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[25] Protein RNA-Binding Activity Measured by Northwestern Blot Analysis: The Interferon-Inducible RNA-Dependent Protein Kinase PKR

Stephen J. McCormack and Charles E. Samuel

Introduction

The Northwestern RNA blot assay is an important method for the identification and initial characterization of RNA-binding proteins. Several viral RNA-binding proteins have been characterized using the Northwestern assay, including proteins encoded by bovine rotavirus (1), human hepatitis delta virus (HDV) (2, 3), mouse coronavirus (4, 5), human immunodeficiency virus (HIV) (6), and human reovirus (7, 8). Cellular RNA-binding proteins likewise have been characterized using the Northwestern assay, including two proteins that bind HIV TAR RNA: a cellular RNA-binding protein of unknown function (9), and the interferon-inducible RNA-dependent protein kinase PKR (also known as P1/eIF-2α, p68, DAI, or dsI) (10, 11). For these viral and cellular RNA-binding proteins (1–11), like the well-established situation for DNA-binding proteins (12), multiple structural motifs exist that constitute functionally different binding domains (13).

The Northwestern RNA blot assay provides an efficient approach for the identification of regions of a protein responsible for its RNA-binding activity. This assay has been utilized in our laboratory at the University of California, Santa Barbara, to localize the RNA-binding subdomains of two proteins: the reovirus $\sigma3$ protein and the cellular protein kinase PKR. Truncation of cDNA clones encoding $\sigma3$ and PKR and subsequent expression with efficient inducible bacterial systems permitted the production of deletion mutant $\sigma3$ and PKR proteins that could be analyzed rapidly for RNA-binding activity (8, 10).

The $\sigma3$ protein is the major capsid protein of human reovirus, a segmented double-stranded (ds) RNA virus (14). The 41-kDa $\sigma3$ protein binds dsRNA, but not ssRNA or dsRNA (15). However, there is no evidence that the dsRNA binding activity of $\sigma3$ is selective for either sequence- or structure-specific dsRNAs (16). Northwestern blot analysis of *Staphylococcus aureus* V8 protease-derived fragments of $\sigma3$ localized the dsRNA-binding activity to the C-terminal 16-kDa peptide fragment (7, 8). Northwestern analysis of truncated $\sigma3$ proteins expressed from the s4 cDNA in *Escherichia coli* using a modified pET3 vector led to the identification of an 85-amino-acid domain responsible for RNA-binding activity (8).

PKR is an interferon-inducible RNA-dependent protein kinase of central impor-

tance in the antiviral actions of interferon (17). Activation of PKR requires the presence of a suitable activator RNA and involves the autophosphorylation of PKR. The RNA-binding activity of PKR appears to be selective for structure-specific ssRNAs such as adenovirus VA_1 RNA, HIV TAR RNA, and reovirus s1 mRNA (18, 19). Northwestern analysis of substitution and deletion mutant PKR proteins expressed in *E. coli* as TrpE fusion proteins clearly defined the N-terminal 98 amino acids of PKR as both necessary and sufficient for RNA-binding activity (10, 11). Furthermore, a repeated subdomain core of 20 amino acid residues, predicted by computer algorithms to be an amphipathic α-helix, was identified within the N-terminal RNA-binding region of PKR. This core sequence, designated motif R, was present at amino acid positions 55–75 and 145–166 of PKR (10). The RNA-binding R motif is conserved in several viral and cellular proteins from both prokaryotic and eukaryotic sources, proteins now known to be dsRNA-binding proteins (10, 20).

We describe in this article the procedure for analysis of RNA-binding activity by the Northwestern RNA blot assay. The procedure is illustrated with the human RNA-dependent protein kinase PKR.

Strategy

The strategy for measurement of RNA-binding activity by Northwestern analysis involves immobilization of target proteins on a filter membrane. Proteins fractionated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) are electroblotted onto a nitrocellulose filter membrane by standard techniques. The fiber-bound proteins are then analyzed for RNA-binding activity using a radioactive RNA probe; this RNA–protein blot analysis constitutes the Northwestern assay. Subsequently, a Western immunoblot analysis is carried out using the same filter membrane as was used for the Northwestern analysis in order to verify that comparable amounts of test proteins were present. The Western analysis is especially important in the cases of proteins that do not register as RNA-binding proteins in the Northwestern assay.

Materials and Methods

Reagents

The chemicals used in the following procedures are of reagent grade. Solutions are prepared with deionized glass-distilled water. The pH of buffers is measured at 25°C.

Tris–HCl (1 *M*, pH 7.9)
Tris–HCl (1 *M*, pH 7.4)
HEPES (0.5 *M*, pH 7.5)

NaCl (5 *M*)
KCl (3 *M*)
NaOAc (3 *M*)
MgCl$_2$ (1 *M*)
EDTA (0.5 *M*)
2-Mercaptoethanol (14.4 *M*)
Dithiothreitol (DTT; 0.1 *M;* Sigma, St. Louis, MO)
Phenylmethylsulfonyl fluoride (PMSF; 0.2 *M* in ethanol)
Spermidine (0.1 *M;* Sigma)
Nonidet P-40 [NP-40; 10% (w/v); Sigma]
Triton X-100 [10% (w/v); Sigma]
Ficoll 400 [20% (w/v); Sigma]
Polyvinylpyrrolidone [20% (w/v); Sigma]
ATP, GTP, UTP, and CTP (each 0.1 *M;* Sigma)
[α-^{32}P]UTP (650 Ci/mmol; ICN, Costa Mesa, CA)
[^{125}I]Protein A (ICN)
Pansorbin *S. aureus* cells (Calbiochem, La Jolla, CA)
Restriction endonucleases (United States Biochemical, Cleveland, OH)
T4 DNA ligase (Bethesda Research Laboratories, Gaithersburg, MD)
RNase-free DNase (RQ1, Promega, Madison, WI)
RNasin (Promega)
SP6 RNA polymerase (New England Biolabs, Beverly, MA)
Sequenase version 2.0 (United States Biochemical)
T7 RNA polymerase (United States Biochemical)
Thermus aquaticus DNA polymerase I (Boehringer-Mannheim, Indianapolis, IN)
Yeast total RNA (5 Prime–3 Prime, Boulder, CO)
Bovine serum albumin (Sigma)
Nitrocellulose filter membranes (0.45 μm; Schleicher & Schuell, Keene, NH)
Dulbecco's modified Eagle's medium (DMEM; GIBCO, Grand Island, NY)
Fetal bovine serum (FBS; HyClone, Logan, UT)

Vector Constructions

The transcription vector construction pSP6S1(416–576) was previously described (21). The vector construction pT7TAR was provided courtesy of N. Hernandez (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). The P1 KIN cDNA clone of human PKR (22) was used for the construction of the pSV(PKR) (11, 23) and pATH–PKR expression plasmids (10, 11).

Expression of TrpE–PKR Fusion Proteins

Freshly transformed *E. coli* C600 cells were used for the expression of the TrpE–PKR fusion proteins essentially as previously described (22, 24). Colonies were grown at 37° C in 2 ml of minimal medium (24) supplemented with 200 μg/ml am-

picillin and 20 μg/ml tryptophan. When the cells were at an optical density of 0.5 measured at 600 nm, a 0.5-ml aliquot of the culture was transferred to a 1.5-ml microfuge tube and the cells were collected by centrifugation at 4000 g for 5 min at 4°C. The supernatant solution was discarded and the cells were suspended in 3 ml of minimal medium supplemented with 200 μg/ml ampicillin, but lacking tryptophan. The cells were starved for 3 hr at 37°C in a New Brunswick (New Brunswick, Sci. Co., Edison, NJ) incubator shaker (250 rpm). Indoleacrylic acid (IAA; 5 mg/ml in ethanol) was then added to yield a final concentration of 100 μg/ml, and incubation was continued at 37°C for an additional 3 hr. The IAA-induced cells were collected by centrifugation as above, washed in 1 ml of phosphate-buffered saline (136 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$, and 2 mM KH$_2$PO$_4$), and then used to prepare cell-free extracts. Both whole-cell lysates and the insoluble fraction were analyzed for the presence of expressed PKR protein product.

Isolation of TrpE–PKR Fusion Proteins

To analyze whole-cell lysates for protein expression, 100 μl of either induced or uninduced cells was pelleted and resuspended in 200 μl of 1 × Laemmli buffer (25). The cell suspension was sonicated (three times for 5 sec each) and then boiled for 10 min. Insoluble material was removed by centrifugation for 5 min at 4°C with a microfuge and the supernatant solution was analyzed on an SDS–polyacrylamide (10%, w/v) gel. Proteins were visualized by staining the gel [0.06% (w/v) Coomassie brilliant blue R-250, 10% (v/v) acetic acid, and 25% (v/v) isopropanol] or by Western analysis.

  To isolate the insoluble proteins, the whole-cell pellets obtained above were resuspended at 4°C in 20 mM Tris–Cl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 0.5% (w/v) NP-40, 5 mM PMSF, and 5 mM 2-mercaptoethanol, sonicated (three times for 5 sec each), and microfuged at 14,000 g for 10 min at 4°C. The supernatant solution was removed by aspiration, and the insoluble pellet was then resuspended by sonication in 500 μl of the extraction buffer [20 mM Tris–Cl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, 5 mM PMSF, and 5 mM 2-mercaptoethanol]. This procedure was repeated four times and the final insoluble pellet was then suspended in 500 μl of 1 × Laemmli buffer.

Cell Maintenance and Transfection

Monkey COS-1 cells were grown as monolayer cultures in DMEM supplemented with 5% (v/v) FBS. Transfection was by the diethylaminoethyl (DEAE)–dextran/chloroquine phosphate method (26, 27).

Preparation of the PKR Protein Kinase from Transfected COS Cells

Cell-free extracts were prepared from monkey COS cells at 48 hr after transfection with plasmid constructions prepared using the simian virus 40 (SV40)-based pJC119 vector. Transfected monolayer cell cultures (60-mm dishes) were rinsed twice with 1 ml of PBS, and the cells were lysed at 4°C by the addition of 600 μl of modified

NP-40 lysis buffer [20 mM HEPES (pH 7.5), 50 mM KCl, 5 mM EDTA, 5 mM
2-mercaptoethanol, and 1 mM PMSF]. PKR proteins were immunoprecipitated using
rabbit antiserum raised against recombinant PKR; immune complexes were collected
using formalin-fixed *S. aureus* cells. Dilutions of the immunoprecipitated PKR pro-
teins were then fractionated on an SDS–polyacrylamide (10%, w/v) gel and subse-
quently processed for the Northwestern and Western analyses.

Preparation of RNA Probes

[32]P-Labeled reovirus s1(416–576) mRNA, adenovirus VA$_I$ RNA, and HIV TAR
RNA were prepared as *in vitro* transcripts with bacteriophage polymerases (28) as
previously described (21), except that the standard transcription mixture was modi-
fied to contain 40 mM Tris–Cl buffer (pH 7.9), 6 mM MgCl$_2$, 2 mM spermidine, 10
mM NaCl, 0.5 mM ATP, 0.5 mM CTP, 0.5 mM GTP, 0.2 mM UTP, [α-^{32}P]UTP (500
μCi/ml), 10 mM DTT, 0.25 mg/ml bovine serum albumin, 250 U/ml RNasin, and
400 U/ml either T7 RNA polymerase or SP6 RNA polymerase as indicated. The
vector pSP6S1(416–576) and SP6 RNA polymerase were used to prepare the reovi-
rus s1(416–576) RNA; the vector pT7VA$_I$ and T7 RNA polymerase were used to
prepare adenovirus VA$_I$(1–160) RNA; and the vector pT7TAR and T7 RNA poly-
merase were used to prepare the HIV TAR(1–82) RNA. Following incubation of the
transcription reaction mixture at 37°C (T7 polymerase) or 40°C (SP6 polymerase)
for 90 min, RNase-free DNase (5 U) was added and the incubation was continued for
an additional 15 min at 37°C, after which the reaction mixtures were extracted with
phenol–chloroform (1:1) and then chloroform. RNA was chromatographed on a
Sephadex (Pharmacia Fine Chemicals, Sweden) scolumn (G150-40) to further re-
move unincorporated nucleotides. The amount of RNA synthesized was quantitated
by trichloroacetic acid precipitation on Whatman (Clifton, NJ) GF/C filter disks; the
radioactivity was measured in Aquasol II (DuPont, Boston, MA) with a Beckman LS
1801 liquid scintillation system. The integrity of the RNA was ascertained by elec-
trophoresis under denaturing conditions, using formaldehyde–agarose gels and au-
toradiography (21, 29).

Northwestern Gel Blot Analysis

Northwestern gel blot analysis for the RNA-binding activity of wild-type and mutant
PKR proteins was carried out essentially as described by Boyle and Holmes (1).
Briefly, PKR-containing protein samples were fractionated by discontinuous SDS–
polyacrylamide slab (1.5 mm thick) gel electrophoresis (25). Proteins were electro-
blotted to nitrocellulose filter membranes at 4°C (>10 hr at 500 mA) in buffer con-
taining 25 mM Tris, 192 mM glycine, and 20% (v/v) methanol. Following transfer
the filter membranes were washed with water and enclosed in Seal-a-meal bags con-
taining RNA binding buffer [RBB: 50 mM HEPES (pH 7.5), 50 mM KCl, 0.05%
(v/v) Triton X-100, 0.04% (v/v) Ficoll, 0.04% (v/v) polyvinylpyrrolidone, 0.08%
(w/v) bovine serum albumin, and 2.5 mM EDTA] and 20 μg/ml either human U-cell
or *E. coli* total RNA. The filter membranes were pretreated in RBB containing an

RNA blocker at room temperature for 3 hr, with gentle agitation using New Bruns-wick gyratory shaker G-2. The filters were then incubated at room temperature in RBB with ^{32}P-labeled reovirus s1(416–576) mRNA, ^{32}P-labeled adenovirus RNA, or ^{32}P-labeled HIV TAR RNA as indicated (>1 × 10^5 cpm/ml). Following incubation with the probe, the nitrocellulose membrane filters were removed from the Seal-a-meal bags, washed four times with RBB for 30 sec at room temperature, and then air-dried. Autoradiography was carried out with a screen at − 80° C using Fuji RX film (Fuju Photo Film Co., Japan).

For the competition experiments filters were pretreated with RBB lacking U-cell RNA; the competitor RNAs were then added at the same time as the probe. Following incubation with the probe–competitor RNA mixture, the nitrocellulose membrane filters were washed (four times for 30 sec each) in RBB at room temperature and then air-dried. Autoradiography was carried out with a screen at − 80° C.

Western Immunoblot Analysis

Following Northwestern analysis using ^{32}P-labeled TAR RNA or ^{32}P-labeled s1(416–576) RNA, the nitrocellulose filters were routinely washed with water for 30 min at room temperature to remove the RNA probe, which was verified by autoradiography. The filter membrane was then subjected to Western analysis using anti-PKR antibody.

Western immunoblots were performed by the method of Towbin *et al.* (30) essentially as previously described (31). Filter membranes were blocked in PBS containing 0.05% (v/v) Tween 20 (Sigma Chem. Co.) and 5% (w/v) nonfat dry milk at room temperature for 1 hr. Binding of antibody was performed overnight at 4° C with antiserum diluted 1/750 in PBS containing 0.05% (v/v) Tween 20. The filters were washed three times for 20 min at room temperature with PBS containing 0.05% (v/v) Tween 20, then probed with [^{125}I]protein A diluted 1/5000 in PBS containing 0.05% (v/v) Tween 20. The filters were then washed (three times for 20 min each) at room temperature with PBS containing 0.05% (v/v) Tween 20 and air-dried. Autoradiography was then carried out at − 80° C with a screen. Western signals detected with [^{125}I]protein A and antibody against PKR correlated with the intensity of staining by Coomassie brilliant blue R-250 of the various TrpE–P1 fusion proteins.

Preparation of PKR for Gel Mobility-Shift Analysis

Recombinant PKR protein for RNA gel mobility-shift analysis was prepared from transfected COS cells. COS cells grown as monolayer cultures in 150-mm dishes (Nuclon) with DMEM containing 5% (v/v) FBS were transfected by the DEAE–dextran/chloroquine phosphate method using 40 μg of expression vector DNA in 4 ml of serum-free DMEM. Three culture dishes (150 mm) were routinely transfected with each pSV(PKR) expression vector construction; harvest was at 48 hr posttransfection. Monolayer cultures were washed three times with 5 ml of ice-cold PBS before cell lysis with 3 ml of kinase lysis buffer [containing 20 mM HEPES (pH 7.5), 50 mM KCl, 5 mM EDTA, 0.65% (v/v) NP-40, 5 mM 2-mercaptoethanol, and 5 mM

PMSF]. All subsequent steps were carried out at 4°C. Ribosomes were separated from S10 extracts and crude 0.8 M ribosome salt-wash fractions were prepared essentially as previously described (32), except that the A.12 and A.8 buffers were modified to contain 20 mM HEPES (pH 7.5), 120 mM or 800 mM KCl, 5 mM EDTA, 0.1% (w/v) Triton X-100, 5 mM 2-mercaptoethanol, and 1 mM PMSF. Eluate fractions from the DEAE–cellulose step containing PKR were pooled, frozen in liquid nitrogen, and stored at $-80°$C until further analysis.

Gel Mobility-Shift Analysis

Gel mobility-shift analysis (32a) was performed essentially as described by Judware and Petryshyn (33) and Gatignol *et al.* (9). The final reaction volume (10 μl) contained approximately 1 ng of the recombinant PKR protein and varying concentrations of ^{32}P-labeled probe RNA (specific activity, $>1 \times 10^7$ cpm/pmol) in 15 mM Tris–HCl (pH 7.8), 70 mM NaCl, 10 mM KCl, 1 mM EDTA, 6% (v/v) glycerol, 0.01% (v/v) Triton X-100, 25 ng of ovalbumin, 2 U of RNasin, and 10 ng of yeast total RNA. Reaction mixtures were incubated for 20 min at room temperature before loading onto a native acrylamide (10%) gel (80:1 acrylamide–bisacrylamide) in 0.5$\times$ Tris–borate–EDTA buffer. The gel was prerun at 4°C for over 1 hr at 200 V; 40 μl of 2% (v/v) glycerol solution was added to the wells prior to loading the samples. A dye mixture containing 0.05% (w/v) xylene cyanol, 0.05% (w/v) bromphenol blue, and 10% (v/v) glycerol was loaded on the outside lanes of the gel. Electrophoresis was conducted at 280 V for 5 hr or until the xylene cyanol dye was approximately 3 cm from the bottom of the gel. The gels were then dried and subjected to autoradiography.

Discussion

The Northwestern assay is an effective approach both for identifying RNA-binding proteins and for characterizing the subdomain regions within them necessary for RNA-binding activity. The procedures described here for analysis of protein RNA-binding activity measured by the Northwestern blot assay have been used in our laboratory to identify the RNA-binding domain within the interferon-inducible RNA-dependent protein kinase PKR from human cells (10, 11).

Identification of an RNA-Binding Domain within the N-Terminal Region of PKR

The availability of cDNA clones of the PKR kinase provided an opportunity to elucidate the region of PKR responsible for its RNA-binding activity. As an approach to determining the residues of PKR necessary and sufficient for RNA-binding activity, deletion mutants of the P1 KIN cDNA encoding PKR were expressed in *E. coli* as TrpE–P1 fusion proteins (10, 22). The strategy was to determine whether a contiguous subdomain region expressed from the PKR cDNA could convert the bacterial TrpE protein (24) into an RNA-binding protein (10). A similar strategy involving

TrpE fusion proteins was also used to characterize the binding of hepatitis delta antigen to HDV RNA (2).

TrpE–P1 fusion proteins of PKR were examined at varying protein concentrations, as measured by Western immunoblot analysis, for their ability to bind ^{32}P-labeled reovirus s1(415–576) RNA. Reovirus s1(416–576) RNA is known to activate the autophosphorylation of PKR (21), and thus was presumed to bind PKR. As shown by Fig. 1, ^{32}P-labeled reovirus s1 RNA selectively bound to TrpE–P1 fusion
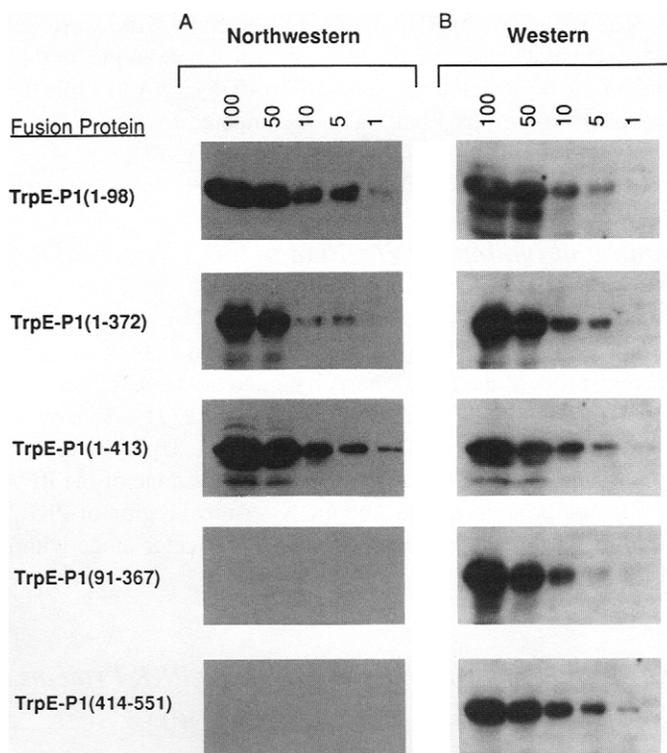

FIG. 1   Effect of protein concentration on the RNA-binding activity of PKR deletion mutant proteins expressed in *E. coli* as TrpE–P1 fusion proteins. (A) Northwestern RNA blot assay using ^{32}P-labeled reovirus s1(416–576) RNA as the probe. (B) Western immunoblot assay carried out with rabbit immune serum generated against recombinant human PKR. Different regions of the P1 KIN cDNA open reading frame, which encodes the human PKR protein kinase (22), were expressed as fusion proteins with TrpE (10). The nitrocellulose fiber membrane containing the TrpE–P1 fusion proteins analyzed in the Northwestern RNA binding assay shown under (A) was stripped and subsequently analyzed in the Western immunoblot assay shown under (B). The amount of fusion protein analyzed (expressed in picomoles) is indicated above the gel lanes. [From S. J. McCormack, D. C. Thomis, and C. E. Samuel, *Virology* **188,** 47 (1992). Reprinted with permission.]

proteins that included the N-terminal region of PKR. The TrpE–P1 fusion proteins that possessed PKR amino acid residues 1–98, 1–372, or 1–413, when immobilized on nitrocellulose, all bound the ^{32}P-labeled reovirus s1 RNA probe. By contrast, TrpE–P1 fusion proteins that lacked the N-terminal 98 amino acid residues but included either the internal or C-terminal regions of PKR or amino acids 91–367 or 414–551, respectively, did not bind the reovirus s1 activator RNA (Fig. 1). In the case of HDV, the middle one third of delta antigen expressed as TrpE fusion was necessary and sufficient for binding of HDV RNA in the Northwestern RNA blot assay (2).

The specificity of RNA-binding detected by Northwestern RNA blot assay can be assessed by the use of competitor RNAs. For example, in the case of PKR, the binding of the ^{32}P-labeled reovirus s1(416–576) RNA to TrpE–P1(1–98) was competed by poly(rI)–poly(rC) but was not competed by total RNA isolated from human U cells (10).

Both Activator and Inhibitor RNAs Bind to the N-Terminal Region of PKR

As shown by Fig. 2, both adenovirus VA$_I$ RNA, an antagonist of PKR kinase activation (34, 35) and HIV TAR RNA, described as both an agonist (36, 37) and an antagonist (38) of the PKR kinase, bound to the same region of PKR, as did the reovirus s1(416–576) RNA, an activator of the kinase (21). Adenovirus VA$_I$ RNA, HIV TAR RNA, and reovirus s1 RNA all bound to the TrpE–P1 fusion proteins that included the N-terminal 98 residues of PKR, whereas none of the RNAs bound to the TrpE–P1 fusion proteins that lacked the N-terminal region of PKR. Finally, the TrpE portion of the fusion protein expressed from vector alone without PKR insert did not bind RNA.

RNA-Binding Activity of Mutant and Wild-Type PKR Proteins Measured by Northwestern Blot and Gel-Mobility Shift Assays

The RNA-binding activity of PKR measured by Northwestern analysis maps to the N-terminal half of PKR within a region that includes the repeated subdomain R motif, the core of which is about 20 amino acids (10). The R motif first identified in human PKR is conserved in many other RNA-binding proteins, is often repeated, and represents the prototype for a new RNA-binding motif with the consensus sequence G–X–G–S/T–K–X–X–A/S–K–X–X–A–A–X–X–A–X–X–X–L (10, 17, 20).

Among the most highly conserved residues of the core R motif is a lysine residue, present at amino acid position 64 within the N-terminal proximal copy of R in PKR (10). In order to assess the importance of the conserved core motif R in the RNA-binding activity of human PKR, lysine-64 was converted to glutamic acid by site-
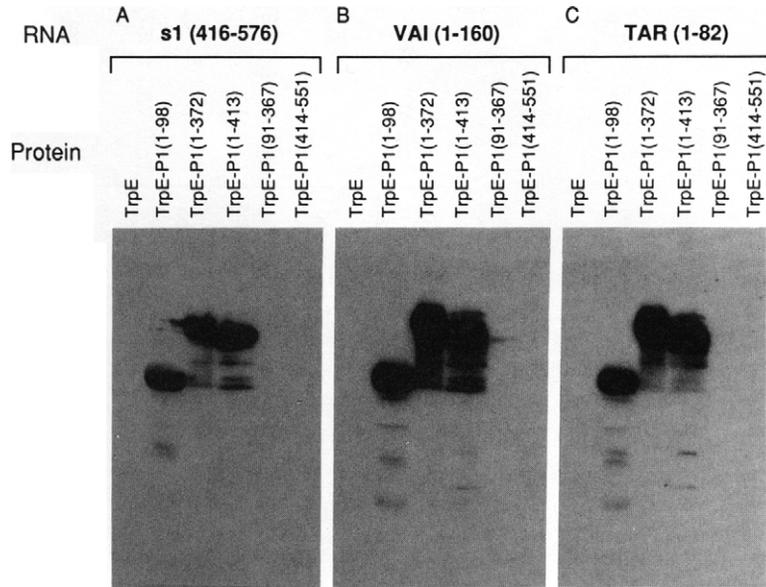
Fig. 2   PKR RNA-binding activity measured with reovirus, adenovirus, and HIV RNA probes. PKR deletion mutant proteins expressed from P1 KIN cDNA as TrpE–P1 fusion proteins were analyzed for RNA-binding activity. (A) Northwestern RNA blot assay using as the probe ^{32}P-labeled reovirus s1(416–576) RNA, an activator of the PKR kinase (21). (B) Northwestern RNA blot assay using as the probe ^{32}P-labeled adenovirus VA$_{\mathrm{I}}$(1–160) RNA, an inhibitor of PKR kinase activation (19). (C) Northwestern RNA blot assay using as the probe ^{32}P-labeled HIV TAR(1–82) RNA, described as both an activator and an inhibitor of the PKR kinase (18). [From S. J. McCormack, D. C. Thomis, and C. E. Samuel, *Virology* **188,** 47 (1992). Reprinted with permission.]

directed mutagenesis (11). Serial dilutions of TrpE fused with the N-terminal 98 amino acids of PKR, either wild-type (Wt) PKR or the K64E substitution mutant, were first analyzed by the Northwestern assay. Although the TrpE–Wt(1–98) fusion protein efficiently bound RNA (Fig. 3, lanes 1–5), the K64E substitution mutant protein TrpE–K64E(1–98) did not bind RNA, even at concentrations at least 20-fold higher than those of the TrpE–Wt(1–98) protein (Fig. 3, lanes 6–10). The TrpE–Wt(91–367) fusion protein lacking the N-proximal copy of R, but retaining the more C-terminal variant repeat of the R motif, likewise was unable to bind RNA as measured by the Northwestern assay (Fig. 3). The TrpE–Wt(1–372) fusion protein that included both copies of the core motif R efficiently bound RNA (Fig. 3, lane 16). These results suggest that the N-proximal copy of the R motif is both necessary and sufficient to confer RNA-binding activity to TrpE as measured by the Northwestern assay. This result was confirmed by gel-mobility shift analysis of the full-length 551-amino-acid PKR protein (Fig. 4).
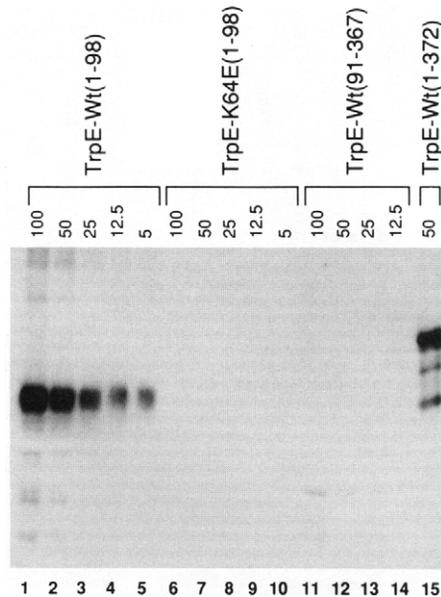
FIG. 3   RNA-binding activity of wild-type (Wt) and mutant PKR proteins expressed in *E. coli* as TrpE–P1 fusion proteins. The Northwestern RNA blot assay was performed with ^{32}P-labeled HIV TAR RNA as the probe. Recombinant PKR proteins expressed from P1 KIN cDNA as TrpE fusion proteins were analyzed as a function of protein concentration; the amount of each protein (in picomoles) is indicated above the gel lanes. [From S. J. McCormack, L. G. Ortega, J. P. Doohan, and C. E. Samuel, *Virology* **198,** 92 (1994). Reprinted with permission.]

The full-length catalytic subdomain II K296R(1–551) mutant, which lacks kinase catalytic activity (23, 39), bound ^{32}P-labeled adenovirus VA$_1$ RNA in the gel mobility-shift assay (Fig. 4, lane 2), whereas the ability of the K64E/K296R(1–551) double mutant to bind RNA was greatly diminished (Fig. 4, lane 4). Thus, the K64E substitution was sufficient to severely impair RNA-binding activity of the PKR protein, measured both by the Northwestern assay using truncated PKR TrpE fusion protein immobilized on nitrocellulose (Fig. 3) and by the gel mobility-shift assay with full-length PKR protein in solution (Fig. 4).

RNA-Binding Activity of Wild-Type and Mutant PKR Proteins Expressed in Mammalian Cells

The Northwestern blot assay may also be utilized to analyze the RNA-binding activity of proteins present in the crude extracts prepared from mammalian cells. This
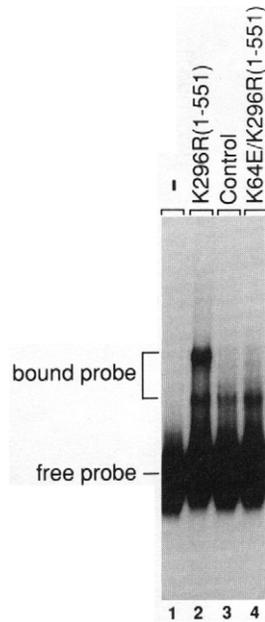
FIG. 4   Gel mobility-shift analysis of recombinant PKR proteins expressed in transfected monkey COS cells. PKR proteins K296R(1–551) and K64E/K296R(1–551), expressed from human P1 KIN cDNAs in monkey COS cells using the pJC119 vector, were analyzed for their ability to bind ^{32}P-labeled VA$_I$ RNA in a gel mobility-shift assay. The PKR proteins were isolated from the 0.8 M KCl ribosomal salt-wash fraction by DEAE–cellulose chromatography. Lane 1, ^{32}P-labeled adenovirus VA$_I$ RNA probe (6 × 10^{-9} M) alone; lane 2, PKR K296R(1–551) catalytic activity-deficient mutant, with an arginine substituted for the catalytic subdomain II lysine at amino acid position 296 (23); lane 3, control protein sample prepared from COS cells transfected with pJC119 vector containing no PKR cDNA insert; and lane 4, PKR K64E/K296R(1–551) double mutant, with a glutamic acid substituted for the conserved lysine at amino acid position 64 (11) as well as the K296R mutation (23). Comparable amounts (5 × 10^{-10} M) of PKR proteins were present in lanes 1 and 3, as determined by Western immunoblot analysis and comparison to a HIS–K296R(1–551) standard of known concentration.

is exemplified by the results shown in Fig. 5. PKR proteins isolated by quantitative immunoprecipitation from extracts of vector-transfected COS cells were fractionated by SDS–PAGE, transferred to nitrocellulose, and subjected to sequential Northwestern and Western blot analyses. The same nitrocellulose filter blot was first used to measure the RNA-binding activity of PKR proteins by Northwestern RNA blot analysis with ^{32}P-labeled RNA probe; then, after removal of the RNA probe, the amount of PKR protein present on the filter membrane was quantitated by Western analysis with [^{125}I]protein A and polyclonal antibody probe against PKR.
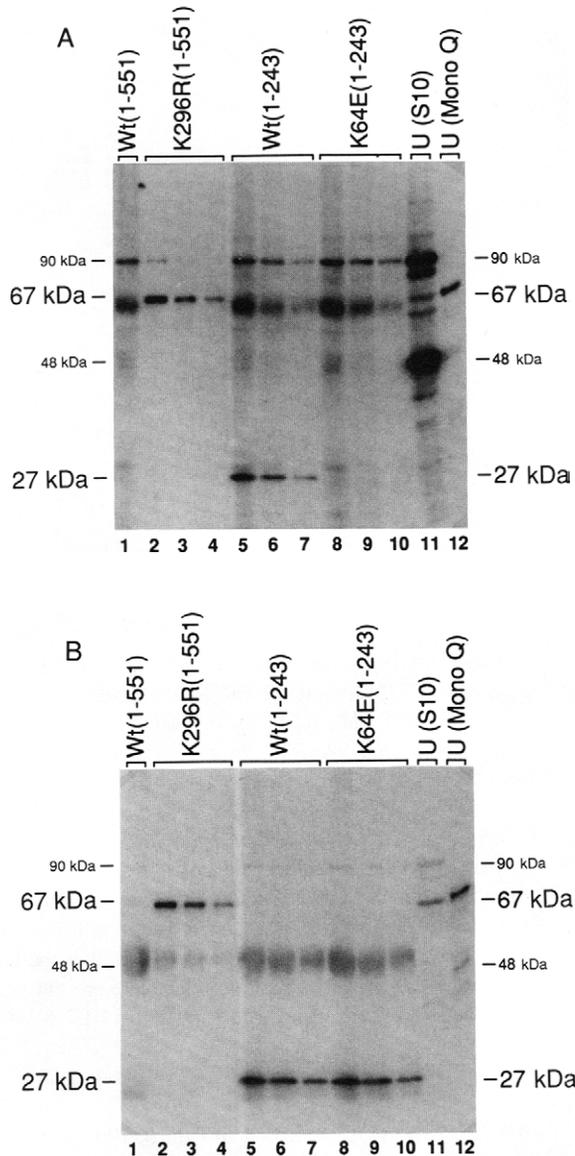
FIG. 5   Effect of protein concentration on the RNA-binding activity of wild-type and mutant PKR proteins expressed in transfected monkey COS cells. The SV40-based vector pJC119 was used to express wild-type (Wt) and mutant forms of the P1 KIN cDNA in monkey COS cells. At 48 hr after transfection, cell-free extracts were prepared and the PKR proteins were immunoprecipitated with anti-PKR antibody. The immunoprecipitates were then analyzed by (A) Northwestern RNA blot assay for RNA-binding activity and (B) Western immunoblot assay for amounts of PKR protein. *Dilution* refers to the fold-dilution prior to SDS–PAGE of

As shown in Fig. 5A, the 67-kDa catalytic subdomain II K296R(1–551) mutant PKR protein (lanes 2–4) and the 27-kDa C-truncated Wt(1–243) PKR protein (lanes 5–7) both retained RNA-binding activity in the Northwestern assay. By contrast, the 27-kDa C-truncated K64E(1–243) substitution mutant did not bind RNA (lanes 8–10). The Northwestern RNA-binding signal (Fig. 5A) was dependent on the concentration of the K296R(1–551) and Wt(1–243) PKR proteins and correlated with their Western signals (Fig. 5B). The K64E(1–243) mutant protein did not display detectable RNA-binding activity at concentrations comparable to those of Wt(1–243) that efficiently bound RNA. Because full-length Wt(1–551) is expressed inefficiently due to translational autoregulation (23, 40), the Western and Northwestern signals are difficult to discern over background (Fig. 5, lane 1). As internal standards, crude S10 extract prepared from interferon-treated human U cells (lane 11) and PKR isolated from the ribosomal salt wash from interferon-treated U cells (lane 12) were included. Both samples contained a 67-kDa RNA-binding protein. The relatively abundant immunologically cross-reactive 90-kDa RNA-binding protein present in crude extracts (lane 11) from human cells (23, 41) was not detected with the partially purified PKR preparation (Fig. 5, lane 12). Other investigators, using the Northwestern assay to analyze homogenates of human brain tissue, also detected a 90-kDa RNA-binding protein (42).

Concluding Comments

The Northwestern RNA–protein blot assay represents one approach to identifying RNA-binding proteins. The Northwestern assay also represents an efficient method for rapidly screening mutant proteins, with the objective of defining the subdomain responsible for RNA-binding activity. This is especially true when a cDNA encoding the protein of interest is available. The RNA-binding analysis of proteins can be facilitated by the use of inducible bacterial systems that permit the overexpression of nested-set deletion mutants of the target protein (2, 8, 10). The Northwestern assay may also be used to examine the *in vitro* RNA-binding specificity of proteins (2, 3,

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the immunoprecipitate derived from 100 μl of lysate. Lane 1, Wt(1–551) at 1/1 (v/v) dilution; lanes 2–4, K296R(1–551) mutant at 1/10, 1/20, and 1/40 (v/v) dilutions, respectively; lanes 5–7, truncated Wt(1–243) at 1/1, 1/2, and 1/4 (v/v) dilutions, respectively; lanes 8–10, truncated K64E(1–243) at 1/1, 1/2, and 1/4 (v/v) dilutions, respectively; lane 11, S10 cell-free extract prepared from interferon-treated human U cells; and lane 12, PKR kinase purified by Mono Q FPLC chromatography (Waters-Millipore, Bedford, MA) from the ribosomal salt-wash fraction of interferon-treated U cells. The positions of the 67-kDa full-length PKR protein and the truncated 27-kDa PKR protein, as well as the positions of the endogenous 90-kDa and 48-kDa RNA binding proteins, are indicated at the sides of the autoradiograms. [From S. J. McCormack, L. G. Ortega, J. P. Doohan, and C. E. Samuel, *Virology* **198**, 92 (1994). Reprinted with permission.]

10). However, because RNA-binding activity as measured by the Northwestern assay is sensitive to pH, false-positive responses may occur at pH below 6.5 (8). Also, because the candidate target proteins first are resolved by SDS–PAGE, the Northwestern assay may not be suitable for proteins whose RNA-binding activity depends on a dimeric structure, particularly a heterodimeric structure in which the two subunits possess different mobilities in SDS–PAGE gels. Therefore, it is important to attempt to confirm RNA-binding results obtained by the Northwestern RNA blot assay with results obtained by a second independent approach, for example, the gel mobility-shift assay (32a).

Acknowledgments

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