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Paradoxical Interactions between Human Delta Hepatitis Agent RNA and the Cellular Protein Kinase PKR

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The genome of the human delta hepatitis agent is a circular, highly structured single-stranded RNA lacking regular runs of RNA-RNA duplex longer than 15 bp. We have tested the ability of delta agent RNA to participate in reactions with a protein containing a motif which confers the ability to bind double-stranded RNA (dsRNA). Surprisingly, highly purified delta agent RNA preparations from which all traces of contaminating dsRNA have been removed activate PKR, the dsRNA-dependent protein kinase activity of mammalian cells (also known as DAI, P1-eIF-2, and p68 kinase). This behavior is in marked contrast to the interaction of PKR with a number of other highly structured viral single-stranded RNAs, which inhibit, rather than stimulate, activation of this kinase. PKR activation leads to inhibition of protein synthesis in the rabbit reticulocyte lysate system. Paradoxically, delta RNA failed to elicit the expected PKR-mediated inhibition of cell-free translation. Instead, delta RNA interfered with PKR activation and the translational block induced by dsRNA. We conclude that the interaction of PKR and delta agent RNA may represent a new category of protein-RNA interactions involving the dsRNA binding motif.

The human delta hepatitis agent is, so far, the sole representative of the viroid-like pathogen group known to replicate in mammalian cells. Members of this group, which are found principally in the plant kingdom, are defined by their circular, highly structured single-stranded RNA (ssRNA) genomes, the lack of mRNA activity in their genomic strands, and their replication by a rolling-circle pathway (4–7, 18, 50). While the RNAs of the delta agent are likely to interact with host components (reviewed in reference 44), decisive evidence is lacking in this area. We wished to test the ability of full-length and subgenomic transcripts of the delta agent RNA to interact with the double-stranded RNA (dsRNA)-activated protein kinase of mammalian cells called PKR (also known as DAI, P1-eIF-2, and p68 kinase), a key cellular regulator which mediates the interferon-induced antiviral response as well as tumor suppression and other functions (11, 22, 24, 28, 30–32, 35, 46).

The class of RNA-protein interactions represented by PKR-RNA binding uses one or more copies of a motif found in proteins that bind dsRNA (17, 48). Organisms ranging from bacteria to mammals yield cases of such dsRNA-binding proteins, so that more than 10 occurrences are now known (1, 48). Well-studied examples include the RNA processing enzyme RNase III of *Escherichia coli*, which recognizes dsRNA or highly structured ssRNA substrates (39–42), and PKR itself, which also interacts with RNAs containing extensive, perfect RNA-RNA duplex regions (8, 27, 30, 47), as well as those containing specific ssRNA structures (16, 30, 33). The pattern that is beginning to emerge includes different responses observed in both proteins to extensive perfect RNA-RNA duplexes compared with highly structured ssRNA sites. The latter response, in fact, may also require sequence specificity. In the case of RNase III, dsRNA directs cleavage in a non-sequence-dependent manner (39, 42), while the enzyme's ssRNA sites direct highly specific cleavage (39–42). PKR binds to dsRNA in a manner independent of the base sequence. Such binding activates PKR, resulting in the phosphorylation of initiation factor eIF-2, sequestration of the recycling factor eIF-2B, and consequent protein synthesis inhibition (21, 23, 30, 31, 36, 43). The binding of PKR to highly structured regions of viral ssRNAs blocks kinase activation which would otherwise occur in the presence of dsRNA (30). Among viral ssRNA species which can block PKR activation by dsRNA are adenovirus VA (virus-associated) RNA (33, 34), human immunodeficiency virus TAR RNA (19, 20), and the EBER-1 RNA of Epstein-Barr virus (10). A recent study used RNA footprinting and other techniques to pinpoint the domain of adenovirus VA RNA_I responsible for interacting with a dsRNA-binding motif in the PKR protein (8).

We have extended studies of the interaction between PKR and highly structured ssRNAs to the human hepatitis delta agent. Although single stranded by several well-established criteria, such as treatment with various RNases (2, 4) and chromatography on cellulose CF-11 (14), delta RNA has extensive regions of secondary structure as evidenced by its sequence (50) and modeling studies (3, 4, 50). We report here that delta agent genomic RNA—and in particular a subdomain containing the conserved, viroid-like sequences thereof—can activate the protein kinase activity of PKR despite lacking stable dsRNA as defined by conventional tests (42, 43). On the other hand, in an assay that relates the action of PKR more closely to in vivo conditions, the delta RNA subdomain failed to inhibit protein synthesis in a cell-free translation system. Paradoxically, the delta RNA instead interfered with the activation of PKR by fully duplexed dsRNA. We believe that this novel delta RNA-PKR interaction may be the first example of a new category of protein-RNA interaction involving the dsRNA-protein binding motif, with implications for the interaction of both viral and cellular RNAs with this class of regulatory proteins.

MATERIALS AND METHODS

Materials. PKR was purified through the MonoS stage (25) and assayed as previously described (30, 34). The p20 fragment of PKR, containing amino acid residues 1 to 184 of PKR overexpressed in and purified from *E. coli* (47) retains

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the dsRNA-binding domains of the protein (17, 30). For gel shift assays, monoclonal immunoglobulin G directed against PKR (antibody from A. Hovanessian [26]) was utilized. *E. coli* RNase III, purified through stage VI as previously described (42, 43), was used to pretreat RNA preparations for the removal of dsRNA.

^{32}P-labeled RNAs were prepared by using [α-^{32}P]GTP from ICN or New England Nuclear and bacteriophage T7 and SP6 RNA polymerase from Promega. Reactions were carried out in 20-μl volumes under the enzyme supplier's recommended conditions or those described previously (3), and incubations were performed so as to yield RNA specific activities of 4×10^7 dpm/μg. Full-length genomic and antigenomic transcripts of delta agent RNA, as well as delta 13/3, a delta transcript of genomic polarity spanning residues 482 to 963 of the conserved, viroid-like region, were prepared from DNA templates amplified and utilized as described previously (2). RNAs used in the experiments shown in Fig. 1 and 2 were purified by polyacrylamide gel electrophoresis in 7 M urea, followed by cellulose CF-11 chromatography (14) and RNase III treatment, as indicated in the figure legends. Transcripts of adenovirus type 2 VA RNA$_I$ were synthesized as described by Mellits et al. (34). Synthetic dsRNA, labeled with [α-^{32}P]GTP, of 85 bp was prepared as described by Manche et al. (30). Unlabeled reovirus RNA was a gift of A. Shatkin. Cellulose CF-11 for the purification of ssRNA and dsRNA fractions (14) was obtained from Whatman and prepared for chromatography as previously described (3). Carrier RNA was *E. coli* tRNA (Sigma) extracted with phenol and purified by cellulose CF-11 chromatography as before (3).

Gel shift assays. Each ^{32}P-labeled RNA (55.5 ng/ml) synthesized as described under "Materials" was incubated in the presence of 0.1 mg of carrier tRNA per ml for 20 min on ice, alone or with 1 μl (3.5 μg/ml) of p20 protein as described previously (30, 47). For supershift assays, monoclonal immunoglobulin G directed against PKR was added at the end of the 20-min incubation period described above, and the reaction continued for 5 min on ice. Complexes were resolved in a nondenaturing 5% polyacrylamide gel (run from top to bottom) and visualized by autoradiography.

PKR activation. As described before (30, 34), kinase assays for PKR activity utilized [γ-^{32}P]ATP (ICN). The samples were incubated in a final volume of 10 to 20 μl for 20 min at 30°C, and then an equal volume of a sodium dodecyl sulfate (SDS)-containing gel sample buffer was added. Samples were heated at 100°C for 2 min and then fractionated in 12.5% polyacrylamide–SDS gels. The 68,000-Da ^{32}P-labeled PKR protein was detected by autoradiography. Phosphorylation of the α- subunit of eIF-2 in the presence of PKR was monitored by inclusion of 0.68 mg of eIF-2 (kindly provided by J. W. B. Hershey) in the kinase assay.

RNase III pretreatment. Samples were treated with RNase III, purified through stage VI (42, 43), to digest dsRNA contaminants by incubating in 10-μl reaction volumes containing 0.02 M Tris-HCl, (pH 7.6), 0.01 M $MgCl_2$, 2 U of RNase III per ml, and up to 2 μg of RNA per reaction.

Multistep purification to remove dsRNA contaminants from RNA. The procedure developed by Mellits et al. (34) was used for the RNAs used in the experiments shown in Fig. 3 to 6. RNA was subjected to electrophoresis in denaturing gels containing 7 M urea and standard Tris-borate-EDTA buffer, followed by nondenaturing gels lacking urea. RNA was eluted after each gel electrophoretic step by overnight agitation at room temperature in the presence of 0.01 M Tris-HCl, (pH 7.5)–0.001 M EDTA followed by ethanol precipitation, as before (34). dsRNA contamination was further minimized by cellulose CF-11 chromatography carried out by the method of Franklin (14) in order to separate dsRNA from ssRNA. RNase III treatment of the resulting transcripts was employed as an optional further step.

Cell-free translation. Assays were conducted by using the two-step approach of Gunnery et al. (19) employing micrococcal nuclease-treated reticulocyte lysate (38). The lysate was incubated with the test RNAs for 15 min at 30°C and then supplemented with globin mRNA and ^{35}S-methionine (ICN). Incubation was continued for 30 min, and globin synthesis was monitored by electrophoresis in SDS-polyacrylamide gels, which were scanned with a Fuji Phosphorimager. Translation efficiency in the presence of various ssRNA, dsRNA, and delta RNA concentrations was determined as before (38). PKR phosphorylation in the presence of the same three RNA types was ascertained by addition of 5 or 10 μCi [γ-^{32}P]ATP to similar translation reaction mixtures. The labeled nucleotide was present from the first step of the reaction. Globin mRNA and ^{35}S-methionine were omitted from the second step. PKR was immunoprecipitated with polyclonal anti-PKR antibody (17). Phosphorylated PKR was detected by SDS-polyacrylamide gel electrophoresis and quantitated by using a Fuji Phosphorimager.

RESULTS

RNP complex formation. Initial experiments were aimed at testing the hypothesis that delta agent RNA might belong in the category of highly structured ssRNA which could, like adenovirus VA RNA, interact with PKR and inhibit kinase activation. To determine whether PKR and delta RNA are able to form an RNP complex, we employed gel mobility shift analysis. The RNA binding domain of PKR has been expressed

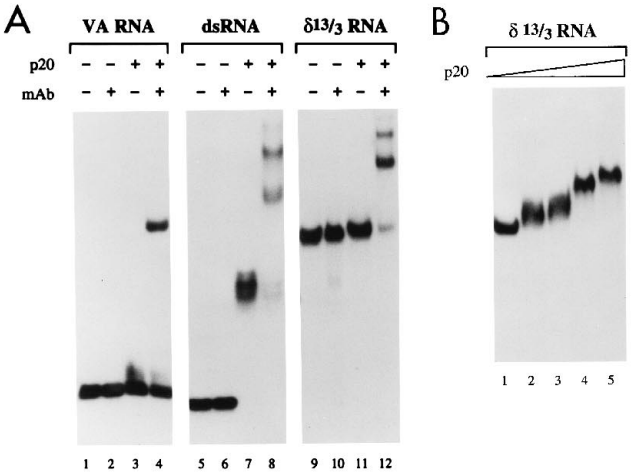

FIG. 1. Gel mobility shift analysis of delta agent RNA. (A) Gel shift and supershift with delta RNA, VA RNA, and dsRNAs. Each ^{32}P-labeled RNA, synthesized and assayed as described in Materials and Methods, was incubated in the presence of 0.1 mg of carrier tRNA per ml for 20 min on ice, alone or with 1 μl (3.5 μg/ml) of p20 protein. Where indicated, monoclonal immunoglobulin G directed against PKR was added, and the incubation continued for 5 min on ice. Complexes were resolved in a 5% nondenaturing polyacrylamide gel and visualized by autoradiography. Lanes 1 to 4, adenovirus type 2 VA RNA$_I$; lanes 5 to 8, synthetic 85-bp dsRNA; lanes 9 to 12, the 13/3 fragment of delta agent RNA, described in Materials and Methods. Reactions lacked or contained p20 and anti-PKR antibody (mAb), as indicated. (B) Progressive retardation of delta 13/3 RNA by increasing concentrations of p20. Lanes 1 to 5 contained ^{32}P-labeled delta 13/3 RNA and 0, 1, 2, 5, or 10 μl of the p20 solution, respectively.

in *E. coli* as a 184-amino-acid polypeptide containing tandem copies of the RNA binding motif (30, 47). The purified p20 protein bound efficiently to a perfectly duplexed 85-bp synthetic dsRNA, as illustrated by the gel shift experiment of Fig. 1A (lanes 5 and 7). In the presence of monoclonal anti-PKR antibody, the complex was supershifted (compare lanes 7 and 8). Adenovirus type 2 VA RNA$_I$, a highly structured ssRNA of 160 bases which at high concentrations serves to block PKR activation by dsRNA (33, 34), bound less efficiently than dsRNA and gave only a small shift under these conditions, but it also gave a distinct supershifted complex in the presence of antibody (lanes 1 to 4). The relatively weak ability of VA RNA to produce a gel shift reflects its relatively weak affinity for p20 (47). A 482-base fragment of the delta genomic RNA designated 13/3 behaved similarly: it was shifted weakly at this concentration of protein p20 (lanes 9 and 11), but in the presence of antibody the interaction was clearly seen (lane 12). At higher p20 levels, several shifted complexes were visualized in the absence of antibody (Fig. 1B), as also shown in Fig. 1A in the presence of antibody. It is seen from Fig. 1A that the affinity of p20 is higher for dsRNA than for either VA RNA or delta 13/3 RNA. The apparent dissociation constants for dsRNA and VA RNA are 4×10^{-9} and 3.5×10^{-7} M, respectively (47). Preliminary estimates suggest that the affinity of p20 for the delta 13/3 RNA is somewhat lower, on the order of 10^{-6} M (data not shown).

Activation of the kinase. PKR phosphorylates the α subunit of mammalian protein synthesis initiation factor eIF-2, leading to inhibition of protein synthesis (21). Recent studies have confirmed the dsRNA size dependence (>30 bp [30, 36]) and sequence independence (30, 36, 43) of this phenomenon. The ability of small viral RNAs, such as VA RNA (33, 34, 37) and perhaps TAR RNA of human immunodeficiency virus type 1 and EBER-1 RNA of Epstein-Barr virus (9, 19, 20, 31), to inhibit PKR activation seems to be based on their binding to

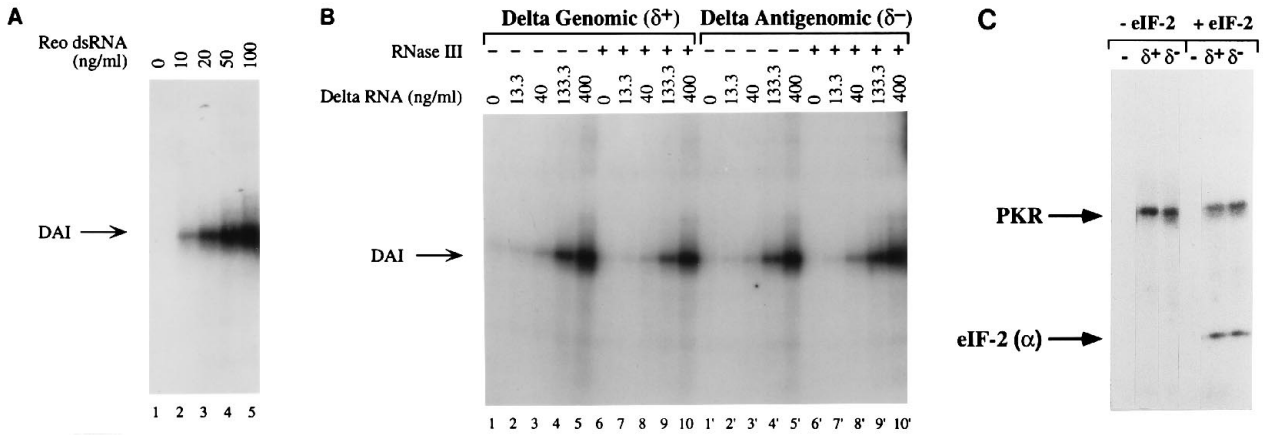

FIG. 2. Activation of PKR by reovirus and delta agent RNAs. PKR purification and kinase assay conditions were as described in Materials and Methods. The 68,000-D ^{32}P-labeled PKR (arrow) was detected by autoradiography. (A) Titration of reovirus (Reo) RNA. Reactions contained 0 to 100 ng of reovirus RNA per ml as indicated at the top. (B) Titration of delta agent RNAs. RNA concentrations were calculated by including low levels of [α-^{32}P]GTP in the transcription reactions. Lanes 1 to 10, delta genomic RNA transcripts (δ^+) with (lanes 6 to 10) or without (lanes 1 to 5) RNase III pretreatment carried out as described in Materials and Methods. Lanes 1′ to 10′, delta antigenomic RNA (δ^-) with (lanes 6′ to 10′) or without (lanes 1′ to 5′) RNase III pretreatment. (C) PKR-specific phosphorylation of eIF-2α. Kinase reaction mixtures contained no RNA (−), delta 13/3 genomic (δ^+) or antigenomic (δ^-) RNA pretreated with RNase III, or eIF-2 as indicated. Positions of PKR (DAI in panels A and B) and the α subunit of eIF-2 are indicated.

PKR at specific structures or sequences. Like these species, delta agent RNA is imperfectly duplexed (50), as also confirmed below, and we therefore tested its behavior in kinase activation assays. Since the activation of PKR is accompanied by its autophosphorylation, the standard assay for PKR activation monitors the transfer of label from γ-^{32}P-labeled ATP to the kinase, which migrates in SDS-polyacrylamide gels with an apparent molecular mass of 68 kDa. As shown in Fig. 2A, perfect RNA-RNA duplexes from reovirus activated the kinase, causing the appearance of a 68-kDa ^{32}P-labeled protein band of increasing intensity with increasing dsRNA concentration. Initial studies with delta genomic (Fig. 2B, lanes 1 to 10) or antigenomic (Fig. 2B, lanes 1′ to 10′) RNA transcripts showed that, contrary to expectation, they activated PKR kinase activity. The data suggest that, like the synthetic dsRNA poly(I · C) (36), the delta RNAs are 10 to 20% as active as reovirus RNA in this assay. Further experiments confirmed that PKR activated by delta RNA of either polarity can phosphorylate the enzyme's substrate, the α subunit of eIF-2 (Fig. 2C).

Even though the delta RNAs still activated the kinase after treatment with RNase III (Fig. 2), which is capable of removing dsRNA contaminants from other RNAs tested in this fashion (20, 34), the sensitivity of PKR to very low levels of dsRNA obliged us to exclude rigorously such potential contamination. To this end we employed a purification technique (34), which involves sequential electrophoresis and elution of candidate RNAs under denaturing and then nondenaturing conditions (to ensure maximal separation of ssRNA from dsRNA), cellulose CF-11 chromatography by the method of Franklin (14) (which separates even highly structured ssRNAs from dsRNA), and, as an optional further treatment, *E. coli* RNase III digestion. This nuclease specifically cleaves perfect duplex RNA regions containing ≥20 bp, while ignoring even highly structured ssRNA regions other than its own prokaryotic recognition signals (39, 43). The highly structured mammalian viral ssRNAs known to inhibit activation of PKR are not cleaved by RNase III nor do they contain extended duplexes (6, 19, 20, 34, 37, 39). Likewise, sequence-based secondary structure projections as well as CF-11 chromatography and RNase III treatment (unpublished observations) indicate that no region of delta RNA contains perfect double-stranded regions approaching 20 consecutive base pairs.

Both genomic and antigenomic delta RNAs were able to activate the kinase activity of PKR following rigorous purification (Fig. 3, lanes 1 to 4 and 5 to 8, respectively). Furthermore, the ability of the purified delta RNA to activate PKR was resistant to incubation with RNase III (Fig. 3). Similar results were obtained with the 13/3 subgenomic transcript of delta RNA which contains 482 bases of genomic sequence (lanes 9 to 12). We therefore conclude that delta agent RNA itself contains a structure which (although lacking extended duplexes) is able to bind PKR as if it were dsRNA. To confirm this, we carried out similar experiments using delta transcripts

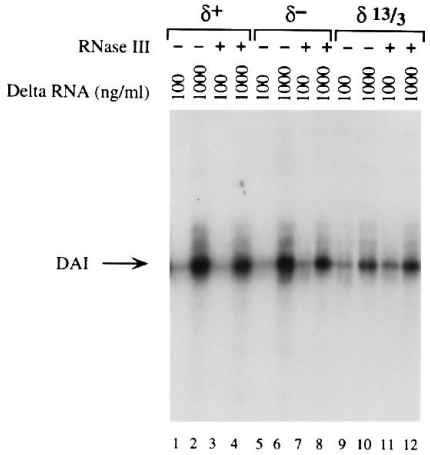

FIG. 3. Activation of PKR (DAI) by delta agent RNAs after multistep purification to remove potential dsRNA contaminants. Transcripts synthesized as described in Materials and Methods and utilized as in Fig. 1 and 2 were purified through denaturing and nondenaturing gels by the procedure of Mellits et al. (34), followed by cellulose CF-11 chromatography. Recovered RNAs were then assayed at two concentrations (100 and 1,000 ng/ml) as in Fig. 2, either with or without RNase III pretreatment. Lanes 1 to 4, delta genomic RNA (δ^+); lanes 5 to 8, delta antigenomic RNA (δ^-); lanes 9 to 12, delta genomic RNA subdomain 13/3 (δ 13/3). Inclusion or omission of RNase III treatment is indicated.

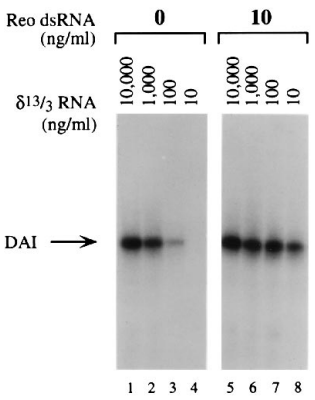

FIG. 4. Tests of PKR (DAI) activation and inhibition by delta 13/3 RNA in the presence or absence of reovirus RNA. PKR kinase activity assays were conducted as described in Materials and Methods in the absence (lanes 1 to 4) or presence (lanes 5 to 8) of 10 ng of reovirus (Reo) RNA per ml and of delta 13/3 RNA at concentrations ranging from 10,000 to 10 ng/ml as indicated.

and control RNAs (both ssRNA and dsRNA) which had been pretreated with either RNase T_1 (specific for ssRNA), RNase III (specific for dsRNA), or buffer alone (control). These protocols had been validated in studies of VA RNA and TAR RNA, both inhibitors of PKR (20, 34). In all cases, the PKR-activating capacity of delta RNAs fulfilled the characteristics expected for ssRNA: they were sensitive to RNase T_1 but resistant to RNase III (data not shown). These findings represent the first such case for a PKR kinase activator; all others tested are RNase T_1 resistant but RNase III sensitive.

In light of this unexpected behavior, we explored the ability of delta RNA to act as an inhibitor of PKR activation, either of its own activating capacity at high concentrations (as dsRNA does [31]), or of reovirus RNA activation (as VA RNA and the other small viral RNAs do [31]). Figure 4 shows that, at concentrations at which dsRNA begins to block its own ability to activate DAI (1,000 ng/ml; assuming that delta RNA is 10% as active as dsRNA, 10,000 ng of delta RNA per ml), delta RNA continued to activate PKR, whether or not reovirus RNA was present. Similarly, Fig. 4 shows that even the highest concentration of delta RNA tested did not begin to suspend activation of PKR or to inhibit activation by its own or reovirus RNA. Unfortunately, quantities of delta 13/3 RNA sufficient to ascertain whether or not it eventually begins to inhibit dsRNA-mediated activation of PKR, as does dsRNA itself at high concentrations (31), were not available.

Protein synthesis inhibition. PKR was discovered as an activity in rabbit reticulocyte lysates that inhibits protein synthesis in response to dsRNA and was subsequently identified as a phosphoprotein present in a variety of interferon-treated cells and demonstrated to be a dsRNA-dependent eIF-2 kinase (reviewed in references 21, 27, 31, and 33). As shown in Fig. 5A, increasing concentrations of dsRNA isolated from reovirus virions progressively inhibit globin synthesis in the reticulocyte translation system, with a minimum residual translation (about 5% of control level) being reached at about 100 ng/ml. High concentrations of dsRNA, in excess of 1 μg/ml, fail to inhibit in accordance with previous studies of PKR activation and of translation in reticulocyte lysates. Because the inhibitory properties of dsRNA are apparent at some concentrations but not others, albeit over a 1,000-fold concentration range, we assayed a broad range of delta 13/3 concentrations for their effects on globin synthesis. No inhibition was observed at any concentration tested, from 1 to 10 μg/ml (Fig. 5A). This finding came as a surprise in view of the ability of delta RNA concentrations within this range to activate the purified kinase, and it led us to revisit our original hypothesis that delta RNA might act as an inhibitor of kinase activation.

Adenovirus type 2 VA RNA_I is such an inhibitor of kinase activation (31, 33, 37). In the reticulocyte lysate, addition of VA RNA alone has no effect on translation (Fig. 5A). As shown previously, when added together with dsRNA, VA RNA can largely overcome the dsRNA-mediated inhibition of

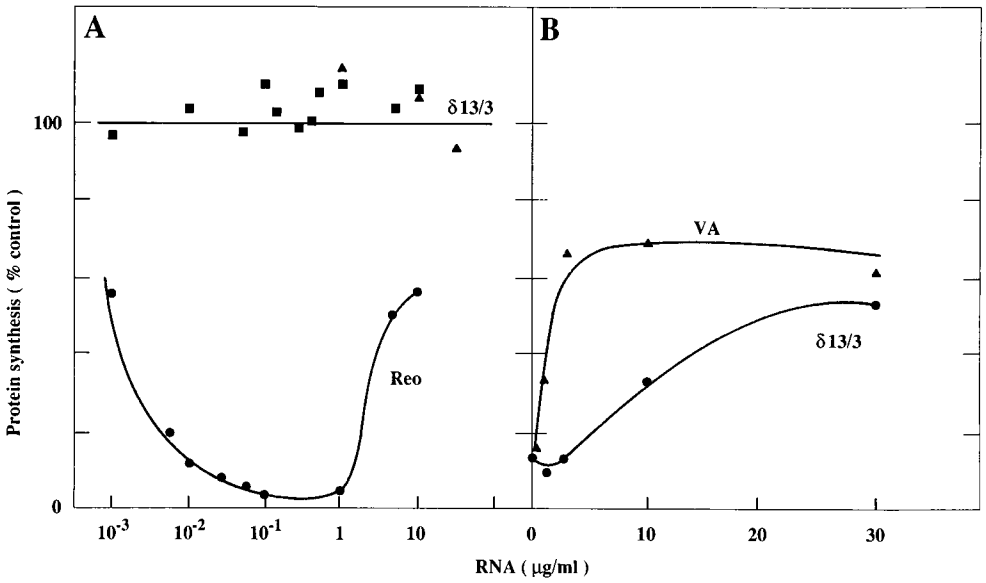

FIG. 5. Activity of delta 13/3 RNA in a cell-free translation system. Globin synthesis was measured in the reticulocyte lysate system after preincubation of the lysate with various RNAs. (A) The lysate was preincubated with various concentrations of dsRNA (●), delta 13/3 RNA (■), or VA RNA (▲). Results from several experiments covering different parts of the concentration range were combined. (B) The lysate was preincubated with dsRNA (20 ng/ml) together with either VA RNA (▲) or delta 13/3 RNA (●) at the concentrations indicated. Results from a single representative experiment are shown.

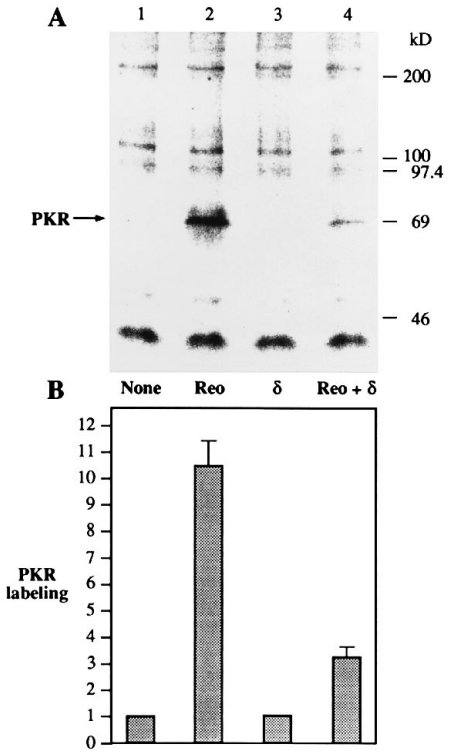

FIG. 6. PKR activation and inhibition in the reticulocyte translation system. Reactions were conducted in the presence of [γ-^{32}P]ATP and either no RNA (None), reovirus dsRNA (20 ng/ml) (Reo), delta 13/3 RNA (20 μg/ml) (δ), or a combination of reovirus dsRNA and delta 13/3 RNA at 20 μg/ml each. The products were immunoprecipitated with anti-PKR antibody and resolved in a 12.5% polyacrylamide–SDS gel. (A) The immunoprecipitated radiolabeled phosphoproteins were detected with a Fuji Phosphorimager. Positions of PKR and of molecular weight markers are indicated. (B) Relative amounts of the γ-^{32}P label in the PKR region of the gel. Labeling is expressed relative to that in the products of reactions conducted in the absence of RNA (None). The results are means of two experiments; error bars are equal to half the range.

translation by preventing the activation of PKR. Similarly, delta 13/3 RNA was able to rescue protein synthesis in the reticulocyte lysate from inhibition by dsRNA (Fig. 5B). Both of these ssRNAs restored translation to about 60% of the control level obtained in the absence of dsRNA, but relative to VA RNA approximately 10-fold greater concentrations of delta 13/3 RNA were required. When the sizes of the two species are taken into account (483 nucleotides for delta 13/3 and 160 nucleotides for VA RNA), VA RNA is only three- to fourfold more efficient than delta RNA in rescuing translation. Controls using ^{32}P-labeled delta 13/3 RNA demonstrated that this species is stable in reticulocyte lysates under these conditions during the incubation times used. Thus, delta RNA displays opposite properties in the two assay systems tested. In direct kinase assays it activated PKR at moderate concentrations and continued to activate at high concentrations. In the reticulocyte translation assay it did not inhibit protein synthesis at any concentration tested, suggesting that it does not activate the kinase present in reticulocyte lysates, whereas it relieved inhibition at high concentration, implying that it interferes with kinase activation.

To test these inferences directly, we developed an assay to measure the activation of PKR in reticulocyte lysate translation reactions. Phosphoproteins were labeled by supplementing the reactions with [γ-^{32}P]ATP, and PKR phosphorylation was detected by immunoprecipitation with polyclonal antiserum followed by gel electrophoresis (Fig. 6A). PKR was strongly labeled in the presence of reovirus dsRNA, as expected, whereas no band was discernible in this position of the gel in the absence of added RNA. When added alone, delta 13/3 RNA failed to give rise to a detectable PKR band, and it suppressed PKR labeling when added together with reovirus dsRNA. Several other unidentified phosphoproteins were also precipitated by the antibody, but their labeling did not display an RNA dependence. Quantitation of two such experiments confirmed that delta RNA did not increase PKR phosphorylation to any measurable extent, whereas reovirus dsRNA elicited a 10-fold increase that was reduced to about 3-fold when both RNAs were present simultaneously (Fig. 6B). These results substantiate the long-held assumption that PKR phosphorylation correlates with the inhibition of translation in the reticulocyte lysate and corroborate the conclusion that, in this system, delta RNA can inhibit PKR but not activate it.

DISCUSSION

We have shown here that rigorously purified delta agent genomic, subgenomic, and antigenomic RNAs all contain one or more subdomains which activate the kinase activity of PKR in vitro without inhibiting translation in rabbit reticulocyte lysates. Although the nuclease sensitivity and dsRNA content of the RNA tested were not examined, preparations of potato spindle tuber viroid RNA were recently reported to activate PKR (12). It would be premature to speculate that this is a general characteristic of such agents, but it is intriguing to consider whether host factors could recognize the sites we have discovered in delta agent RNA which activate PKR. While such experiments will require additional research, it would be interesting to know whether PKR, or some other cellular proteins which are members of the family of dsRNA-binding proteins (17, 48), exerts any effects on the life cycle of the delta agent in liver cells.

The major significance of our findings here, however, must certainly be the paradox in which rigorously purified ssRNAs, defined as such by a number of assays, act like dsRNAs when assayed for their ability to activate purified PKR but like VA RNA in the cell-free translation assay. The delta 13/3 genomic subdomain lacks stretches of dsRNA even 20 bp long. When freed of possible dsRNA contaminants, delta RNA activates the kinase activity in vitro with an efficiency which is 10 to 20% of that observed with reovirus or other authentic dsRNAs (Fig. 2 and 3). In contrast to these latter species, however, the PKR-activating capacity of delta RNA is sensitive to ssRNA-specific nuclease treatment but resistant to dsRNA-specific digestion. Furthermore, as shown in Fig. 5 and 6, behavior characteristic of VA RNA, structured ssRNA, is displayed by delta RNA in assays of PKR activation and protein synthesis initiation in a complex translation system.

The paradoxical interaction between delta RNA and PKR is open to several explanations. There could, for example, be systematic differences between the human kinase and rabbit reticulocyte translation systems, depending upon species specificity or variations in ionic or other conditions. Furthermore, the effective form of PKR in the reticulocyte lysate is probably ribosome associated (13). In addition, RNA-binding proteins in the lysate could mask the region of delta RNA normally capable of PKR activation.

An explanation which we favor for delta RNA's paradoxical behavior is based on the ability of the conserved domain of delta genomic RNA to adopt two conformations, one resembling the "collapsed rod" originally predicted by the sequence of Wang et al. (50) and a cruciform structure in which an

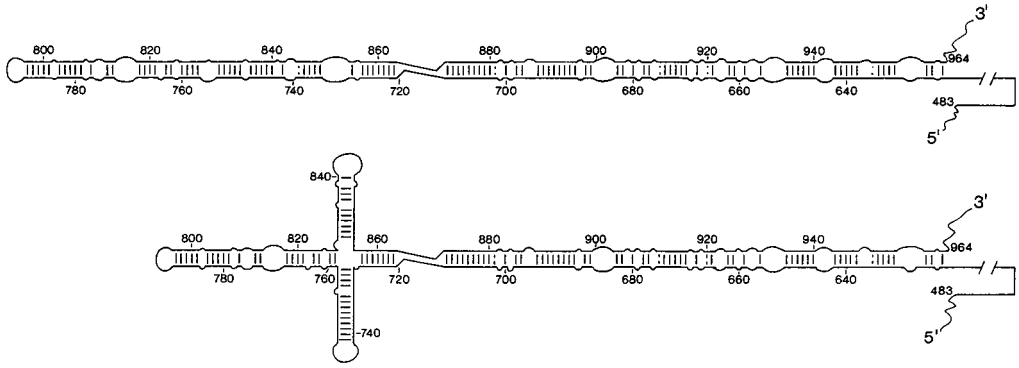

FIG. 7. Conformations of the conserved region of delta agent RNA. In the upper diagram, the collapsed-rod structure of delta agent RNA is shown as proposed by Wang et al. (50) and numbered according to these authors' system. The element of local tertiary structure is shown as a constriction to the right of residues 720 and 860. In the lower diagram, the same region of delta agent RNA is shown in its proposed alternate conformation (6), which includes the cruciform element mentioned in the text. Proposed Watson-Crick base pairs are indicated by solid vertical lines, while G · U pairs are indicated by vertical dashed lines. Regions containing one or more unpaired bases are depicted "looped out" from the overall structure. The delta 13/3 RNA transcript depicted here (2) has vector sequences of 10 to 15 bases at both its 5′ end and its 3′ end (wavy lines) and covers bases 483 to 964 of the genomic sequence. Most of the region from 483 to 620 is omitted because its counterpart from the "top" strand is absent, and thus its pairing to produce the structure in this region proposed by Wang et al. (50) is not possible.

alternate folding takes place on opposite sides of the circular delta RNA's structure (6). These two structures for delta 13/3 RNA are shown in Fig. 7. The ability of delta RNA to adopt two conformations, both of which were detected in RNA preparations with partial RNase T_1 digestion (6, 40a), suggests that both might remain functional. The delta RNA conformation normally favored in purified preparations, the collapsed rod, might thus be viewed as responsible for interacting with, and activating, purified human PKR activity. When the additional proteins and factors of the reticulocyte lysate are introduced, on the other hand, some could bind to delta RNA and stabilize the alternative cruciform structure, which (according to this model) would be unable to activate the PKR activity, thus explaining the results in Fig. 5A. The remainder of the delta RNA molecule remains a highly structured ssRNA, however, and could still, in this model, interact with the kinase at higher concentrations in the same way as for VA RNA, explaining the results in Fig. 5B. The direct protein kinase assays in Fig. 6 fully corroborate these expectations.

The structural-transition model described above implies that discrimination between activators and inhibitors of PKR may be more delicately poised than previously thought. Such a view might help to explain claims that TAR RNA (29, 45), certain mRNAs (21, 49), and even VA RNA (15) can sometimes activate PKR, although possible dsRNA contamination was not excluded and could also account for some or all of the observations reported. This view of the interactions between PKR and its ligands is compatible with the close relationship, if not identity, between the PKR sites that bind VA RNA and dsRNA (16, 17, 25).

While further research will be needed to pinpoint any region of delta RNA which could be responsible for binding of delta RNA to PKR, further studies (8) suggest strongly that unique domains of delta RNA may be involved, in parallel to the recent findings of Clarke and Mathews (9) defining a specific PKR-binding subdomain of VA RNA by RNA footprinting. It may be relevant, also, that delta 13/3 RNA contains a characteristic structural element which can undergo UV-induced RNA-RNA cross-linking (2, 44). Intriguingly, the cross-link site closely neighbors the cruciform structure mentioned above (Fig. 6), although without overlapping it or restricting its formation. Mapping of the RNA protected by PKR protein in delta 13/3 RNA should allow us to discover whether this unusual region, which contains tertiary as well as secondary structure, is involved in the recognition of PKR by delta agent RNA.

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