2672–2678 *Nucleic Acids Research, 1997, Vol. 25, No. 13*

Characterization and mapping of the double-stranded regions involved in activation of PKR within a cellular RNA from 3T3-F442A cells

Raymond A. Petryshyn[1,2,*], Andrea G. Ferrenz[1] and Jinhe Li[3]

[1]Center for Cancer and Transplantation Biology, Children's National Medical Center, Washington, DC, USA, [2]Department of Biochemistry and Molecular Biology, George Washington University School of Medicine, Washington, DC, USA and [3]Department of Neurobiology, Harvard Medical School, Boston, MA, USA

Received February 10, 1997; Revised and Accepted May 9, 1997

DDBJ/EMBL/GenBank accession no. M93663

ABSTRACT

PKR is a doubled-stranded RNA-dependent protein kinase which is implicated in the regulation of several cellular processes, including cell proliferation. PKR undergoes phosphorylation and activation in mouse embryonic 3T3-F442A cells in response to endogenous RNA(s). Activation of PKR is related to growth and differentiation of these cells. A cellular regulatory RNA (R-RNA) which activates PKR has been isolated from these cells and its cDNA partially sequenced. Here we have characterized the R-RNA transcript with respect to nuclease sensitivity and the extent of double-stranded structure involved in activation of PKR. The location of the activating sequence was mapped to a contiguous 226/252 nt region of the R-RNA transcript by hybridization to its cDNA fragments. Hybridization with a panel of short oligodeoxynucleotides complementary to the R-RNA, coupled with protein kinase analysis, was used to probe the 252 nt region for critical sequences. Three short non-contiguous sequences which appear most important for activation of PKR were identified within the 252 nt region. Thus, these studies have identified specific sequences most important for activation of PKR. Furthermore, since the above antisense oligodeoxynucleotides inhibit enzyme activation, our results exemplify an unusual mode of action of antisense sequences on the activation of PKR by disruption of RNA secondary structure.

INTRODUCTION

The double-stranded (ds)RNA-dependent eIF-2α kinase (PKR) is an interferon-induced serine/threonine protein kinase (1–3). It is well recognized for its role in translational control through phosphorylation of eIF-2α (4–7) and in establishing a state of resistence to viral infection in cells (8,9). Other studies have indicated that PKR plays an important role in the regulation of cell growth and differentiation (10–13), tumor suppression (14,15) and in signal transduction (16–18). The conversion of PKR from a latent to active eIF-2α kinase requires ATP, divalent cations and low levels of dsRNA (4,5,19). Activation of PKR is dependent on its autophosphorylation (4,5,20). Paradoxically, autophosphorylation and activation of PKR can also be prevented by high concentrations of dsRNA (4,21).

The dynamic mechanism by which PKR interacts with dsRNA is not fully understood. RNA binding motifs present in the N-terminal portion of PKR have been identified (for a review see 7) and their interaction with dsRNA structures have been partially characterized (22,23). However, the structural and spatial constraints within RNA necessary for interaction with the RNA binding motifs and their location remain to be fully resolved. What is known is that duplex RNAs such as poly(I)·poly(C) and reovirus dsRNA are well-known activators of PKR (4,8,24). Several single-stranded (ss) viral RNAs, such as adenovirus VAI RNA (25,26), Epstein–Barr virus EBER I RNA (27) and HIV-1 mRNA (28–31), have been shown to interact with PKR through secondary structure elements and modulate activity of the enzyme.

The findings that eIF-2α undergoes phosphorylation in response to RNAs prepared from uninfected cells (32,33) and that altered eIF-2α phosphorylation and PKR activity have been reported in cells subjected to heat stress (34) indicate that there are non-viral cellular RNAs which also activate PKR. Evidence for such cellular RNAs is supported by the findings that the mRNA for PKR itself may regulate its activation (20) and that accumulation of dsRNAs capable of activating PKR occurs in embryonal carcinoma cells which have been induced to differentiate (12). However, the range of cellular RNAs involved in regulating this important enzyme is unknown. Moreover, a detailed characterization of the structural features involved in PKR activation within any cellular RNA has yet to be carried out. We have reported that poly(A)$^+$ RNA from mouse embryonic fibroblasts contains RNA(s), termed regulatory RNA (R-RNA), which can activate PKR (35). An R-RNA has been isolated from fibroblast mRNA and its partial cDNA cloned and sequenced (36).

In this study we have characterized the double-stranded features of this R-RNA which is involved in activation of PKR. The location of the double-stranded regions necessary for PKR activation have been mapped to the R-RNA transcript using novel liquid hybridization methods, employing DNA restrictions

*To whom correspondence should be addressed at: Center for Cancer and Transplantation Biology, Children's National Medical Center, 111 Michigan Avenue NW, Washington, DC 20010, USA. Tel: +1 202 884 3078; Fax: +1 202 884 3929

fragments and antisense oligodeoxynucleotides. The nucleic acid sequence of these critical regions have been determined. This study represents an initial characterization of a cellular RNA involved in activation of PKR and also describes an approach for regulating activation of PKR using antisense molecules.

MATERIALS AND METHODS

Preparation of plasmids and oligodeoxynucleotides

The pGEM-3zf(±) plasmid (Promega) containing the 847 bp R-RNA cDNA insert was prepared as previously described (36). Phosphorothioate oligodeoxynucleotides (Oligos Etc. Inc.) were selected for optimal hybridization and minimal self-folding and homodimer annealing. The sequence and size of the oligos used were: OL1A, 5′-CCTCATCTAGCTTATCCATT-3′ 20mer; OL2A, 5′-ATCCGGTTCATACGCCTCATC-3′ 21mer; OL1, 5′-CCTGC-AATGATTCCAATTCC-3′ 20mer; OL3A 5′-ATTTAATTCAATA-GATGCATAT-3′ 22mer; OL2, 5′-TATTGGACGAATGCATT-TTG-3′ 20 mer; OL4A, 5′-TCTTCCTTTGATAGCGACCT-3′ 20mer; OL3, 5′-GAGTAATCAGGATCTTCCTT-3′ 20mer; OL5A, 5′-CCTTATGACTTGACCCTCTATA-3′ 22mer; OL4, 5′-AGGA-GTTGTGCCCAGTCCCA-3′ 20mer; OL6A, 5′-TTCATTCTT-TTAGCTGACAGC-3′ 21mer; OL7A, 5′-CTCAATATCTAGCT-TAAATG-3′ 20mer; OLRS, 5′-GTTTTACGTAAGCAGGTTAT-3′ 20mer; OLRD, 5′-AATGTGTAGTTTCGTACTGA-3′ 20mer.

Protein kinase assays

Protein kinase assays (20 µl) using PKR purified from rabbit reticulocytes (0.5 ng) (5) were performed under conditions as described (10). Other additions are as indicated in the figure legends. Proteins were separated by electrophoresis on 7.5% SDS–polyacrylamide gels and phosphoprotein profiles were analyzed following autoradiography. Poly(I)·poly(C) was obtained from Pharmacia and [γ-^{32}P]ATP (4500 Ci/mmol) was obtained from Dupont. The extent of PKR phosphorylation was quantitated from autoradiograms by scanning densitometry (Shimadzu, Kyoto, Japan).

RNA/DNA liquid hybridization

For RNA/DNA hybridization assays (10 µl) reactions contained 100 mM KCl, 0.1 mM EDTA, pH 7.0, R-RNA transcript (5 ng) or poly(I)·poly(C) (3 ng) and were supplemented with a 10-fold molar excess of *Alu*I DNA fragments or 50–100 ng complementary oligo as indicated in the figures. RNase/DNase-free non-specific *Escherichia coli* DNA was added to reactions as a carrier to minimize loss of RNA transcript during hybridization. Hybridization reactions were for 5 min at 95°C in tightly capped tubes. The mixtures were allowed to cool slowly (30–60 min) to room temperature. Reactions were placed on ice and used directly for protein kinase assays. Controls included parallel hybridization reactions containing hybridization buffer alone, *Alu*I fragments alone, oligos alone, poly(I)·poly(C) alone, poly(I)·poly(C) with excess *Alu*I fragments or poly(I)·poly(C) with oligos (100 ng) as indicated in the figures. All control reactions contained carrier DNA.

Synthesis and purification of the R-RNA transcript

The R-RNA transcript was transcribed *in vitro* from the recombinant pGEM-3zf(±) plasmid after linearization with *Sma*I (Promega). Transcription from the T7 promoter was carried out using the MegAscript kit (Ambion) according to the manufacturer's instructions. The reaction mix was separated by electrophoresis on 0.75 mm, 4% acrylamide/bisacrylamide (19:1) TBE gels containing 7 M urea. The quantity of RNA synthesized allowed visualization of bands using shadow casting. The advantage of this method is that ethidium bromide, which may interfere with kinase assays, can be avoided. The gel was covered in plastic wrap and placed on top of an intensifying screen (Kodak). The gel was exposed to short wavelength UV light (254 nm). The shadow cast by the RNA was clearly visible. Gel slices containing the discrete 847 nt R-RNA transcript were excised and the RNA eluted overnight in a solution (300 µl) containing 2 M CH_3COONH_4, 0.1% SDS and 0.5 mM EDTA. The RNA was precipitated in ethanol, resuspended in DEPC-treated H_2O and stored under liquid nitrogen.

Enzymatic treatment of the R-RNA transcript

The gel-purified R-RNA transcript was digested with RNase T1 (Pharmacia) and RNase V1 (Pharmacia) and utilized for protein kinase assays under conditions previously described (36). Additions are as indicated in Figure 2.

Preparation of *Alu*I DNA fragments

To prepare a highly purified R-RNA cDNA insert, the isolated pGEM-3zf(±) recombinant plasmid was digested with *Eco*RI (36) and the R-RNA cDNA (1 mg) insert was separated by electrophoresis on 1.2% low melting temperature agarose (NuSieve, FMC Bioproducts). The 847 bp R-RNA cDNA was purified from gel slices using the QIAEXII extraction kit (Qiagen). The purified cDNA was digested at 37°C with *Alu*I (NEB). The reaction products were separated by electrophoresis on 1.5% agarose gels and the expected 284, 226, 173 and 133 bp fragments were individually purified as described above. The expected 26 bp fragment was not recovered. The purified cDNA fragments were resuspended in DEPC-treated H_2O and stored at –20°C at a concentration of 20–25 µg/ml.

RESULTS

Analysis of the double-stranded content of the R-RNA

Previous studies have shown that activation of PKR by cytoplasmic RNA obtained from 3T3-F442A cells or by the R-RNA transcript obtained from 3T3-F442A cell cDNA is dependent on ssRNA containing double-stranded structure (35,36). To determine the relative extent of double-stranded structure in the R-RNA transcript involved in activation of PKR, its activity was examined over a broad concentration range and compared with activation with poly(I)·poly (C). The R-RNA transcript and poly(I)·poly(C) were added to the protein kinase assays at the final concentrations indicated in Figure 1 and the extent of activation was determined by quantitation of the level of PKR phosphorylation. The results show that the profile of activation observed with the R-RNA transcript was similar to that observed with poly(I)·poly(C) (Fig. 1). Both RNAs show a transient effect on PKR, i.e. a dose-dependent activation at low concentration followed by a dose-dependent inhibition of activation at higher concentrations. The transient effect of poly(I)·poly(C) observed is consistent with previous reports (4,21). However, the minimum concentration of the R-RNA transcript (0.64 µg/ml, Fig. 1) necessary for optimal activation of PKR is 16-fold higher than the minimal concentration of poly(I)·poly(C) required for a similar

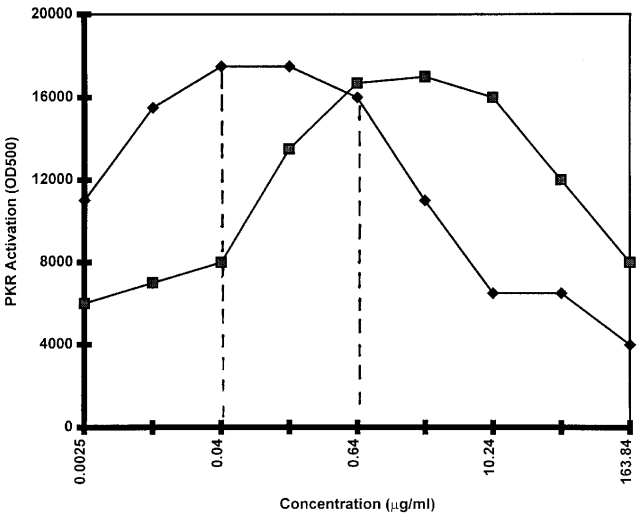

Figure 1. A Comparison between poly(I)·poly(C) and the R-RNA in the autophosphorylation of PKR. Poly(I)·poly(C) and the R-RNA transcript were added to protein kinase assays at the final concentrations indicated in the figure. Phosphoproteins were resolved by 7.5% SDS–PAGE and analyzed by autoradiography. Protein kinase assays (20 µl), containing purified PKR (0.5 ng), SDS–PAGE and autoradiography were carried out as described in Materials and Methods. The extent of phosphorylation of PKR was determined by scanning densitometry (OD 500 nm) of autoradiograms. One assay contained no added RNA and served as a control for PKR and for correction of background during scanning. Poly(I)·poly(C), ◆; R-RNA, ■.

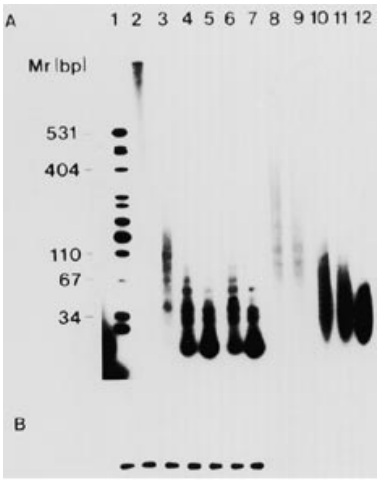

Figure 2. Effect of RNase digestion on the activity of the R-RNA transcript. Radiolabeled or unlabeled R-RNA transcript (4 µg/ml) was digested with either RNase T1 or RNase VI (10 U/ml each) at 30°C for 10 min in a total of 5 µl. (**A**) Digestion reactions (1 µl) using radiolabeled transcript were subjected to electrophoresis on 5% polyacrylamide gels in 0.5× TBE buffer. Lane 1, radiolabeled pGEM-3zf(–) digested with *Hpa*II; lane 2, R-RNA transcript without RNase digestion; lanes 3–6, digestion with RNase T1 at final activities of 0.78, 1.56, 3.13 and 12.5 U/ml respectively; lanes 7–11, digestion with RNase VI at final activities of 0.05, 0.10, 0.19, 0.39 and 0.78 U/ml respectively. The figure is an autoradiogram. (**B**) The digestion reactions (5 µl) were added to protein kinase (20 µl) containing purified PKR as described in Figure 1. The final concentration of the R-RNA transcript added to protein kinase assays was 1 µg/ml. Lane 1, no added RNA; lane 2, 150 ng/ml poly(I)·poly(C); lanes 3–7, RNase T1 digestion reactions (2–6); lanes 8–12, RNase VI digestion reactions (7–11). The final activity of the RNases in the digestion reactions was as in (A). The figure is an autoradiogram.

level of activation (0.04 µg/ml, Fig. 1). Since poly(I)·poly(C) can be assumed to be entirely composed of double-stranded structure (21) and the R-RNA transcript contains both single-stranded and double-stranded structures (36), the difference in the relative minimum concentrations needed for optimal activation may represent differences in the extent of double-stranded structure between the two molecules. This implies that the double-stranded region involved in activation of PKR constitutes a small portion of the secondary structure of the R-RNA. This is further supported by the observation that digestion of the transcript with ssRNA-specific RNase T1 resulted in formation of RNA fragments of ~60 bp or less in length (Fig. 2A, lanes 3–7). These fragments were still capable of activating PKR (Fig. 2B, lanes 3–7), indicating that they retained the critical double-stranded structure(s) even after digestion with high levels of RNase T1. However, digestion with dsRNA-specific RNase VI results in formation of fragments of larger average size (Fig. 2A, lanes 8–12) that were unable to facilitate activation of PKR (Fig. 2B, lanes 8–12).

Mapping of secondary structure involved in activating PKR

To delineate the location of the region of secondary structure involved in activation of PKR and its sequence, RNA:DNA hybridization reactions were carried out utilizing the R-RNA transcript and specific restriction fragments from its cDNA. Because RNA:DNA duplexes are stable once they form and disrupt the natural secondary structure within the R-RNA, the critical structures important for activation of PKR can be mapped by determining which of the known restriction fragments interfer with phosphorylation of PKR. Digestion with *Alu*I was selected because this gave rise to five asymmetric fragments which could be conveniently separated and four of the fragments were easily purified. Figure 3A is a schematic representation indicating the known restriction map of the 847 bp R-RNA cDNA and the orientation of the five predicated *Alu*I fragments. Following digestion with *Alu*I, the predicted 284, 226, 178 and 133 bp fragments were obtained and highly purified by gel electrophoresis. The purity of the individual fragments is shown in Figure 3B. Each cDNA fragment was hybridized to the R-RNA transcript individually or in combination. The effect of hybridization on activation of PKR was analyzed utilizing protein kinase assays. It was found that the 226 bp fragment alone could prevent the R-RNA transcript from activating PKR (Fig. 3C, compare lanes 3 and 5). The 284, 178 and 133 bp fragments had little or no effect on R-RNA activity (Fig. 3C, lanes 4, 6 and 7). Moreover, in mixing experiments containing all possible combinations of the *Alu*I fragments, PKR activation was prevented in only those assays which contained the 226 bp fragment (Fig. 3C, lanes 8–14). No phosphorylation of PKR was observed in the absence of dsRNA (Fig. 3C, lane 1). Controls for hybridization/kinase assays included reactions which contained only poly(I)·poly(C) (Fig. 3C, lane 2) or the R-RNA transcript (Fig. 3C, lane 3). The phosphoproteins observed on autoradiograms of lower molecular weight than PKR represent trace amounts of contaminating proteins that vary in PKR preparations. Their phosphorylation is not dependent on RNA and is not affected by the *Alu*I fragments. The effect of the 226 bp fragments was specific because

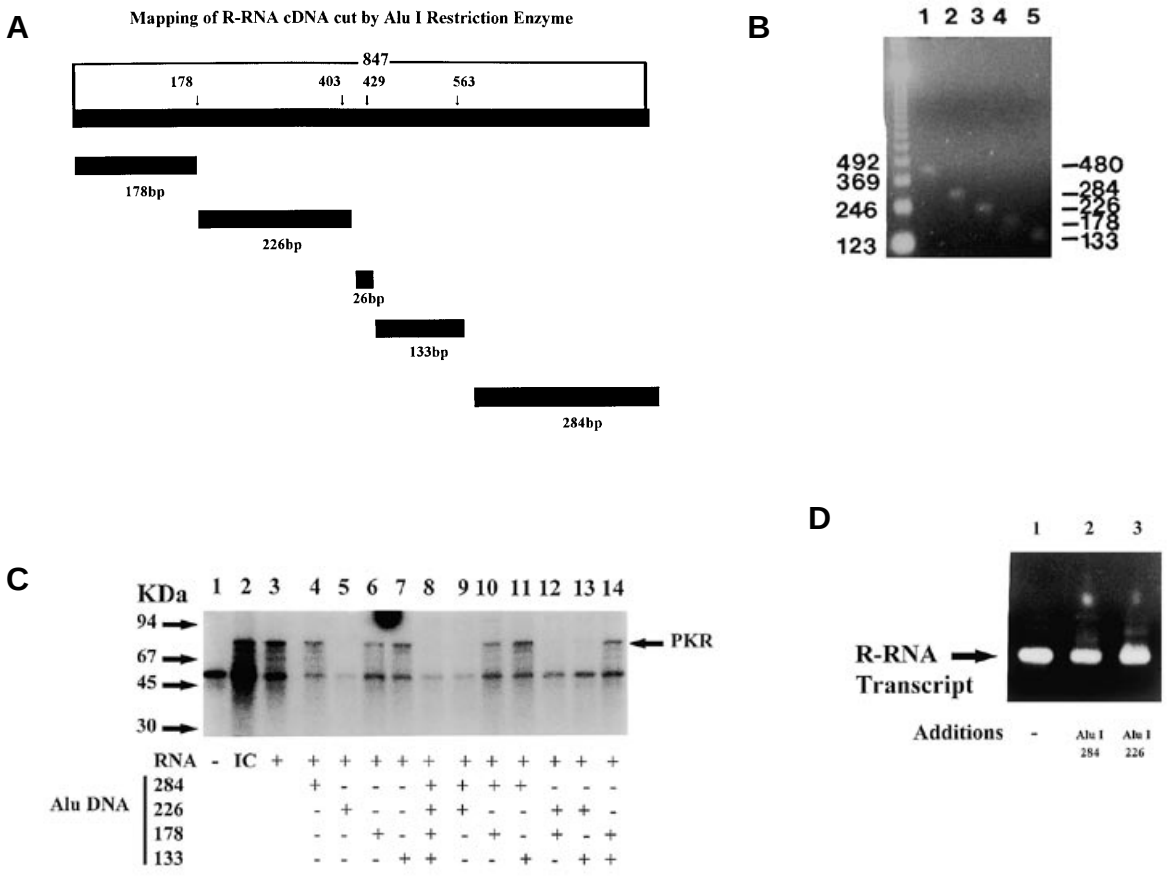

Figure 3. (**A**) Mapping of the R-RNA cDNA by restriction enzyme digestion. Shown is a schematic diagram indicating the*Alu*I restriction map of the 847 bp R-RNA cDNA. Five *Alu*I fragments were predicted from the sequence. (**B**) Purification of *Alu*I fragments. Agarose gel (1.5%) indicating the migration position and purity of *Alu*I fragments obtained from the R-RNA cDNA digest. The 26 bp fragment is not shown. Lane 1, a 480 bp DNA marker; lane 2, Alu 284 bp; lane 3, Alu 226 bp; lane 4, Alu 178 bp; lane 5, Alu 133 bp. The migration position of DNA of a known size ladder is shown in the far left lane. DNA was visualized following ethidium bromide staining. (**C**) Effect of *Alu*I fragments on phosphorylation of PKR. Hybridization reactions (10 µl) containing purified R-RNA transcript (5 ng) and a 10-fold molar excess of *Alu*I cDNA fragments were carried out as described in Materials and Methods. Additions were as indicated in the figure. Protein kinase assays containing the hybridization reaction and purified PKR were as described in Figure 1. As controls for PKR, one assay contained no added RNA (lane 1), while another assay was supplemented with 150 ng/ml poly(I)·poly(C) (lane 2) in the kinase reaction. The migration position and molecular weights ($\times 10^3$) of protein standards, phosphorylase (94 kDa), bovine serum albumin (67 kDa), ovalbumin (45 kDa) and carbonic anahydrase (30 kDa) are indicated on the left. The arrow on the right indicates the position of PKR. The figure is an autoradiogram. (**D**) Effect of *Alu*I fragments on the stability of the R-RNA. Gel-purified R-RNA transcript (500 ng/reaction) was incubated in the presence of a 10-fold molar excess of the*Alu*I 284 fragment (lane 2) and *Alu*I 226 fragment (lane 3). One assay contained only R-RNA transcript (lane 1). Incubations were carried out under the hybridization conditions described in Materials and Methods. Reaction mixtures were resolved by electrophoresis using 1% agarose gels. RNA was visualized following treatment with ethidium bromide.

activation of PKR by poly(I)·poly(C) was not affected by addition of the 226 bp fragment to hybridization reactions and the 226 bp fragment did not effect phosphorylation of PKR by the R-RNA without being subjected to hybridization conditions (data not shown). Moreover, no measurable ribonuclease activity was detected in the 226 bp *Alu*I fragment to account for loss of RNA activity during hybridization (Fig. 3D, lane 3). As an additional control, the 284 bp *Alu*I fragment was also examined and found to contain no ribonuclease activity (Fig. 3D, lane 2). The 26 bp fragment could not be recovered from gels and directly tested in hybridization reactions. However, in reactions where limited digestion of the 847 bp R-RNA cDNA with *Alu*I was carried out, a 252 bp fragment identified by additional restriction mapping with *Xho*I (IBI) as consisting of the 226 + 26 bp (Fig. 3A) fragment was obtained and purified. The 252 bp fragment was equally effective in preventing PKR activation as the 226 bp fragment following hybridization to the R-RNA (data not shown). These findings indicate that the secondary structure(s) important for activation of PKR is localized to the 226/252 nt region within the R-RNA transcript consisting of nucleotides 179–430 (36).

Effect of antisense oligodeoxynucleotides on activity of the R-RNA

To further define the region(s) within the 252 nt fragment of the R-RNA involved in activation of PKR, we developed a novel antisense approach. Eleven short phosphorothioate oligodeoxynucleotides (20–22 nt in length) complementary to the 252 nt stretch of the R-RNA were synthesized. These were selected for

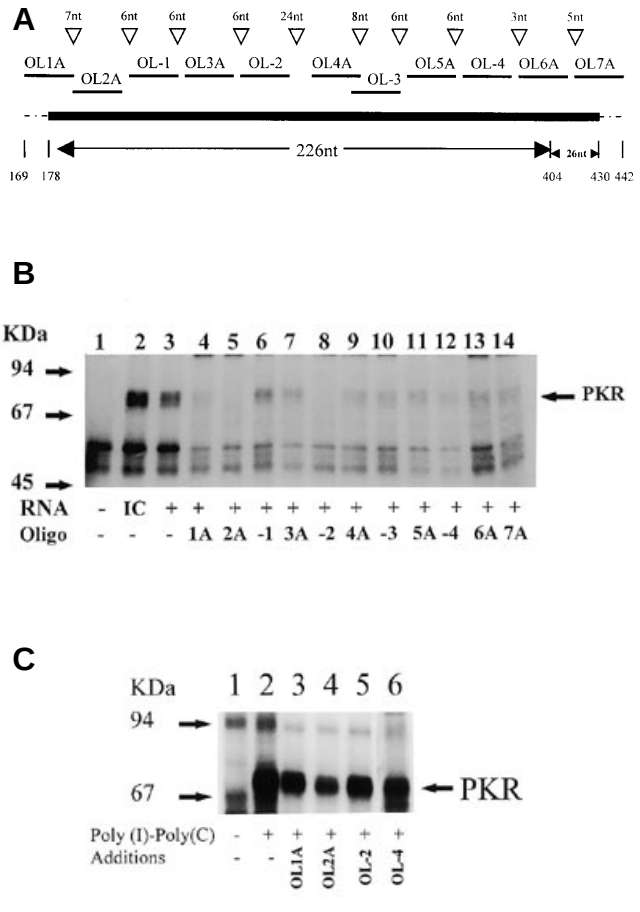

Figure 4. (**A**) Mapping of the 252 nt region with oligos. Shown is a schematic diagram indicating the location and spatial distribution of 11 oligos complementary to the 252 nt region of the R-RNA. The size and sequence of each oligo is described in Materials and Methods. Also indicated are the gaps and overlaps in nucleotides between oligos. The numbers in the lower portion of the figure represent the position of the nucleotide in the R-RNA sequence, which has been published elsewhere (36). (**B**) Effect of oligos on phosphorylation of PKR. Hybridization reactions and protein kinase assays were carried out as described in Figure 3C, except that oligos (50 ng) were added to hybidization reactions as indicated. Controls included hybridization reactions containing: no added RNA (lane 1), 3 ng poly(I)·poly(C) (lane 2) and 5 ng R-RNA transcript alone (lane 3). The figure is an autoradiogram. (**C**) Effect of oligos on phosphorylation of PKR by poly(I)·poly(C). Hybridization reactions and protein kinase assays were carried out as described in Figure 3C except poly(I)·poly(C) (3 ng) and oligos (100 ng) were added to reactions as shown. One hybidization contained no added RNA. The figure is an autoradiogram.

optimal binding properties (Materials and Methods). Figure 4A depicts the location and spatial arrangement of the oligos with respect to the R-RNA. The effect of hybridization of each of these oligos to the R-RNA on activation of PKR was examined using protein kinase assays. The results indicate that oligos OLIA (Fig. 4B, lane 4), OL2A (lane 5), OL2 (lane 8) and OL4 (lane 12) were effective in blocking activation of PKR. Analysis of autoradiograms by scanning densitometry indicates that each of these oligos blocked activation of PKR by >95%. The remaining seven oligos had considerably lower but variable inhibitory effects, ranging from 30 to 40% at identical concentrations, which may be due to non-specific inhibition because similar levels of reduction were observed with non-complementary oligos. The marked inhibitory

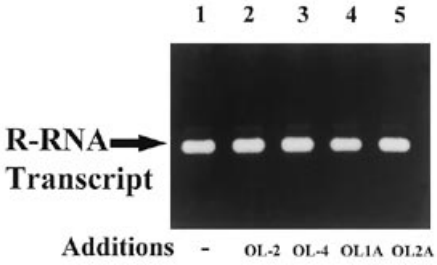

Figure 5. Effect of oligos on the stability of the R-RNA. Gel-purified R-RNA transcript (500 ng/reaction) was incubated in the presence of the indicated complementary oligo (200 ng), as described in Figure 3D. One incubation contained RNA alone (lane 1) as a control. Gel electrophoresis and staining was as described in Figure 3D.

effects of OL1A, OL2A, OL2 and OL4 relative to the other oligos were observed in three separate experiments. In some experiments oligos OL1, OL2, OL3 and OL4 were from separate preparations. Inhibition of PKR activation by the blocking oligos did not appear to be the result of differential G+C content among the oligos. This is evident because the G+C content of each of the oligos tested varied from 18 to 60%. However, this did not correlate with blockage of PKR activation. For example, OL2, the most potent inhibtor, has only a 35% G+C content. This is less than OL1 (45%), OL4A (45%), OL3 (40%) and OL5A (41%), which were considerably less effective in blocking PKR activation. On the other hand, OL4 contains 60% G+C content and effectively inhibited PKR activation. Moreover, the two control oligos, OLRS (reverse polarity) and OLRD (randomized), contain G+C content identical to that of OL2 but are considerably less inhibitory (30–45%). The largest gap between oligos was 24 nt (OL2→OL4A), which alone is insufficient in length for activation of PKR (37). The possibility, however, that nucleotides in the gap sequence contributed to other sequences in the 226/252 region for activation of PKR cannot be absolutely excluded. PKR was not phosphorylated in the absence of RNA (Fig. 4B, lane 1). Controls included one assay which contained only poly(I)·poly(C) (Fig. 4B, lane 2) and one assay which contained only the R-RNA transcript (Fig. 4B, lane 3). The effects of these blocking oligos appears specific because they had no effect on activation by poly(I)·poly(C) (Fig. 4C, compare lane 2 and lanes 3–6). Moreover, no ribonuclease activity could be detected in the blocking oligo preparations or loss of R-RNA due to addition of these oligos (Fig. 5, compare lane 1 and lanes 2–5). These findings suggest nucleotide sequences 178–202, 263–283 and 374–393 within the R-RNA transcript are involved in activation of PKR, which can be prevented by hybridization to corresponding complementary oligos.

DISCUSSION

Regulation of growth and differentiation in 3T3-F442A cells involves, in part, activation of PKR (10,11,13). These cells contain a cellular regulatory RNA (R-RNA) which has been shown to activate PKR (35,36). The aim of this study was to begin to characterize the secondary structure(s) within the R-RNA involved in activation of PKR. A comparison of the activation profiles of the R-RNA transcript and poly(I)·poly(C) established that each RNA activated PKR at low concentrations but prevented

activation at high concentrations (Fig. 1). A considerably higher amount of R-RNA was required to achieve the same optimal level of activation as seen with poly(I)·poly(C). Quantitation of the data indicates that the activation level of the R-RNA was only ~6% of the activation level of poly(I)·poly(C). Assuming that the double-stranded structures involved in activation within each RNA are equally efficient in binding and activating PKR, the lower efficiency of the R-RNA is likely to reflect fewer relevant double-stranded structures. Nevertheless, the data indicate that the single chain R-RNA (36) modulates activation of PKR with similar characteristics to those observed with a synthetic duplex RNA and as seen with natural duplex RNAs such as reovirus RNA (24). Thus, it is possible that within cells PKR could be both activated and prevented from undergoing activation depending on the level of R-RNA present. This intriguing possibility is physiologically unlikely, however, because the amount of R-RNA necessary to prevent activation is greater than is likely to be attainable intracellularly.

Digestion of the R-RNA transcript with low levels of dsRNA-specific RNase completely abolished its capacity to activate PKR (Fig. 2B). In contrast, digestion with ssRNA-specific RNase had no effect even after exhaustive treatment (Fig. 2A). These findings indicate that: (i) the integrity of the complete R-RNA transcript is not essential for activation of PKR; (ii) a short double-stranded element(s) of ~60 bp or less which is extensively free of single-stranded regions retains its capacity for activation. This observation is consistent with other studies demonstrating that the 57–83 nt stem–loop stretch of the TAR element of HIV-1 RNA is sufficient to complex with PKR and alter its activation (28,29,38).

Hybridization of the R-RNA transcript with restriction fragments prepared from its cDNA mapped the location of the secondary structure(s) involved in activation of PKR to a contiguous 226/252 nt region consisting of nucleotides 179–420 (36; Fig. 3). The remaining region of the R-RNA transcript was neither active nor contributed sequences necessary for activation. Moreover, because inhibition of PKR activation by hybridization to the 226 nt segment of the R-RNA was specific, it suggests that disruption of secondary structure in this region accounts for inhibition. It is also of interest that the predicted secondary structure (program RNA-Fold) of the 226 nt region contains more extensive and thermodynamically favorable structure than the 284, 178 and 133 nt regions mapped by the other *Alu*I fragments (data not shown).

Further analysis of the 252 nt region was carried out using our novel hybridization method but employing short complementary oligos to achieve greater resolution of the important sequences (Fig. 4). Three discrete and discontinuous RNA sequences consisting of nucleotides 178–202, 263–283 and 374–393 appear to be involved in the structures necessary for activation of PKR. Sequence 178–202 was the longest sequence and is defined by the blocking effect of two overlapping oligos (Fig. 4). It is not possible to determine from the current data whether these sequences represent a portion of the region(s) of secondary structure directly involved in binding of PKR or if they contribute to formation of nearby secondary structures required for interaction with PKR. However, the delineation of these sequences provides a basis for further analysis focused on the 252 nt region of the R-RNA transcript.

A significant number of studies have provided evidence for activated PKR and elevated phosphorylation of eIF-2α in non-virally infected cells (10,12,13,32–34). This is not surprising in the light of the variety of biological processes reported to involve PKR (10–18). However, to date the only cellular RNAs identified as interacting specifically with PKR are its own mRNA (20) and the cellular R-RNA isolated from mouse embryonic fibroblasts (36). The results in this study represent an initial characterization of the latter, the R-RNA, with respect to activation of PKR. This partial characterization is an important step toward understanding the cellular mechanism by which PKR is regulated by RNA. This study also suggests an approach for controlling the activity of PKR by altering the structure of relevant cellular RNAs. This is of interest because of the potential of PKR for regulating cell proliferation (10,13) and suppressing tumors (14,15). The approach itself is interesting as well, since we show enzymatic activity to be altered by disruption of secondary structure in RNA with antisense molecules. This mechanism of action of the antisense molecules is distinct from the usual mechanisms implicated in most applications of antisense molecules, the targeting of specific mRNAs to block their translation into protein (39). Therefore, the findings presented here raise the possibility of a strategy for modifying cell responses by designing antisense molecules directed to RNA sequences that interact with regulatory proteins. Such an approach has already been used to identify the HIV-1 Rev response element sites necessary for interactions with rev protein (40). This is particularly interesting since RNA:protein interaction is now recognized as an important step in the regulation of a variety of biological processes (41).

ACKNOWLEDGEMENTS

We thank Drs Stephan Ladisch and Eliel Bayever for their helpful discussion and suggestions. This work was supported by grants CA421717 from the National Cancer Institute, NIH, the Elaine H.Snyder Cancer Research Trust and the Discovery Fund.

REFERENCES

1 Petryshyn,R.A., Li,J. and Judware,R. (1994) In Müller,W.E.G. and Schröder,H.C. (eds), *Progress in Molecular and Subcellular Biology.* Springer-Verlag, Berlin, Germany, Vol. 14, pp. 1–14.
2 Jarmillo,M.L., Abraham,N. and Bell,J.C. (1995) *Cancer Invest.*, **13**, 327–338.
3 Kalvakolanu,D. and Borden,E. (1996) *Cancer Invest.*, **14**, 25–53.
4 Farrell,P.J., Balkow,K., Hunt,T., Jackson,R.J. and Trachsel,H. (1977) *Cell*, **11**, 187–200.
5 Petryshyn,R.A., Levin,D.H. and London,I.M. (1983) *Methods Enzymol.*, **99**, 346–362.
6 Samuel,C.E. (1993) *J. Biol. Chem.*, **268**, 7603–7606.
7 DeHaro,C., Mendez,R. and Santoyo,J. (1996) *FASEB J.*, **10**, 1378–1387.
8 Pestka,S., Langer,J.A., Zoon,K.C. and Samuel,C.E. (1987) *Annu. Rev. Biochem.*, **56**, 727–777.
9 Sen,G.S. and Lengyel,P. (1992) *J. Biol. Chem.*, **267**, 5017–5020.
10 Petryshyn,R., Chen,J.-J. and London,I.M. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 1427–1431.
11 Judware,R. and Petryshyn,R. (1991) *Mol. Cell. Biol.*, **11**, 3259–3267.
12 Belkumeur,P., Lanoix,J., Blais,Y., Forget,D., Steyaert,A. and Skup,D. (1993). *Mol. Cell. Biol.*, **13**, 2846–2857.
13 Petryshyn,R.A., Chen,J.-J., Danley,L. and Matts,R.L. (1996) *Arch. Biochem. Biophys.*, **328**, 290–297.
14 Koromilas,A.E., Roy,S., Barber,G.N., Katze,M.G. and Sonenberg,N. (1992) *Science*, **257**, 1685–1689.
15 Barber,G.N., Wambach,M., Thompson,S., Jagus,R. and Katze,M.G. (1995) *Mol. Cell. Biol.*, **15**, 3138–3196.
16 Kumar,A., Haque,J., Locoste,J., Hiscott,J. and Williams,B.G.R. (1994) *Proc. Natl. Acad. Sci. USA*, **91**, 6288–6292.

17 Maran,A., Maitra,R.K., Kumar,A., Dong,B., Xiao,W., Li,G., Williams,B.R.G., Torrence,P.F. and Silverman,R.H. (1994) *Science*, **265**, 789–792.
18 Mundschau,L.J. and Faller,D.W. (1995) *J. Biol. Chem.*, **270**, 3100–3106.
19 Lebleu,B., Sen,G.C., Shaila,S., Cabrer,B. and Lengyel,P. (1976) *Proc. Natl. Acad. Sci. USA*, **73**, 3107–3111.
20 Thomis,D.C. and Samuel,C.E. (1993) *J. Virol.*, **67**, 7695–7700.
21 Hunter,T., Hunt,T., Jackson,R.J. and Robertson,H.D. (1975) *J. Biol. Chem.*, **250**, 409–417.
22 Nekhai,S., Kumar,A., Bottaro,D.P. and Petryshyn,R. (1996) *Virology*, **222**, 193–200.
23 Bevilacqua,P.C. and Cech,T.R. (1996) *Biochemistry*, **35**, 9983–9994.
24 Bischoff,J.R. and Samuel,C.E. (1989) *Virology*, **172**, 106–115.
25 Kitajewski,J., Schneider,R.J., Safer,B., Munemitsu,S.M., Samuel,C.E., Thimmapaya,B. and Shenk,T. (1986) *Cell*, **45**, 195–200.
26 Ghadge,G.D., Malhatra,P., Furtado,M.R., Dhar,R. and Thimmapaya,B. (1994) *J. Virol.*, **68**, 4137–4151.
27 Clarke,P.A., Schwemmle,M., Schickinger,J., Hilse,K. and Clemens,M.J. (1991) *Nucleic Acids Res.*, **19**, 243–248.
28 Edery,J., Petryshyn,R.A. and Sonenberg,N. (1989) *Cell*, **56**, 303–312.
29 Sen Gupta,D.N. and Silverman,R.H. (1989) *Nucleic Acids Res.*, 17, 969–978.
30 Roy,S., Agy,M., Hovanessian,A.G., Sonenberg,N. and Katze,M.G. (1991) *J. Virol.*, **65**, 632–640.
31 Judware,R., Li,J. and Petryshyn,R.A. (1993) *J. Interferon Res.*, **13**, 153–160.
32 Baum,E.Z. and Ernst,V.G. (1983) *Biochem. Biophys. Res. Commun.*, **114**, 41–49.
33 Pratt,G., Galpine,A., Sharp,N., Palmer,S. and Clemens,M.J. (1988) *Nucleic Acids Res.*, **16**, 3497–3510.
34 Dubois,M.F., Hovanessian,A.G. and Bensaude,O. (1991) *J. Biol. Chem.*, **266**, 9707–9711.
35 Li,J. and Petryshyn,R.A. (1991) *Eur. J. Biochem.*, **195**, 41–48.
36 Li,J., Yu,Z-P., Slomiany,D.J. and Petryshyn,R.A. (1997) *Int. J. Oncol.*, in press,.
37 Manche,L., Green,S.R., Schmedt,C. and Mathews,M.B. (1992) *Mol. Cell. Biol.*, **12**, 5238–5248.
38 Maitra,R.K., McMillan,A.J., Desai,S., McSwiggen,J., Hovanessian,A.G., Sen,G., Williams,B.R.G. and Silverman,R.H. (1994) *Virology*, **204**, 823–827.
39 Tonkinson,J.L. and Stein,M.D. (1996) *Cancer Invest.*, **14**, 54–65.
40 Fenster,S.D., Wagner,R.W., Forehler,B.C. and Chin,D.J. (1994) *Biochemistry*, **33**, 8391–8398.
41 McCarthy,J.E.G. and Kollmus,H. (1995) *Trends Biochem. Sci.*, **20**, 191–197.