*, similar to the critical splicing factor ASF/SF-2, further supports a role for the NFARs in RNA splicing/processing (Fig. 9). Collectively, our analyses strongly implicate a role for the NFARs downstream from the initiation of transcription in the cell.

In summary, we have isolated and characterized two alternatively spliced, dsRNA-binding nuclear proteins and provide evidence that the NFARs may function in the processing of mRNA. Our data further indicate that the antiviral protein kinase PKR may interact with NFAR to influence the protein's function, thus potentially coupling the regulation of post-transcriptional processes with host defense mechanisms in the cell.

REFERENCES

1. Stark, G. R., Kerr, I. M., Williams, B. R., Silverman, R. H., and Schreiber, R. D. (1998) *Annu. Rev. Biochem.* **67,** 227–264
2. Meurs, E., Chong, K., Galabru, J., Thomas, N. S., Kerr, I. M., Williams, B. R., and Hovanessian, A. G. (1990) *Cell* **62,** 379–390
3. Bass, B. L., Hurst, S. R., and Singer, J. D. (1994) *Curr. Biol.* **4,** 301–314
4. St. Johnston, D., Brown, N. H., Gall, J. G., and Jantsch, M. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 10979–10983
5. Levin, D., and London, I. M. (1978) *Proc. Natl. Acad. Sci. U. S. A.* **75,** 1121–1125
6. Galabru, J., and Hovanessian, A. (1987) *J. Biol. Chem.* **262,** 15538–15544
7. Manche, L., Green, S. R., Schmedt, C., and Mathews, M. B. (1992) *Mol. Cell. Biol.* **12,** 5238–5248
8. Panniers, R., and Henshaw, E. C. (1983) *J. Biol. Chem.* **258,** 7928–7934
9. Choi, S. Y., Scherer, B. J., Schnier, J., Davies, M. V., Kaufman, R. J., and Hershey, J. W. (1992) *J. Biol. Chem.* **267,** 286–293
10. Rice, A. P., and Kerr, I. M. (1984) *J. Virol.* **50,** 229–236
11. Samuel, C. E. (1994) *Hokkaido Igaku Zasshi* **69,** 1339–1347
12. Jimenez-Garcia, L. F., Green, S. R., Mathews, M. B., and Spector, D. L. (1993) *J. Cell Sci.* **106,** 11–22
13. Balachandran, S., Roberts, P. C., Kipperman, T., Bhalla, K. N., Compans, R. W., Archer, D. R., and Barber, G. N. (2000) *J. Virol.* **74,** 1513–1523
14. Chang, H. W., Watson, J. C., and Jacobs, B. L. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 4825–4829
15. Davies, M. V., Chang, H. W., Jacobs, B. L., and Kaufman, R. J. (1993) *J. Virol.* **67,** 1688–1692
16. 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. Virol.* **71,** 1992–2003

[2] L. R. Saunders and G. N. Barber, unpublished results.

17. Sharp, T. V., Moonan, F., Romashko, A., Joshi, B., Barber, G. N., and Jagus, R. (1998) *Virology* **250,** 302–315
18. Beattie, E., Tartaglia, J., and Paoletti, E. (1991) *Virology* **183,** 419–422
19. Carroll, K., Elroy-Stein, O., Moss, B., and Jagus, R. (1993) *J. Biol. Chem.* **268,** 12837–12842
20. Mathews, M. B., and Shenk, T. (1991) *J. Virol.* **65,** 5657–5662
21. Brand, S. R., Kobayashi, R., and Mathews, M. B. (1997) *J. Biol. Chem.* **272,** 8388–8395
22. Gale, M. J., Jr., Korth, M. J., Tang, N. M., Tan, S. L., Hopkins, D. A., Dever, T. E., Polyak, S. J., Gretch, D. R., and Katze, M. G. (1997) *Virology* **230,** 217–227
23. Taylor, D. R., Shi, S. T., Romano, P. R., Barber, G. N., and Lai, M. M. (1999) *Science* **285,** 107–110
24. Lee, S. B., and Esteban, M. (1993) *Virology* **193,** 1037–1041
25. Lee, S. B., and Esteban, M. (1994) *Virology* **199,** 491–496
26. Yeung, M. C., Liu, J., and Lau, A. S. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 12451–12455
27. Der, S. D., Yang, Y. L., Weissmann, C., and Williams, B. R. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 3279–3283
28. Balachandran, S., Kim, C. N., Yeh, W. C., Mak, T. W., Bhalla, K., and Barber, G. N. (1998) *EMBO J.* **17,** 6888–6902
29. Kumar, A., Yang, Y. L., Flati, V., Der, S., Kadereit, S., Deb, A., Haque, J., Reis, L., Weissmann, C., and Williams, B. R. (1997) *EMBO J.* **16,** 406–416
30. Chu, W. M., Ostertag, D., Li, Z. W., Chang, L., Chen, Y., Hu, Y., Williams, B., Perrault, J., and Karin, M. (1999) *Immunity* **11,** 721–731
31. Kumar, A., Haque, J., Lacoste, J., Hiscott, J., and Williams, B. R. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 6288–6292
32. Clemens, M. J., and Elia, A. (1997) *J. Interferon Cytokine Res.* **17,** 503–524
33. Barber, G. N., Tomita, J., Hovanessian, A. G., Meurs, E., and Katze, M. G. (1991) *Biochemistry* **30,** 10356–10361
34. Polyak, S. J., Tang, N., Wambach, M., Barber, G. N., and Katze, M. G. (1996) *J. Biol. Chem.* **271,** 1702–1707
35. Laurent, A. G., Krust, B., Galabru, J., Svab, J., and Hovanessian, A. G. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 4341–4345
36. Abraham, N., Stojdl, D. F., Duncan, P. I., Methot, N., Ishii, T., Dube, M., Vanderhyden, B. C., Atkins, H. L., Gray, D. A., McBurney, M. W., Koromilas, A. E., Brown, E. G., Sonenberg, N., and Bell, J. C. (1999) *J. Biol. Chem.* **274,** 5953–5962
37. Hanamura, A., Caceres, J. F., Mayeda, A., Franza, B. R., Jr., and Krainer, A. R. (1998) *RNA* **4,** 430–444
38. Fields, S., and Song, O. (1989) *Nature* **340,** 245–246
39. Dever, T. E., Chen, J. J., Barber, G. N., Cigan, A. M., Feng, L., Donahue, T. F., London, I. M., Katze, M. G., and Hinnebusch, A. G. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 4616–4620
40. Barber, G. N., Wambach, M., Wong, M. L., Dever, T. E., Hinnebusch, A. G., and Katze, M. G. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 4621–4625
41. Saunders, L. R., Jurecic, V., and Barber, G. N. (2001) *Genomics* **71,** 256–259
42. Kharrat, A., Macias, M. J., Gibson, T. J., Nilges, M., and Pastore, A. (1995) *EMBO J.* **14,** 3572–3584
43. Matsumoto-Taniura, N., Pirollet, F., Monroe, R., Gerace, L., and Westendorf, J. M. (1996) *Mol. Biol. Cell* **7,** 1455–1469
44. Kao, P. N., Chen, L., Brock, G., Ng, J., Kenny, J., Smith, A. J., and Corthesy, B. (1994) *J. Biol. Chem.* **269,** 20691–20699
45. Patel, R. C., Vestal, D. J., Xu, Z., Bandyopadhyay, S., Guo, W., Erme, S. M., Williams, B. R., and Sen, G. C. (1999) *J. Biol. Chem.* **274,** 20432–20437
46. Dubois, M. F., and Hovanessian, A. G. (1990) *Virology* **179,** 591–598
47. Schwemmle, M., Clemens, M. J., Hilse, K., Pfeifer, K., Troster, H., Muller, W. E., and Bachmann, M. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 10292–10296
48. Jeffrey, I. W., Kadereit, S., Meurs, E. F., Metzger, T., Bachmann, M., Schwemmle, M., Hovanessian, A. G., and Clemens, M. J. (1995) *Exp. Cell Res.* **218,** 17–27
49. Besse, S., Rebouillat, D., Marie, I., Puvion-Dutilleul, F., and Hovanessian, A. G. (1998) *Exp. Cell Res.* **239,** 379–392
50. Thomis, D. C., and Samuel, C. E. (1995) *J. Virol.* **69,** 5195–5198
51. Barber, G. N., Jagus, R., Meurs, E. F., Hovanessian, A. G., and Katze, M. G. (1995) *J. Biol. Chem.* **270,** 17423–17428
52. Wu, S., and Kaufman, R. J. (1997) *J. Biol. Chem.* **272,** 1291–1296
53. Lefebvre, S., Burglen, L., Reboullet, S., Clermont, O., Burlet, P., Viollet, L., Benichou, B., Cruaud, C., Millasseau, P., and Zeviani, M. (1995) *Cell* **80,** 155–165
54. Pellizzoni, L., Kataoka, N., Charroux, B., and Dreyfuss, G. (1998) *Cell* **95,** 615–624
55. Buhler, D., Raker, V., Luhrmann, R., and Fischer, U. (1999) *Hum. Mol. Genet.* **8,** 2351–2357
56. Crozat, A., Aman, P., Mandahl, N., and Ron, D. (1993) *Nature* **363,** 640–644
57. Rabbitts, T. H., Forster, A., Larson, R., and Nathan, P. (1993) *Nat. Genet.* **4,** 175–180
58. Zinszner, H., Sok, J., Immanuel, D., Yin, Y., and Ron, D. (1997) *J. Cell Sci.* **110,** 1741–1750
59. Yang, L., Embree, L. J., Tsai, S., and Hickstein, D. D. (1998) *J. Biol. Chem.* **273,** 27761–27764
60. Baechtold, H., Kuroda, M., Sok, J., Ron, D., Lopez, B. S., and Akhmedov, A. T. (1999) *J. Biol. Chem.* **274,** 34337–34342
61. Kuroda, M., Sok, J., Webb, L., Baechtold, H., Urano, F., Yin, Y., Chung, P., de Rooij, D. G., Akhmedov, A., Ashley, T., and Ron, D. (2000) *EMBO J.* **19,** 453–462
62. Bentley, D. (1999) *Curr. Opin. Cell Biol.* **11,** 347–351
63. Caceres, J. F., and Krainer, A. R. (1997) in *Eukaryotic mRNA Processing* (Krainer, A. R., ed) pp. 174–212, IRL Press, Oxford
64. Katze, M. G., Wambach, M., Wong, M. L., Garfinkel, M., Meurs, E., Chong, K., Williams, B. R., Hovanessian, A. G., and Barber, G. N. (1991) *Mol. Cell. Biol.* **11,** 5497–5505
65. Green, S. R., and Mathews, M. B. (1992) *Genes Dev.* **6,** 2478–2490
66. McMillan, N. A., Chun, R. F., Siderovski, D. P., Galabru, J., Toone, W. M., Samuel, C. E., Mak, T. W., Hovanessian, A. G., Jeang, K. T., and Williams, B. R. (1995) *Virology* **213,** 413–424
67. Buaas, F. W., Lee, K., Edelhoff, S., Disteche, C., and Braun, R. E. (1999) *Mamm. Genome* **10,** 451–456
68. Langland, J. O., Kao, P. N., and Jacobs, B. L. (1999) *Biochemistry* **38,** 6361–6368
69. Coolidge, C. J., and Patton, J. G. (2000) *Nucleic Acids Res.* **28,** 1407–1417
70. Ting, N. S., Kao, P. N., Chan, D. W., Lintott, L. G., and Lees-Miller, S. P. (1998) *J. Biol. Chem.* **273,** 2136–2145
71. Liu, Y., Emeson, R. B., and Samuel, C. E. (1999) *J. Biol. Chem.* **274,** 18351–18358
72. Lai, F., Chen, C. X., Carter, K. C., and Nishikura, K. (1997) *Mol. Cell. Biol.* **17,** 2413–2424
73. Polson, A. G., Bass, B. L., and Casey, J. L. (1996) *Nature* **380,** 454–456
74. Micklem, D. R., Adams, J., Grunert, S., and St. Johnston, D. (2000) *EMBO J.* **19,** 1366–1377
75. Corthesy, B., and Kao, P. N. (1994) *J. Biol. Chem.* **269,** 20682–20690
76. Sakamoto, S., Morisawa, K., Ota, K., Nie, J., and Taniguchi, T. (1999) *Biochemistry* **38,** 3355–3361
77. Benkirane, M., Neuveut, C., Chun, R. F., Smith, S. M., Samuel, C. E., Gatignol, A., and Jeang, K. T. (1997) *EMBO J.* **16,** 611–624
78. Patel, R. C., and Sen, G. C. (1998) *EMBO J.* **17,** 4379–4390
79. Ito, T., Yang, M., and May, W. S. (1999) *J. Biol. Chem.* **274,** 15427–15432
80. Yeakley, J. M., Tronchere, H., Olesen, J., Dyck, J. A., Wang, H. Y., and Fu, X. D. (1999) *J. Cell Biol.* **145,** 447–455
81. Osman, F., Jarrous, N., Ben-Asouli, Y., and Kaempfer, R. (1999) *Genes Dev.* **13,** 3280–3293
82. Yang, L., Embree, L. J., and Hickstein, D. D. (2000) *Mol. Cell. Biol.* **20,** 3345–3354
83. Fischer, U., Liu, Q., and Dreyfuss, G. (1997) *Cell* **90,** 1023–1029

Characterization of Two Evolutionarily Conserved, Alternatively Spliced Nuclear Phosphoproteins, NFAR-1 and -2, That Function in mRNA Processing and Interact with the Double-stranded RNA-dependent Protein Kinase, PKR

Laura R. Saunders, Darren J. Perkins, Siddharth Balachandran, Rebecca Michaels, Ryan Ford, Akila Mayeda and Glen N. Barber

Access the most updated version of this article at doi: 10.1074/jbc.M104207200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 82 references, 51 of which can be accessed free at
http://www.jbc.org/content/276/34/32300.full.html#ref-list-1