Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 508–513, January 1996
Cell Biology

In vitro activation of the interferon-induced, double-stranded RNA-dependent protein kinase PKR by RNA from the 3′ untranslated regions of human α-tropomyosin

SUSAN DAVIS AND JULIA C. WATSON*

RiboGene, Inc., Hayward, CA 94545

Communicated by Richard D. Klausner, National Institutes of Health, Bethesda, MD, October 6, 1995

ABSTRACT **The cellular kinase known as PKR (protein kinase RNA-activated) is induced by interferon and activated by RNA. PKR is known to have antiviral properties due to its role in translational control. Active PKR phosphorylates eukaryotic initiation factor 2α and leads to inhibition of translation, including viral translation. PKR is also known to function as a tumor suppressor, presumably by limiting the rate of tumor-cell translation and growth. Recent research has shown that RNA from the 3′ untranslated region (3′UTR) of human α-tropomyosin has tumor-suppressor properties *in vivo* [Rastinejad, F., Conboy, M. J., Rando, T. A. & Blau, H. M. (1993) *Cell* 75, 1107–1117]. Here we report that purified RNA from the 3′UTR of human α-tropomyosin can inhibit *in vitro* translation in a manner consistent with activation of PKR. Inhibition of translation by tropomyosin 3′UTR RNA was observed in a rabbit reticulocyte lysate system, which is known to contain endogenous PKR but was not seen in wheat germ lysate, which is not responsive to a known activator of PKR. A control RNA purified in the same manner as the 3′UTR RNA did not inhibit translation in either system. The inhibition of translation observed in reticulocyte lysates was prevented by the addition of adenovirus virus-associated RNA_I ($VA\ RNA_I$), an inhibitor of PKR activation. Tropomyosin 3′UTR RNA was bound by immunoprecipitated PKR and activated the enzyme in an *in vitro* kinase assay. These data suggest that activation of PKR could be the mechanism by which tropomyosin 3′UTR RNA exerts its tumor-suppression activity *in vivo*.**

The interferon-induced protein kinase PKR [also designated DAI, eukaryotic initiation factor 2α (eIF-2)α) kinase, p68] was first characterized as a part of the mammalian interferon-mediated antiviral response (1–4). Although interferon induces increased expression of PKR, the newly synthesized protein is inactive. Activation of PKR occurs after binding to double-stranded RNA (dsRNA), which can be produced during a viral infection (5, 6). PKR is autophosphorylated concomitantly with activation. Active PKR phosphorylates eIF-2α, and this phosphorylation event blocks the GDP–GTP exchange cycle required for eIF-2 function (7–9). Functional eIF-2 is required for every translation initiation event; therefore, phosphorylation of this factor leads to a general inhibition of translation (10). Because viruses are obligate intracellular parasites that depend on their host cell for translation, the viral life cycle can be blocked by this inhibition of translation. Many viruses encode or induce inhibitors of PKR that allow them to replicate in the presence of interferon and potentially active PKR (11, 12). The best characterized of these is adenovirus $VA\ RNA_I$, a specialized RNA that binds to PKR and inhibits its activation (13, 14). Vaccinia virus (15–18), rotavirus (19), reovirus (20), influenza virus (21, 22), and poliovirus (23, 24) also inhibit PKR. The fact that most of the best-characterized pathogenic animal viruses have inhibitors of PKR suggests that inhibition of this enzyme is important for viral replication.

In addition to its role in antiviral response, PKR has been implicated as a tumor suppressor. NIH 3T3 cells expressing an inactive mutant of human PKR displayed morphological and growth pattern alterations consistent with a transformed phenotype (25, 26). When these cells were injected into nude mice, tumor formation occurred in all animals. In cells expressing the mutant human PKR, endogenous murine PKR was present at normal levels but could not be activated by the addition of dsRNA. This latter observation is consistent with earlier reports that mutants of PKR that are kinase-deficient act in a trans-dominant manner to inhibit activation of wild-type PKR (27, 28). Expression of mutant, catalytically inactive forms of the enzyme in transfected cells could act in a trans-dominant manner to inhibit endogenous wild-type PKR. This suppression of PKR activation may lead to an increased rate of cellular protein synthesis and the increased rate of cell growth characteristic of neoplasia.

The genes encoding cytoskeletal proteins such as α-tropomyosin, troponin, and α-cardiac actin are expressed in muscle tissues during differentiation. Complementation studies with murine cell lines have demonstrated that expression of these genes can rescue differentiation-deficient cell lines and trigger the expression of tissue-specific gene products (29). The complementation activities of α-tropomyosin, troponin, and α-cardiac actin were found to map to their 3′ untranslated regions (3′UTRs). Further study of α-tropomyosin showed that a plasmid encoding the 3′UTR sequence can also act as a tumor suppressor and that RNA expression is required for this activity (30). Expression of cytoskeletal proteins such as tropomyosin, troponin, and α-cardiac actin may be incompatible with neoplasia. The intracellular concentrations of mRNAs encoding isoforms of tropomyosin are known to decrease in neoplasmic cells and increase when cells revert to normal (31). Constitutive expression of tropomyosin 1 cDNA in cells transformed by *ras* was found to suppress neoplastic growth (32).

In view of the evidence that PKR may act as a tumor suppressor and the possible role of some 3′UTR RNAs in differentiation and tumor suppression, we investigated the effects of purified 3′UTR RNA of human α-tropomyosin on translation. We found that purified RNA from the 3′UTR of α-tropomyosin (designated Tm1-3 RNA) inhibited translation in a manner analogous to a prototypical viral PKR activator, reovirus dsRNA. Namely, Tm1-3 RNA inhibited translation in a PKR-positive system but did not do so in a PKR-deficient system, and inhibition in the PKR-positive system could be prevented by the addition of $VA\ RNA_I$. A control RNA transcribed *in vitro* and purified by the same method did not

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: PKR, protein kinase RNA-activated; 3′UTR, 3′ untranslated region; eIF-2α, eukaryotic initiation factor 2α; dsRNA, double-stranded RNA.

*To whom reprint requests should be addressed.

inhibit translation in either system. Tm1-3 RNA bound specifically to immunoprecipitated PKR and led to autophosphorylation of PKR purified from human cells, whereas control RNA did not.

MATERIALS AND METHODS

Cloning of the 3′UTRs. The 3′UTR of α-tropomyosin was cloned by PCR from a human skeletal muscle cDNA library in λ gt10 (Clontech). Three PCR primers were synthesized on the basis of GenBank sequences of the α-tropomyosin gene (33). The primer designated here as Tm1 (5′-GGGGGGAGCTC*TGA*AAACCTAGCTGCG-3′; *Sac* I site underlined, termination codon italicized) was used in combination with the primer Tm3 (3′-CTGATACCTAGGGCGTACGGATTCGAAGGGGGG-5′; *Hin*dIII site underlined) to amplify a 515-bp sequence from the tropomyosin 3′UTR, starting at the termination codon. Primer Tm2 (5′-AAGTAGAGCTCAAGGTCCCCCTGTGG-3′; *Sac* I site underlined) was used in combination with the primer Tm3 to amplify a 133-bp sequence from the tropomyosin 3′UTR, starting 380 nt downstream from the termination codon. PCR fragments of the correct sizes were cut with *Sac* I and *Hin*dIII restriction enzymes and then cloned into Bluescript II KS (Stratagene) oriented for transcription by the T7 RNA polymerase promoter. The two clones, designated Tm1-3 and Tm2-3, were sequenced to confirm insertion of tropomyosin sequences. Some slight differences from published sequences were noted. In the GenBank sequence for human α-tropomyosin, there are four adjacent repeats of the motif GTTTT, beginning 36 bases downstream from the translation termination codon. In the clone designated here as Tm1-3, there are five repeats of this sequence. In addition, Tm1-3 contains one point substitution (A → G, 280 nt from the termination codon), and one base deletion (at position 324 from the termination codon) as compared to the GenBank sequence. No differences from the GenBank sequence were found in Tm2-3.

Transcription and Purification of 3′UTR RNAs. Plasmid DNAs from Tm1-3 and Tm2-3 were cut at the unique *Hin*dIII site (from the common primer Tm3) for run-off transcription of the inserted sequences. Tm1-3 RNA and Tm2-3 RNA were produced by *in vitro* transcription using a Megascript T7 RNA polymerase kit (Ambion, Austin, TX). For radiolabeled RNAs, [α-^{32}P]UTP (Amersham) was included in the transcription reaction. The 3′UTR RNAs, as well as the control RNA LS1 (see below), were double gel purified by the following modification of the method of Mellits *et al.* (34). After transcription, the reaction mixtures were extracted with 24:24:1 phenol/chloroform/isoamyl alcohol, 24:24:1, extracted with chloroform/isoamyl alcohol, 1:1, and then precipitated with isopropanol. RNAs were resuspended in Tris/EDTA and then run on a native gel (6% acrylamide for LS1, Tm2-3; 3.5% acrylamide for Tm1-3). RNA bands were visualized by UV shadowing, excised, and loaded directly on a 6% acrylamide/6% urea gel. Bands were excised, eluted from the gel matrix in Tris/EDTA containing 0.5% SDS (3 hr at 30°C for LS1, Tm2-3; 18 hr at 30°C for Tm1-3), and then precipitated in ethanol and 300 mM sodium acetate. Precipitated RNAs were resuspended in Tris/EDTA and quantitated by absorption at 260 nm.

LS1 RNA. pT7LS1 encodes LS1, a mutant of adenovirus VA RNA$_I$ (35). LS1 RNA was transcribed from pT7LS1 cut with *Dra* I and then purified by the same method as for the 3′UTR RNAs.

Luciferase, VA RNA$_I$, and Reovirus RNAs. Luciferase mRNA was prepared from a plasmid coding for luciferase under the control of a T7 promoter (from N. Sonenberg, McGill University). Purified plasmid DNA was cut with *Sac* I and transcribed *in vitro* by using a Megascript T7 RNA polymerase kit (Ambion). The transcribed RNA was extracted with phenol/chloroform/isoamyl alcohol, 24:24:1, extracted with chloroform/isoamyl alcohol, 24:1, and then precipitated with isopropanol. Luciferase RNA was resuspended in Tris/EDTA, quantitated by absorption at 260 nm, and used in rabbit reticulocyte lysate translation reactions. In wheat germ lysate translation reactions, luciferase mRNA supplied with the lysate was used. VA RNA$_I$ was transcribed and purified by the same method as for the 3′UTR RNAs, using the plasmid pT7VA, cut with *Dra* I (34). Purified reovirus dsRNA was from A. Shatkin (Center for Advanced Biotechnology and Medicine, Piscataway, NJ).

Rabbit Reticulocyte Lysate *in Vitro* Translation. Micrococcal nuclease-treated rabbit reticulocyte lysate (Promega) was supplemented with all essential amino acids and used in translation reactions at 55% of the final volume. Purified RNAs were added at the indicated final concentrations and then incubated at 30°C for 15 min to allow activation of PKR before the addition of luciferase mRNA at a final concentration of 15 μg/ml. After addition of the reporter mRNA, reactions were incubated for 30 min at 30°C, and then translation was stopped by the addition of cycloheximide to a final concentration of 50 μg/ml. Luciferase activity was quantitated using an enhanced luciferase assay kit (Analytical Luminescence Laboratory, San Diego) and a Dynatech model ML3000 luminometer.

Wheat Germ Lysate *in Vitro* Translation. Micrococcal nuclease-treated wheat germ lysate (Promega) was supplemented with all essential amino acids and potassium acetate to 10 mM and then used in translation at 60% of the final volume. Purified RNAs were added at the indicated final concentrations and then incubated at 27°C for 15 min before the addition of luciferase mRNA (Promega) at a final concentration of 30 μg/ml. After addition of the reporter mRNA, reactions were incubated for 60 min at 27°C, and then translation was stopped by the addition of cycloheximide to a final concentration of 50 μg/ml. Luciferase activity was quantitated by using an enhanced luciferase assay kit (Analytical Luminescence Laboratory) and a Dynatech model ML3000 luminometer.

RNase T1 Digestion. Purified RNAs were digested with 0.01 unit of RNase T1 (Calbiochem) per μg of RNA in 10 mM Tris, pH 7.4/1 mM EDTA/300 mM NaCl, at 37°C for 1 hr. After digestion, RNAs were extracted twice with phenol/chloroform/isoamyl alcohol, 24:24:1, extracted with chloroform/isoamyl alcohol, 24:1, and then precipitated in ethanol with 300 mM sodium acetate and 16 μg of glycogen. Precipitated RNAs were resuspended in Tris/EDTA and quantitated by absorption at 260 nm.

Binding to Immunoprecipitated PKR. Binding of radiolabeled RNAs to immunoprecipitated PKR was assessed by using a modification of the technique of Katze *et al.* (36). Lysates were prepared from *Escherichia coli* strains expressing wild-type PKR [*E. coli* BL21(DE3) pSRG2 (37)], a carboxyl-truncated form of PKR [*E. coli* BL21(DE3) pSRG2BN (38)], or the parental strain [*E. coli* BL21(DE3)]. The truncated form of PKR, designated p20 for its molecular weight, lacks kinase activity but binds dsRNA (38). Expression was induced with isopropyl β-D-thiogalactoside during logarithmic-phase growth; cells were harvested 2.5 hr after induction. Cells were lysed by incubation in 50 mM Tris, pH 8.0/1 mM EDTA/lysozyme at 133 μg/ml/1 mM phenylmethylsulfonyl fluoride for 20 min at 4°C, followed by addition of Triton X-100 to 1% and incubation for 5 min at 37°C. The lysed cell solution was treated with DNase I for 30 min at room temperature. DNA and cell wall debris were removed by centrifugation; the resulting supernatant was used in binding experiments. A control lysate was also prepared from the parent strain, *E. coli* BL21(DE3).

Monoclonal antibody to PKR was provided by A. Hovanessian (Institut Pasteur) (39). Monoclonal antibodies were coupled to protein A–Sepharose (Calbiochem) in phosphate-

buffered saline for 15 min at 4°C. The resin was washed three times in buffer I [400 mM NaCl/50 mM KCl/20 mM Tris, pH 7.5/1% Triton X-100/1 mM EDTA/1 mM dithiothreitol/0.2 mM 4-(2-aminoethyl)benzenesulfonylfluoride/HCl (AEBSF; Calbiochem)/20% glycerol]; then lysates from *E. coli* BL21(DE3) pSRG2, *E. coli* BL21(DE3) pSRG2BN, or the parental strain *E. coli* BL21(DE3) were added to aliquots of the washed resin. Reactions were incubated for 1 hr at 4°C. Resin aliquots were washed four times in buffer I, then three times in binding buffer (25 mM Hepes, pH 7.4/10 mM $MgCl_2$/100 mM KCl/1 mM dithiothreitol/10 μM AEBSF/0.1 mM EDTA/bovine serum albumin at 0.1 mg/ml) containing tRNA at 0.1 mg/ml. The final pellet was resuspended in binding buffer with tRNA at 0.1 mg/ml. Radiolabeled RNAs were added to separate aliquots of washed resin, and reactions were incubated for 5 min at 30°C and then for 30 min at 4°C. The resins were washed once in binding buffer containing tRNA at 0.1 mg/ml and then washed three times in binding buffer without tRNA. Bound radiolabeled RNAs were quantitated by using liquid scintillation counting.

PKR Kinase Assay. PKR purified by column chromatography (40) from interferon-treated human Daudi cells was from M. Mathews (Cold Spring Harbor Laboratory). Kinase activity was assayed by standard methods (40) using 0.5 μl of purified kinase per 10-μl reaction. Reactions contained final concentrations of 75 mM KCl, 25 mM Hepes (pH 7.5), 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM EDTA, 0.1 mM ATP, protease inhibitors, and 5 μCi (1 Ci = 37 GBq) of [γ-^{32}P]ATP. Reactions were supplemented with Tm1-3 RNA or LS1 RNA (50 pM–50 nM), or reovirus RNA (1 ng/ml–1 μg/ml), as indicated. Reactions were incubated for 20 min at 30°C and then quenched by the addition of SDS sample buffer. Phosphorylated proteins were separated by SDS/PAGE and visualized by autoradiography.

RESULTS

Purified RNA from the 3′UTR of Tropomyosin Inhibits Translation in Rabbit Reticulocyte Lysate. Purified RNAs were tested for their ability to activate PKR and inhibit translation in rabbit reticulocyte lysate using an assay modified from the technique of Gunnery *et al.* (41). This assay is based on the fact that endogenous PKR in a rabbit reticulocyte lysate can be activated by the addition of exogenous activating RNA such as reovirus dsRNA. Activation of PKR results in a decrease in translation of a reporter mRNA. This assay has been used to detect PKR inhibitors and to compare the relative activities of mutants within inhibitory RNA sequences (41). Using the reporter mRNA luciferase, translation can be assessed by luciferase activity. This technique was used to examine effects on translation of purified α-tropomyosin 3′UTR RNA from the clone Tm1-3 (Tm1-3 RNA). Tm1-3 RNA contains 515 bp from the tropomyosin 3′UTR, starting at the termination codon. LS1, a mutant of adenovirus VA RNA_I, was chosen as a control RNA. In contrast to wild-type VA RNA_I, LS1 does not inhibit activation of PKR (35, 42). Reovirus dsRNA was also included in the assay because it has been well-characterized as an activator of PKR (43, 44).

The results of the *in vitro* translation assay with reovirus dsRNA, Tm1-3 RNA, and LS1 RNA are shown in Fig. 1. As expected, addition of reovirus dsRNA led to an inhibition of luciferase mRNA translation. Addition of purified Tm1-3 RNA also resulted in a decrease in translation. In contrast, addition of purified LS1 RNA had no effect on translation. A shortened tropomyosin RNA, consisting of 133 bases at the 3′ end of Tm1-3 and designated Tm2-3 RNA, was also tested and did inhibit translation (data not shown). The work of Rastinejad *et al.* (30) showed that the *in vivo* tumor-suppressor function of tropomyosin RNA was present in the 3′ half of the

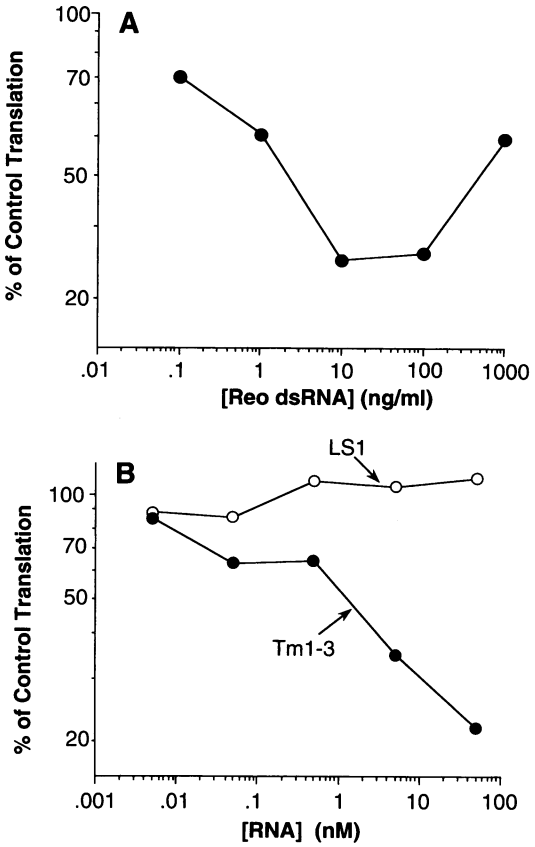

FIG. 1. Effects of RNAs on *in vitro* translation in rabbit reticulocyte lysates. Purified RNAs were added to rabbit reticulocyte lysate at the indicated final concentrations. *(A)* Reovirus dsRNA (●). *(B)* LS1 RNA (○) and Tm1-3 RNA (●). Reactions were incubated at 30°C for 15 min to allow activation of PKR, then luciferase mRNA was added to a final concentration of 15 μg/ml, and reactions were incubated for 30 min at 30°C. Luciferase activity was quantitated using a Dynatech model ML3000 luminometer and normalized to a control translation with only luciferase mRNA. Results shown are the average of three separate experiments.

tropomyosin 3′UTR. Our results, which showed little difference between Tm1-3 RNA and Tm2-3 RNA, are consistent with this observation.

At the highest concentration of reovirus dsRNA, inhibition of translation decreased slightly. This result is in keeping with the previously reported bell-shaped curve of PKR activation by dsRNA. High concentrations of dsRNA are known to be inhibitory to PKR activation (45). It is not clear whether Tm1-3 RNA would follow a similar pattern at higher concentrations.

As shown in Fig. 1, similar levels of inhibition of translation were seen with 5 nM Tm1-3 RNA (corresponding to 800 ng/ml) and reovirus dsRNA at 10 ng/ml. Thus, on a mass basis, Tm1-3 RNA required higher concentrations to cause comparable levels of PKR activation. However, little is known of the percentage of PKR activation required *in vivo* to mediate significant effects including differentiation, growth suppression, and tumor suppression.

Purified RNA from 3′ UTR of Tropomyosin Does Not Inhibit Translation in Wheat Germ Lysate. If the decrease in translation seen in rabbit reticulocyte lysates were due to PKR activation, a PKR-deficient translation system would not be affected. Wheat germ lysate was chosen as a PKR-deficient system. Wheat germ lysate is not responsive to reovirus dsRNA (Fig. 2), suggesting that PKR-like activity is not present or is not responsive to activators of the mammalian enzyme. Moreover, wheat germ lysate contained no proteins reactive in immunoblots using polyclonal or monoclonal antibodies to

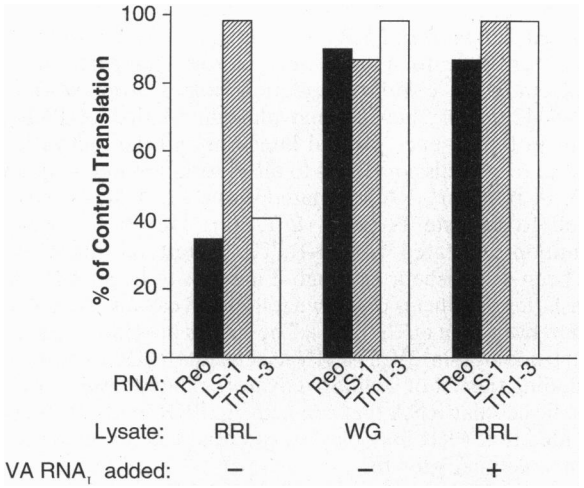

FIG. 2. Effects of RNAs on *in vitro* translation in rabbit reticulocyte lysate and wheat germ lysate. Purified LS1 and Tm1-3 RNAs were added to rabbit reticulocyte lysate (RRL) or wheat germ lysate (WG) to a final concentration of 10 nM. Reovirus dsRNA was added to a final concentration of 10 ng/ml; VA RNA$_I$ was added at a final concentration of 5 μg/ml. Reactions were incubated at 30°C for 15 min to allow activation of PKR; then luciferase mRNA was added to a final concentration of 15 μg/ml. Reactions were incubated for 30 min at 30°C for rabbit reticulocyte or 60 min at 27°C for wheat germ lysate. Luciferase activity was quantitated by using a Dynatech model ML3000 luminometer. Activities were normalized to the appropriate control translation (rabbit reticulocyte lysate, wheat germ lysate, or rabbit reticulocyte lysate with VA RNA$_I$), with only luciferase mRNA. Results shown are the average of two separate experiments.

PKR (data not shown). On the basis of these results, wheat germ lysate was assumed to be effectively PKR-deficient.

The effects of reovirus dsRNA, Tm1-3 RNA, and LS1 RNA on translation in wheat germ as compared with rabbit reticulocyte are shown in Fig. 2. Luciferase mRNA was effectively translated in both systems. Addition of LS1 RNA did not significantly affect translation in either system. Although both reovirus dsRNA and Tm1-3 RNA inhibited translation in rabbit reticulocyte lysate, neither inhibited translation significantly in wheat germ lysate.

Inhibition of Translation in Rabbit Reticulocyte Lysate Can Be Prevented by the Addition of PKR Inhibitor. The observation that Tm1-3 RNA inhibits translation in rabbit reticulocyte lysate but does not do so in wheat germ could be explained by differences in translation factors between the two systems: Tm1-3 RNA might inhibit a translation factor that is not present or is less sensitive in wheat germ. However, if the decrease in translation seen in rabbit reticulocyte lysates was due to PKR activation, a specific PKR inhibitor such as VA RNA$_I$ would be expected to prevent the inhibition. VA RNA$_I$ would not prevent inhibition of translation that was due to putative factors specific to rabbit reticulocyte or more sensitive in rabbit reticulocyte lysate. The results in Fig. 2 show that VA RNA$_I$ blocked the inhibitory effects of Tm1-3 RNA and reovirus dsRNA on translation in rabbit reticulocyte lysate. The presence of VA RNA$_I$ did not affect the wheat germ lysate result for any of the RNAs tested (data not shown).

Inhibition of Translation by the 3′ UTR Is Not Due to dsRNA Contamination from T7 Transcription. There have been reports that T7 RNA polymerase *in vitro* transcription can produce dsRNA contaminants that have the potential to activate PKR and inhibit translation (34). Activation of PKR by these contaminating RNAs was reported to be eliminated by digestion with RNase III, a nuclease specific for dsRNA but unaffected by digestion with RNase T1, a nuclease specific for single-stranded RNA. As described, the 3′UTR RNAs and LS1 RNA were purified through two gels, in accordance with a published protocol for eliminating these dsRNA contaminants (34). To formally exclude dsRNA contaminants as the PKR-activating entity in our RNA preparations, RNAs were digested with RNase T1 and then tested as translational inhibitors in a rabbit reticulocyte lysate assay. If the PKR activating moiety were actually dsRNA contaminants from the T7 transcription reaction, there would be no loss of function after T1 digestion because T1 cannot cut dsRNA. Conversely, if the PKR activating molecule were the 3′ UTR transcripts containing single-stranded regions, T1 digestion would decrease PKR activation. Whereas both reovirus dsRNA and Tm1-3 inhibit translation by ≈80%, only the reovirus inhibition was maintained after RNase T1 digestion. In contrast, nearly 80% of the inhibition seen with Tm1-3 RNA was relieved by pretreatment of the RNA with RNase T1 (data not shown). VA RNA$_I$ function was also assessed with and without T1 digestion. VA RNA$_I$ function was significantly decreased by RNase T1, showing that the RNase T1 could cut single-stranded RNA. These results indicate that the PKR-activating molecule from the T7 transcription of Tm1-3 is not dsRNA but rather contains significant single-stranded RNA.

Tropomyosin 3′ UTR RNA Binds Specifically to PKR. To detect a physical interaction between Tm1-3 RNA and PKR, immunoprecipitated PKR was incubated with radiolabeled RNAs. The results of this assay are shown in Table 1. Purified radiolabeled Tm1-3 RNA bound strongly to PKR immunoprecipitated from a lysate of *E. coli* expressing human PKR but not to a comparable immunoprecipitation using lysate of the parental strain (*E. coli* BL21). VA RNA$_I$, as expected, also bound specifically to PKR, although less strongly than Tm1-3. LS1, as reported (46), bound less strongly than VA RNA$_I$. None of the RNAs bound a control preparation of protein A–Sepharose and monoclonal PKR antibody. Both VA RNA$_I$ and Tm1-3 RNA also bound to a truncated PKR protein, p20, which does not contain the kinase domain but does contain the RNA-binding domain (data not shown). This latter result indicates that the Tm1-3 RNA recognizes the region of PKR known to contain the RNA-binding domain (37).

Tropomyosin 3′ UTR RNA Activates PKR. To confirm that Tm1-3 RNA can activate PKR, *in vitro* kinase assays were done using human PKR purified from Daudi cells. As shown in Fig. 3, addition of increased concentrations of reovirus dsRNA resulted in phosphorylation of PKR. Addition of Tm1-3 RNA also caused phosphorylation of PKR. Autophosphorylation occurred at 5 nM Tm 1-3 RNA, which corresponds to 800 ng/ml. Synthetic dsRNAs such as poly(rI)-poly(rC) are typically added at 1 μg/ml to detect PKR activation (47–49). In contrast, PKR was not phosphorylated in the presence of 50 nM LS1, a concentration that corresponds to 2.7 μg/ml. These *in vitro* kinase activation results indicate that Tm1-3 is a specific RNA activator of PKR. The effects of Tm1-3 RNA on

Table 1. Binding of tropomyosin 3′UTR RNA to immunoprecipitated PKR

Lysate	α-PKR mAb	Input RNA bound, %		
		LS1	VA	Tm
	+	0.1	0.1	0.1
E. coli	+	0.03	0.01	0.2
E. coli (PKR)	+	0.2	1	12

Monoclonal antibodies to PKR (α-PKR mAb) were coupled to protein A–Sepharose. Lysates of *E. coli* expressing human PKR [*E. coli* BL21(DE3) pSRG2; designated here as *E. coli* (PKR)] or parental strain without PKR [*E. coli* BL21(DE3), designated here as *E. coli*], were incubated with the washed resin. Resin was again washed and then incubated with purified radiolabeled RNAs ($1–2 \times 10^7$ cpm) for 5 min at 30°C and then for 30 min at 4°C. Resin was again washed; then bound RNAs were quantitated by scintillation counting. VA, adenovirus VA RNA$_I$; LS1, nonfunctional adenovirus VA RNA$_I$; Tm, Tm1-3 RNA.

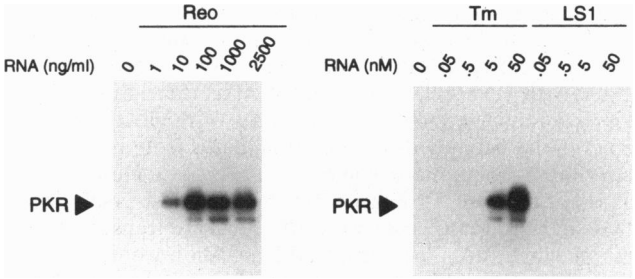

FIG. 3. *In vitro* PKR kinase activity. PKR purified from interferon-treated Daudi cells was incubated with reovirus dsRNA (Reo), or purified Tm1-3 RNA (Tm), or purified LS1 RNA (LS1), at the indicated concentrations, in 75 mM KCl/25 mM Hepes, pH 7.5/10 mM $MgCl_2$/1 mM dithiothreitol/0.1 mM EDTA/0.1 mM ATP/protease inhibitors/5 μCi of [γ-^{32}P]ATP. Reactions were incubated for 20 min at 30°C and then quenched by the addition of SDS sample buffer. Phosphorylated proteins were separated by SDS/PAGE; an autoradiogram of the gel is presented.

translation and the direct demonstration of PKR activation by this RNA suggest that activation of cellular PKR may account for the tumor-suppressor activity of tropomyosin 3′UTR RNA *in vivo*.

DISCUSSION

The results presented here show that RNA from the 3′UTR of tropomyosin inhibited translation in a manner consistent with PKR activation. Translation was inhibited in rabbit reticulocyte lysate, a mammalian system known to contain PKR but was not inhibited in wheat germ lysate. Inhibition of translation by Tm1-3 RNA was prevented by VA RNA_I, an inhibitor of PKR activation. In short, RNA from the 3′UTR of tropomyosin functions in a manner analogous to reovirus dsRNA, the prototypical activator of PKR. Our results also show a direct interaction between PKR and Tm1-3 RNA. Tm1-3 RNA bound to PKR *in vitro* (Table 1) and activated purified PKR *in vitro*, as assessed by autophosphorylation in a kinase assay (Fig. 3). It seems unlikely that this RNA would bind and activate PKR and yet inhibit translation through a mechanism totally unrelated to PKR. There are several significant implications in the finding that PKR is activated *in vitro* by tropomyosin RNA. (*i*) It extends the characteristics of PKR activators to include single-stranded RNA. (*ii*) It adds to growing evidence that cellular RNAs can activate PKR. (*iii*) Since tropomyosin 3′UTR RNA has been shown to act as a tumor suppressor *in vivo*, it suggests that PKR may actually mediate the tumor-suppressor activity observed with tropomyosin 3′UTR RNA. Each of these points warrant further discussion.

Activation of PKR by dsRNA is thought to be length dependent but not sequence dependent, requiring at least 30 perfectly duplexed base pairs. Efficiency of activation increases with increased duplex length, reaching a maximum at 85 bp (38). However, the results presented here, as well as recent work with hepatitis delta virus RNA (50), indicate that PKR activation may also occur with RNAs that would not be predicted to have double-stranded regions of this length. Tm1-3 RNA is single-stranded (confirmed by its sensitivity to RNase T1) and not predicted to contain any regions of duplexed bases longer than 20 bp (MacDNASIS analysis, data not shown). Thus our results with Tm1-3 RNA indicate that PKR activation by RNA may not be as length and duplex dependent as was previously thought based on experiments using synthetic dsRNA polymers (38, 51, 52).

PKR was first characterized as part of the interferon-induced antiviral response, and the best-characterized activators are viral and synthetic dsRNAs. PKR has also been shown to act as a tumor suppressor and therefore may have a role in cell growth apart from viral infection. Little is known about nonviral activators of PKR, but cellular activators must exist in order for PKR to assume such a role. Support for PKR involvement in cellular regulation comes from work with 3T3-F442A cells showing that phosphorylation of PKR can occur in the absence of viral infection and that activation of PKR in these cells correlates to differentiation into adipocytes (53). A poly(A)$^+$ RNA isolated from 3T3-F442A cells was found to activate PKR *in vitro* (54). This RNA could be immunoprecipitated with PKR. The identity of this RNA has not been established; although it appears to be an mRNA, its translation product is unknown (54). PKR can also be activated *in vitro* by its own mRNA (55). The results presented here show that tropomyosin 3′UTR RNA activates PKR *in vitro*, thus extending the list of cellular activators. The identification of a specific cellular RNA that can activate PKR lends credence to the idea that PKR may play an essential role in regulation of normal cellular growth.

It is significant that tropomyosin 3′UTR RNA has been implicated as a transacting regulator of growth and differentiation (29) and as a tumor suppressor (30). One intriguing possible mediator of such an activity is PKR, an enzyme that requires activation by RNA. The results presented here suggest that PKR could mediate the tumor-suppressor activity observed with tropomyosin 3′UTR RNA.

While it would not be surprising to find that other cellular RNAs might also activate PKR, translation could not occur *in vivo* or *in vitro* unless most RNAs fail to activate PKR. In support of this idea, recently published work has shown that a cellular mRNA, β_2 microglobulin, does not activate PKR (56). Control of PKR activity may require balancing the activities of cellular activators and inhibitors. Further research in this area will be crucial to understanding the role of cellular RNAs in regulating PKR and will undoubtedly lead to exciting results concerning the role of PKR in tumor suppression.

We thank Joe Harford, Alan Hinnebusch, Simon Green, Michael Katze, Michael Mathews, Charles Moehle, Nahum Sonenberg, and Gary Witherell for helpful discussions and critical review of the manuscript; Michael Mathews and Lisa Manche for the gift of purified PKR and plasmids encoding adenovirus VA RNA_I and LS1; Aaron Shatkin for the gift of reovirus dsRNA; Nahum Sonenberg for the gift of luciferase plasmid; and Michael Mathews for communication of results prior to publication.

1. Lebleu, B., Sen, G. C., Shaila, S., Cabrer, B. & Lengyel, P. (1976) *Proc. Natl. Acad. Sci. USA* **73,** 3107–3111.
2. Lengyel, P. (1982) *Annu. Rev. Biochem.* **51,** 251–282.
3. Roberts, W. K., Hovanessian, A., Brown, R. E., Clemens, M. J. & Kerr, I. M. (1976) *Nature (London)* **264,** 477–480.
4. Zilberstein, A., Federman, P., Shulman, L. & Revel, M. (1976) *FEBS Lett.* **68,** 119–124.
5. Boone, R. F., Parr, R. P. & Moss, B. (1979) *J. Virol.* **30,** 365–374.
6. Maran, A. & Mathews, M. B. (1988) *Virology* **164,** 106–113.
7. Farrell, P. J., Sen, G. C., Dubois, M. F., Ratner, L., Slattery, E. & Lengyel, P. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 5893–5897.
8. Proud, C. G. (1986) *Trends Biochem. Sci.* **11,** 73–77.
9. Zilberstein, A., Kimchi, A., Schmidt, A. & Revel, M. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 4734–4738.
10. Hershey, J. W. B. (1993) *Semin. Virol.* **4,** 201–207.
11. Katze, M. G. (1993) *Semin. Virol.* **4,** 259–268.
12. Mathews, M. B. (1993) *Semin. Virol.* **4,** 247–257.
13. Ghadge, G. D., Swaminathan, S., Katze, M. G. & Thimmappaya, B. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 7140–7144.
14. Mathews, M. B. & Shenk, T. (1991) *J. Virol.* **65,** 5657–5662.
15. Beattie, E., Tartaglia, J. & Paoletti, E. (1991) *Virology* **183,** 419–422.
16. Carroll, K., Elroy-Stein, O., Moss, B. & Jagus, R. (1993) *J. Biol. Chem.* **268,** 12837–12842.
17. Chang, H. W., Watson, J. C. & Jacobs, B. L. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 4825–4829.
18. Watson, J. C., Chang, H. W. & Jacobs, B. L. (1991) *Virology* **185,** 206–216.

19. Langland, J. O., Pettiford, S., Jaing, B. & Jacobs, B. L. (1994) *J. Virol.* **68,** 3821–3829.
20. Imani, F. & Jacobs, B. L. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 7887–7891.
21. Lee, T. G., Tomita, J., Hovanessian, A. G. & Katze, M. G. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 6208–6212.
22. Lee, T. G., Tomita, J., Hovanessian, A. G. & Katze, M. G. (1992) *J. Biol. Chem.* **267,** 14238–14243.
23. Black, T. L., Barber, G. N. & Katze, M. G. (1993) *J. Virol.* **67,** 791–800.
24. Black, T. L., Safer, B., Hovanessian, A. G. & Katze, M. G. (1989) *J. Virol.* **63,** 2244–2251.
25. Koromilas, A. E., Roy, S., Barber, G. N., Katze, M. G. & Sonenberg, N. (1992) *Science* **257,** 1685–1689.
26. Meurs, E. F., Galabru, J., Barber, G. N., Katze, M. G. & Hovanessian, A. G. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 232–236.
27. Chong, K. L., Feng, L., Schappert, K., Meurs, E., Donahue, T. F., Friesen, J. D., Hovanessian, A. G. & Williams, B. R. G. (1992) *EMBO J.* **11,** 1553–1562.
28. Sharp, T. V., Xiao, Q., Jeffrey, I., Gewert, D. R. & Clemens, M. J. (1993) *Eur. J. Biochem.* **214,** 945–948.
29. Rastinejad, F. & Blau, H. M. (1993) *Cell* **72,** 903–917.
30. Rastinejad, F., Conboy, M. J., Rando, T. A. & Blau, H. M. (1993) *Cell* **75,** 1107–1117.
31. Cooper, H. L., Feuerstein, N., Noda, M. & Bassin, R. H. (1985) *Mol. Cell. Biol.* **5,** 972–983.
32. Prasad, G. L., Fuldner, R. A. & Cooper, H. L. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 7039–7043.
33. Lin, C. & Leavitt, J. (1988) *Mol. Cell. Biol.* **8,** 160–168.
34. Mellits, K. H., Pe'ery, T., Manche, L., Robertson, H. D. & Mathews, M. B. (1990) *Nucleic Acids Res.* **18,** 5401–5406.
35. Mellits, K. H. & Mathews, M. B. (1988) *EMBO J.* **7,** 2849–2859.
36. Katze, M. G., DeCorato, D., Safer, B., Galabru, J. & Hovanessian, A. G. (1987) *EMBO J.* **6,** 689–697.
37. Green, S. R. & Mathews, M. B. (1992) *Genes Dev.* **6,** 2478–2490.
38. Manche, L., Green, S. R., Schmedt, C. & Mathews, M. B. (1992) *Mol. Cell. Biol.* **12,** 5238–5248.
39. Laurent, A. G., Krust, B., Galabru, J., Svab, J. & Hovanessian, A. G. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 4341–4345.
40. Kostura, M. & Mathews, M. B. (1989) *J. Biol. Chem.* **9,** 1576–1586.
41. Gunnery, S., Green, S. R. & Mathews, M. B. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 11557–11561.
42. Mellits, K. H., Pe'ery, T. & Mathews, M. B. (1992) *J. Virol.* **66,** 2369–2377.
43. Nilsen, T. W., Maroney, P. A. & Baglioni, C. (1982) *J. Biol. Chem.* **257,** 14593–14596.
44. Samuel, C. E., Duncan, R., Knutson, G. S. & Hershey, J. W. B. (1984) *J. Biol. Chem.* **259,** 13451–13457.
45. Hunter, T., Hunt, T., Jackson, R. J. & Robertson, H. D. (1975) *J. Biol. Chem.* **250,** 409–417.
46. Mellits, K. H., Kostura, M. & Mathews, M. B. (1990) *Cell* **61,** 843–852.
47. Galabru, J. & Hovanessian, A. (1987) *J. Biol. Chem.* **262,** 15538–15544
48. Lasky, S. R., Jacobs, B. L. & Samuel, C. E. (1982) *J. Biol. Chem.* **257,** 11087–11093.
49. Patel, R. C., Stanton, P. & Sen, G. C. (1994) *J. Biol. Chem.* **269,** 18593–18598.
50. Robertson, H. D., Manche, L. & Mathews, M. (1996) *J. Virol.*, in press.
51. Minks, M. A., West, D. K., Benvin, S. & Baglioni, C. (1979) *J. Biol. Chem.* **254,** 10180–10183.
52. Minks, M. A., West, D. K., Benvin, S., Greene, J. J., Ts'o, P. O. P. & Baglioni, C. (1980) *J. Biol. Chem.* **255,** 6403–6407.
53. Judware, R. & Petryshyn, R. (1992) *J. Biol. Chem.* **267,** 21685–21690.
54. Li, J. & Petryshyn, R. A. (1991) *Eur. J. Biochem.* **195,** 41–48.
55. Thomis, D. C. & Samuel, C. E. (1993) *J. Virol.* **67,** 7695–7700.
56. Mordechai, E., Kon, N., Henderson, E. E., and Suhadolnik, R. J. (1995) *Virology* **206,** 913–922.