In Vitro Analysis of Virus-Associated RNA I (VAI RNA): Inhibition of the Double-Stranded RNA-Activated Protein Kinase PKR by 
VAI RNA Mutants Correlates with the In Vivo Phenotype and the Structural Integrity of the Central Domain

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Adenoviruses use the virus-encoded virus-associated RNA (VAI RNA) as a defense against cellular antiviral response by blocking the activation of the interferon-induced, double-stranded RNA-activated protein kinase PKR. The structure of VAI RNA consists of two long, imperfectly base-paired duplex regions connected by a complex short stem-loop at the center, referred to as the central domain. By using a series of adenovirus mutants with linker-scan mutations in the VAI RNA gene, we recently showed that the critical elements required for function in the VAI RNA molecule are in the central domain and that these same elements of the central domain are also involved in binding to PKR. In virus-infected cells, VAI RNA interacts with latent kinase, which is bound to ribosomes; this interaction takes place in a complex milieu. To more fully understand the relationship between structure and function and to determine whether the in vivo phenotype of these mutants can be reproduced in vitro, we have now analyzed these mutant VAI alleles for their ability to block the activation of a partially purified PKR from HeLa cells. We have also derived the structure of these mutants experimentally and correlated the structure with function. Without exception, when the structure of the short stem-loop of the central domain was perturbed, the mutants failed to inhibit PKR. Structural disruptions elsewhere in the central domain or in the long duplex regions of the molecule were not deleterious for in vitro function. Thus, these results support our previous findings and underscore the importance of the elements present in the central domain of the VAI RNA for its function. Our results also suggest that the interaction between PKR and VAI RNA involves a precise secondary (and tertiary) structure in the central domain. It has been suggested that VAI RNA does not activate PKR in virus-infected cells because of mismatches in the imperfectly base-paired long duplex regions. We constructed mutant VAI genes in which the imperfectly base-paired duplex regions were converted to perfectly base-paired regions and assayed in vitro for the activation of PKR. As with the wild-type VAI RNA, these mutants failed to activate PKR in vitro, while they were able to block the activation of PKR better than did the wild type. These results suggest that the failure of VAI RNA to activate PKR is not the result of mismatches in the long duplex regions. Thus, the role of the long duplex regions of VAI RNA is probably to hold the nucleotide sequences of the central domain in a conformation optimal for function.

One way that cells defend against virus infection is by activation of the interferon-induced, double-stranded RNA (dsRNA)-activated protein kinase designated PKR (also known as p68 kinase, eIF-2 alpha kinase, and dsRNA-activated inhibitor), which, when activated, phosphorylates the alpha subunit of the translation initiation factor eIF-2. The phosphorylated eIF-2 does not recycle and is sequestered in the cell, leading to cessation of protein synthesis (reviewed in references 19, 32, and 47). Viruses have evolved various strategies to counteract this cellular antiviral response. The strategy most extensively studied is that used by adenoviruses.

They encode two RNA polymerase III-directed small RNAs, designated virus-associated RNAs I and II (VAI and VAI RNAs), that accumulate to high levels at late stages of infection. In virus infections, VAI RNA is obligatory for efficient translation of viral and cellular mRNAs at late times (51, 56). It binds to and blocks the activation of PKR produced by the cell, thereby enabling protein synthesis to proceed at normal levels (1, 23, 26, 41, 49, 52; reviewed in references 33, 50, and 55). The structure of VAI RNA consists of two long, imperfectly base-paired stems of 20 to 22 bp joined at the center by a domain that is structurally complex and that contains two loops and a short stem-loop (12, 38). This domain is referred to as the central domain. Although initially it was thought that the long duplex regions might be important in blocking the activation of the PKR, recent mutational analysis showed that it is the central domain that is critical for function (12, 38). These results are consistent with VAI RNA-PKR interaction studies in vivo. The VAI mutants with mutations that alter the integrity of the central domain did not bind to PKR in vivo, whereas mutants with mutations that
disrupt other regions of the molecule bound to PKR efficiently (15).

The cellular target of VAI RNA, PKR, is a ribosome-bound enzyme which is present in a latent state. In an infected cell, dsRNA, probably produced both by the cell and by the virus, activates PKR as the infection progresses from early to late stage (30). Accumulation of the VAI RNA most likely precedes the accumulation of dsRNA, and upon accumulation, VAI RNA binds to and prevents the activation of the kinase. All of these events occur in a complex milieu in the context of cellular translation apparatus. Indeed, it has been suggested that the activation of the PKR (and perhaps the inhibition by VAI RNA) may be localized events in the messages that are selectively inhibited by PKR (41). In addition, there is evidence that the VAI RNA binds to viral mRNAs in vitro (31). We were therefore interested in determining whether the phenotype of the mutant VAI RNAs that we analyzed previously in the context of virus could be reproduced in vitro with a partially purified PKR preparation and whether a correlation exists between the in vitro phenotype, the previously reported in vivo studies (12, 15), and the structural alterations in these mutant RNAs as a consequence of mutations. In this report, we describe the properties of the mutant RNAs with regard to their ability to block the activation of partially purified PKR in vitro, present experimentally derived secondary structures of the mutant RNAs, and show that there exists an excellent correlation between the secondary structures and the inhibition of PKR by these mutants. It has been suggested that the VAI RNA does not activate the PKR in virus-infected cells because of mismatches in the imperfectly base-paired long duplex regions (26, 41). We constructed VAI mutants in which the two imperfectly base-paired long duplex regions were converted to perfectly base-paired duplex regions and analyzed in vitro. These mutants also failed to activate PKR in vitro. Thus, it seems that the failure of VAI RNA to activate PKR may not be the result of mismatches in the duplex regions, and the function of the two long duplex regions of the VAI RNA may be to hold the sequences of the central domain in a conformation that is optimal for function.

**MATERIALS AND METHODS**

**Cells, viruses, and plasmids.** HeLa cells suspension cultures were grown in S-MEM with 7% donor calf serum. Mutant VAI RNA genes used in this study have been reported earlier (4, 5, 12). Substitution mutants sub707, sub709, sub741, sub743, and sub745 through sub749 are linker-scan substitution mutants in which HindIII linker sequences are substituted in the following locations: sub707, between nucleotides 18 and 27 (numbering based on the G start site); sub709, between nucleotides 43 and 55; sub741, between nucleotides 76 and 90; sub743, between nucleotides 90 and 105; sub745, between nucleotides 105 and 117; sub746, between nucleotides 116 and 126; sub747, between nucleotides 122 and 134; sub748, between nucleotides 134 and 143; and sub749, between nucleotides 140 and 150. Mutant in708 is an insertion mutant in which a HindIII linker sequence is inserted after nucleotide 27. Mutant VAI-CB was constructed by substituting DNA sequences between 5'p451 (+61) and BsrEI sites (+99) with a duplex oligonucleotide, the sequence of which is 5'CGAACCCCCACCCCCG GTGTCGCCCATGATACCCCAGC3'. VAI-BR was constructed by substituting DNA sequences between BsrEI (+99) and EcoRI (+160) sites with a duplex oligonucleotide with the sequence 5'-GTTACCCCGCCGCTTCAGACCCAGGTG TGGGACACCAAGCACCAGGAAAGAGTCCCTTTTTG3'. Mutant VAI-CR was constructed by recombining appropriate DNA fragments from VAI-CB and VAI-BR plasmids. Mutations in the VAI genes were confirmed by DNA sequence analysis.

**Purification of PKR.** PKR was purified by the procedure of Samuel and coworkers, with modifications (3, 48). The cytoplasmic extracts were prepared from HeLa suspension cultures (10). The postnuclear fraction was centrifuged at 13,000 rpm in a Sorvall RC5B SS34 rotor for 20 min at 4°C. The supernatant (fraction I) was centrifuged at 100,000 × g for 2.5 h at 4°C to pellet ribosomes. The ribosomal pellet was suspended in buffer X (20 mM Tris-HCl [pH 8.0], 100 mM KCl, 5 mM MgCl2, 5 mM 2-mercaptoethanol, 50 μM EDTA, 5% glycerol) containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg of leupeptin per ml, and 500 U of aprotinin per ml and homogenized in a Dounce homogenizer with 15 to 20 strokes. Potassium chloride was then added to the homogenate to a final concentration of 0.7 M, and the volume was adjusted with buffer X to reach an optical density at 280 nm of 150 to 175 U/ml. The homogenate was kept on ice for 90 min and then centrifuged at 100,000 × g for 5 h. The supernatant (fraction II) was subjected to 40 to 60% ammonium sulfate precipitation. The precipitate obtained was dissolved in buffer Y (20 mM Tris-HCl [pH 8.0], 150 mM KCl, 1 mM EDTA, 5 mM 2-mercaptoethanol, 10% glycerol) containing 1 mM PMSF, 500 U of aprotinin per ml, and 1 μg of leupeptin per ml and dialyzed thoroughly against buffer Y containing 1 mM PMSF. The dialyse (fraction III) was centrifuged at 14,000 rpm for 30 min at 4°C and passed through a DEAE-Sephaloc column (1.6 by 20 cm) equilibrated with buffer Y. The column was washed with 2 column volumes of buffer Y, and flowthrough and wash fractions were pooled and concentrated in an Amicon ultrafiltration unit using a YM10 filter. The concentrate was dialyzed against Mono-S buffer (20 mM Tris-HCl [pH 6.7], 50 mM KCl, 1 mM EDTA, 5 mM 2-mercaptoethanol, 10% glycerol, 1 mM PMSF). The dialyzed fraction (fraction IV) was centrifuged by centrifugation at 16,000 rpm for 20 min at 4°C and then passed through a fast protein liquid chromatography Mono-S column (HR10/30) equilibrated with Mono-S buffer. The column was washed with 3 column volumes, and the adsorbed proteins were eluted with a 0.05 to 1.0 M KCl gradient in Mono-S buffer. The column fractions were analyzed for autophosphorylation activity. Fractions eluted between 0.4 and 0.5 M KCl containing PKR activity were pooled and dialyzed against hexylamine-agarose (aminohexyl-agarose) column buffer (HA buffer; 20 mM Tris-HCl [pH 7.5], 20 mM KCl, 5 mM 2-mercaptoethanol, 5 mM MgCl2, 10% glycerol, 1 mM PMSF). The dialyse (fraction V) was centrifuged at 16,000 rpm for 20 min at 4°C and passed through a hexylamine-agarose column (0.5 by 10 cm) equilibrated with HA buffer. The column was washed with 3 column volumes with HA buffer, and the adsorbed proteins were eluted with a salt gradient of 0.02 to 1.0 M KCl in HA buffer, and the fractions were assayed for PKR activity. Fractions eluted at 40 to 150 mM KCl were pooled and dialyzed against HA buffer, bovine serum albumin was added to a final concentration of 100 μg/ml, and the preparation was stored at −70°C until use.

**Autophosphorylation of PKR.** Autophosphorylation of PKR was carried out in a 50-μl reaction mixture consisting of 10 μl of hexylamine-agarose column fraction, 20 mM Tris-HCl (pH 7.5), 2 mM 2-mercaptoethanol, 4 mM magnesium acetate, 10 μCi of [γ-32P]ATP (specific activity, 30 Ci/mmol; NEN) and concentrations of reovirus RNA as dsRNA with concentrations indicated in the figure legends. The reaction mixture was incubated at 30°C for 30 min, the reaction was terminated by the addition of 50 μl of 2× SDS sample buffer (0.125 M Tris-HCl [pH 6.8], 4% SDS, 20% glycerol, 10% 2-mercapto-
The test blocked the activation of DNA using a mutant plasmid containing RNA samples as a short duplex region, and by Pe'ery et al. (45), respectively. Data discussed in this report are based on the secondary structure model presented in panel A. A to D are looped regions.

FIG. 1. Experimentally derived secondary structure of the VAI RNA. (A) Secondary structure reported by us and others in which the sequences of the central domain are presented as a short stem-loop (12, 38). (B and C) Two alternate secondary structures for the central domain reported by Ma and Mathews (28) and by Pe'ery et al. (45), respectively. Data discussed in this report are based on the secondary structure model presented in panel A. A to D are looped regions.

RESULTS

The secondary structure of VAI RNA as determined by us and others consists of two long duplex regions, stems I and III, connected at the center by a short duplex region, stem II (Fig. 1A). Stem I consists of a duplex in which nucleotides 1 to 22 are base paired to nucleotides 134 to 155 (numbers here represent the positions of the nucleotides from the 5' end with a G start site [12]). Stem II consists of a short duplex in which nucleotides 31 to 35 are base paired to nucleotides 128 to 132. In stem III, the longest of these, nucleotides 37 to 62, are base paired with nucleotides 71 to 94. As a result, nucleotides 63 to 71 exist as a loop (loop B). The most important part of the molecule is the central part, referred to as the central domain, the structure of which is complex and poorly defined. This part of the molecule contains a small loop in the 5' side (loop A, nucleotides 23 to 30) and a short stem-loop in the 3' side in which nucleotides 95 to 129 fold such that nucleotides 99 to 102 pair with nucleotides 118 to 121. As a result, nucleotides 103 to 117 exist as a large loop (loop C). In this model, nucleotides 94 to 99 and nucleotides 121 to 129 do not pair and exist as single-stranded regions (nucleotides 121 to 129 represent a minor loop, loop D). Because there is very little base pairing in this region, it has been difficult to determine the precise secondary structure for this region. Two alternate secondary structures have been proposed for this region by Mathews and coworkers (Figs. 1B and C; references 28 and 45, respectively); the structure shown in Fig. 1B is more recent (28). Further mutational analysis of the central domain, as well
as determination of the three-dimensional structure of the VAI RNA molecule, will be necessary to define the structure for this region accurately. In this report, we discuss our results based on the secondary structure model proposed previously by us and others and shown in Fig. 1A. As discussed below, interpretation of our results will not be affected by any minor changes in structure for this region.

We previously constructed and characterized a series of adenovirus mutants in which the VAI gene was mutagenized by deletion, insertion, and linker-scanning mutagenesis (5, 12). These mutants were analyzed for growth yield, for protein synthesis, and in some cases for the phosphorylation of PKR.

These and other results (38) led to the suggest that the elements present in the central domain are critical for the function of VAI RNA. Subsequently, several of these mutants were analyzed for binding with PKR in vivo and in vitro (15). This study revealed that the functional domain and the PKR binding domains are indistinguishable. In the present study, we have analyzed 10 of these mutants in vitro with a partially purified PKR. The locations of these substitution mutants on the secondary structure of VAI RNA are shown in Fig. 2.

**FIG. 2.** Locations of the insertion and linker-scan mutations in the secondary structure of the WT VAI RNA. Insertion mutation is shown by a triangle. Linker-scan mutations are boxed. Further details of these mutants can be obtained from reference 12.

In vitro inhibition of partially purified PKR by the WT VAI RNA. We purified PKR from cytoplasmic extracts prepared from HeLa cell suspension cultures. Ribosomes were pelleted from the cytoplasmic extracts and washed with 1.0 M ammonium chloride, and the ribosome salt wash was fractionated by ammonium sulfate fractionation and passage through DEAE-Sephacel, Mono-S, and hexylamine-agarose columns as detailed in Materials and Methods. At each stage, the PKR activity was monitored in an autophosphorylation assay using reovirus RNA as the activator. The PKR activity could be detected only after the DEAE-Sephacel step, presumably because of an inhibitor that copurifies with PKR until this step. Figure 3A shows an SDS-polycrylamide gel electrophoresis (PAGE) analysis of selected fractions of the hexylamine-agarose column stained with silver. The PKR activity of the corresponding fractions is shown in Fig. 3B. It is clear that the protein at the hexylamine-agarose column stage is considerably purified, and fractions which show PKR activity contain not more than five polypeptides (Fig. 3A). The position of the phosphorylated polypeptide (Fig. 3B) coincides with the major polypeptide at the 68-kDa position (Fig. 3A). Another band that migrated at approximately the 50-kDa position could be a degradation product of PKR (shown by an open triangle in Fig. 3A) (13). The active fractions from this column (abbreviated as HA fractions hereafter) were pooled and used for the enzyme activity assays described here. The PKR has been shown to respond to dsRNA with a bell-shaped curve, activation of the enzyme with a low concentration of dsRNA, and inhibition at high concentrations (11, 20). To ensure that our enzyme preparation shows this property and to determine the dsRNA concentrations required for optimum activation, autophosphorylation was carried out with increasing concentrations of dsRNA. The enzyme showed the activity profile typical of the PKR, with activation between 0.02 to 0.2 μg/ml and inhibition beginning at 2.0 μg/ml (Fig. 3C). This and other PKR preparations of similar quality were used in the VAI RNA-mediated block assays reported here. Each PKR preparation was tested for its response to dsRNA concentrations before use.

An aliquot of the pooled HA fractions was preincubated with various concentrations of in vitro-transcribed, gel-purified WT VAI RNA for 30 min, and then dsRNA was added along with [γ-32P]ATP. The 32P-labeled protein was then analyzed by SDS-PAGE. We also compared the PKR obtained from the Mono-S column fractions in the inhibition assays using the WT VAI RNA. With the HA fractions, activation of PKR was completely inhibited at 500 μg of VAI RNA per ml. However, we consistently observed that the Mono-S column fractions required a 10-fold higher concentration of VAI RNA (5 μg/ml) for complete inhibition (data not shown). This value is comparable to that observed by other investigators (28, 45). The reason for the difference in the sensitivity of the two enzyme preparations for the VAI RNA is not clear. It is possible that the impurities present in the Mono-S enzyme preparations nonspecifically bind to VAI RNA and reduce its effective concentrations. Alternatively, the Mono-S column fractions may contain factors that inhibit the autophosphorylation. This aspect was not investigated further.

**Phenotypically WT VAI mutants sub707, in708, and sub749 block the activation of PKR by the adenovirus VAI RNA in vitro.** Mutants sub707 and in708 contain HindIII linker sequences substituted in the loop A region of the central domain, whereas sub749 contains mutations in the 3' side of stem I (Fig. 2). Earlier, we showed that in the context of the viral chromosome, these three mutants function efficiently. These mutants were transcribed in vitro with T7 RNA polymerase and gel purified. Various concentrations of these RNAs were then assayed in vitro for the inhibition of PKR derived from the HA fractions.
Data from one such experiment are shown in Fig. 4. Each mutant was assayed at least four times, and control experiments with WT VAI RNA were always carried out in parallel. Phosphorylation of PKR in each experiment was quantitated by scanning the autoradiograms with a laser densitometer. Average values with error bars for each mutant are shown in Fig. 5. Mutants sub707, in708, and sub749 inhibited the PKR as efficiently as did the WT. In all cases, significant inhibition was observed at 0.1 μg/ml and the enzyme was inhibited completely at 0.5 μg/ml.

In mutants sub707, in708, and sub749, the short stem-loop of the central domain is intact. An important consequence of mutations in an RNA molecule is the perturbations in their secondary structures. Because the function of the VAI RNA is dependent on the structure, it was important to determine the structural changes that these RNAs may have undergone as a result of nucleotide substitutions. The in vitro-transcribed RNAs were labeled at their 3' ends with [32P]pCp (44) and subjected to low concentrations of single-strand-specific RNase T1 (cleaves after G), U2 (cleaves after A), or BC (cleaves after pyrimidines), such that the majority of the molecules were not cleaved and the remainder of the molecules cleaved only once (12, 38). The cleavage products were resolved on 14% DNA-sequencing gels along with a ladder marker developed by subjecting the 3'-end-labeled RNA (the same as the mutant) to limited alkaline hydrolysis. The RNA sequences were folded by using a computer-assisted folding program (21, 58) such that the unpaired bases identified in the RNase sensitivity experiments were not allowed to base pair. The derived secondary structures of the mutant RNAs and the RNase cleavage sites in the RNAs for sub707, in708, and sub749 are shown in Fig. 6. An important feature of all three RNAs is that the central short stem-loop structure is intact and all alterations are below stem II. Alterations of secondary structures in sub707 and in708 reside in the loop A region. For example, mutations in sub707 lead to enlargement of loop A from its normal size of 8 nucleotides to 11 nucleotides. As a result, nucleotides 132 to 136 also do not base pair. In addition, the size of stem I is also somewhat shortened. In sub708, the alterations are confined to loop A, the size of which is changed from 8 nucleotides to 16 nucleotides. In sub749, unlike the WT, the 5' and the 3' terminal sequences do not base pair and exist as two large loops (Fig. 4). In summary, these results indicate that any structural alterations below stem II do not affect the ability of the RNAs to inhibit PKR in vitro.

The VAI RNA sequence in sub709, sub741, sub743, and sub745 through sub748 do not block autophosphorylation of PKR in vitro. Mutations in sub709 and sub741 contain alterations in the 5' and 3' sides, respectively, of the middle portion of the apical stem-loop (stem III) (Fig. 2). These sequences are critical for the maintenance of the apical stem-loop. In sub743, sub745, sub746, and sub747, mutations reside in the short stem-loop of the central domain. Mutant sub748 contains mutations in a region that is proximal to the short stem-loop of the central domain. As discussed below, all of these mutations are expected to destroy the central domain. Adenovirus mutants which harbor these mutations were shown previously to be defective (4, 12). These mutant RNAs were assayed for the inhibition of PKR activity in vitro with HA fractions as described above. sub709, sub741, sub743, and sub745 to sub747 showed little or no inhibition of kinase activity at 100 ng of RNA per ml and only a modest inhibition at 200 ng/ml. More than 80% inhibition was observed for the WT RNA at 100 ng/ml and inhibition increased to 90% at 200 ng/ml. At 500 ng/ml, inhibition was complete. Thus, at low concentrations, the mutants that failed to function in our virus experiments failed to block the activation of PKR in vitro. As the RNA concentrations increased to 500 ng/ml, some of the RNAs showed a moderate inhibition (sub743, sub745, and sub746). When the RNA concentration was increased to 1.6 μg/ml, nearly total inhibition of kinase activity was observed for all of the mutants except sub709 and sub741. These mutants required much higher concentrations of RNA for the inhibition. Results for mutant sub748 are interesting. Although this RNA did not
These two mutations are similar to another point mutation in the sub745 region, suggesting that substitutions at this location are important for the activation of PKR activity. However, the structural alterations resulting from these substitutions are not as striking as those observed in the sub745 region (Fig. 2). These results indicate that at very high concentrations, the apical stem-loop alone may be able to inhibit the enzyme activity. In sub743, sub745, sub746, and sub747, the nucleotides that are substituted in the short stem-loop region of the central domain (Fig. 2). These mutants undergo different structural alterations depending on the location and the nature of the bases that are substituted (Fig. 6). However, none of these mutants retain the central short stem-loop structure of the central domain. It is noteworthy that most of the nucleotides are contained within the central region of sub745 RNA, and the apical stem-loop is intact in these mutants.

The structural alterations in mutant sub745 are particularly noteworthy. This RNA contains two smaller loops below stem II, roughly in the same positions as those of the mutant sub707. One of these loops (between nucleotides 22 and 37) also resembles the loop between nucleotides 22 and 38 of sub708. The short stem-loop of the central domain of the WT RNA (Fig. 1B) somewhat resembles the large loop present in the 3' side of sub745, between nucleotides 92 and 119. What is more important is that the position and the size of the large loop in the 3' side of sub745 have striking similarities to the large loop present in the same location in the alternate WT structure described by Ma and Mathews (28) (Fig. 1B). In both cases, the size of this loop is about 26 bases. Thus, although the central portion of sub745 RNA has features that in some respects...
FIG. 5. Bar diagrams showing the inhibition of PKR by WT and VAI mutant RNAs. The VAI genes were transcribed in vitro, gel purified, and assayed in in vitro PKR autophosphorylation assays as detailed in Materials and Methods. Bands corresponding to the phosphorylated PKR from the autoradiograms were quantitated by densitometer scanning. The experiments were repeated at least four times, and the average values and error bars are shown. Values shown on the y axis correspond to the percent activity of PKR remaining compared with control experiments in which autophosphorylation was carried out without VAI RNA. Control values were taken as 100%.

resemble the central domain of the WT RNA, sub745 fails to function in vivo as well as in vitro at low concentrations of RNA. These results underscore the importance of the elements present in the short stem-loop of the central domain in the function of the VAI RNA.

At 100-ng/ml concentrations, sub748 RNA can inhibit enzyme activity by about 25%, which is better than the value for other defective mutants but still not close to that of WT RNA, which shows more than 80% inhibition (Fig. 5). However, at 500-ng/ml concentrations, a nearly complete inhibition of enzyme activity can be observed. This property is not reflected in its structure. This RNA shows a much longer apical stem-loop (Fig. 6). However, the folding of the sequences in the other part of the molecule has little resemblance to the central
domain of the WT RNA. It is conceivable that at higher concentrations of RNA, the extended apical stem-loop facilitates the inhibition process. Nonetheless, at low concentrations, the in vivo phenotype of this RNA is in agreement with its in vivo phenotype.

VAI RNA mutants in which the imperfectly base-paired duplex region is changed to perfectly base-paired regions do not activate PKR in vitro. We next examined the role of the G-U pairs and mismatches in the imperfectly base-paired duplex regions in the function of the VAI RNA. We considered this study important for two reasons. First, it has been thought that the VAI RNA would not activate the kinase because of the mismatches and G-U pairs in the long duplex regions (26, 41), although one study did show that the WT VAI RNA can activate PKR in vitro (14). Each of the two long duplex regions (stems I and III) contain five G-U base pairs and two mismatches (Fig. 7). In addition, stem III also contains two stacked bases (nucleotides 42 and 43). Second, we wanted to determine whether these mismatches have any role in blocking the activation of PKR. While the precise mechanism by which VAI RNA functions is not clear, recent evidence suggests that it would bind to PKR in the same region as that of dsRNA and somehow interfere in the dsRNA-mediated activation (16, 24, 36, 43).

To determine whether the imperfectly base-paired long dsRNA segments of the VAI RNA have any biological role, we constructed three VAI RNA mutants. The first mutant, VAI-CB, was constructed by replacing the DNA segment from Csp45I (+61) and BstEII (+99) sites with a chemically synthesized double-stranded oligonucleotide with appropriate nucleotide sequence changes (Fig. 7). Two bases were added to stem III between residues 91 and 92 such that these two bases would pair with the two stacked bases, A and U at 41 and 42, respectively. Changing the U residue at 94 (or 92 of the WT numbering) generates a pentanucleotide sequence, ACCCA (boxed in Fig. 7), that is also present between nucleotides 120 and 126 (or 118 and 124 based on WT numbering). This sequence is conserved in the VAI genes of different serotypes (28). When this gene is transcribed, it should produce an RNA with a perfectly base-paired apical stem-loop. The second mutant, VAI-BR, was constructed by replacing the DNA sequences of the VAI RNA gene between BstEII and EcoRI (+161) sites with a chemically synthesized double-stranded oligonucleotide with appropriate base changes. RNA transcribed by this gene would contain perfectly base-paired stem I. The last mutant, VAI-CR, is a combination of VAI-CB and VAI-BR and contains perfectly base-paired stems I and III.

These mutants were first tested for their ability to activate PKR in vitro with HA fractions. The in vitro phosphorylation assays were carried out by replacing reovirus RNA with various concentrations of the above-described mutant VAI RNAs; the radiolabeled polypeptides were analyzed by SDS-PAGE. Control autophosphorylation assays with various concentrations of reovirus RNA were also carried out in a parallel fashion. Despite repeated efforts, we did not detect the activation of the PKR with WT VAI RNA or with the three mutants, VAI-CB, VAI-BR, and VAI-CR. As expected, only reovirus RNA activated PKR in vitro (data not shown). We conclude that the long duplex regions of VAI RNA are not capable of activating PKR even when the mismatches in the duplex regions are converted to perfectly base-paired regions.

Mutants VAI-CB, VAI-BR, and VAI CR can block activation of PKR in vitro as efficiently as or better than the WT VAI RNA. To determine whether these mutant RNAs are capable of blocking the activation of the kinase with the same efficiency as the WT VAI RNA molecule, the mutant genes were transcribed in vitro, and the RNAs were purified and then assayed for inhibition of the autophosphorylation of PKR with
FIG. 6—Continued.
HA fractions (Fig. 8). Average values obtained for these mutants from three independent experiments with error bars are shown in Fig. 9. Mutant VAI-CB inhibited the PKR as efficiently as the WT VAI RNA did; at an RNA concentration of 100 ng/ml, the VAI-CB inhibited the PKR more than 80%, which is comparable to the value for the WT VAI. Interestingly, the VAI-BR and VAI-CR RNAs were more active than the WT RNA. VAI-BR inhibited the kinase activity two- to threefold more efficiently than the WT RNA. VAI-CR was even more active than VAI-BR. At an RNA concentration of 40 ng/ml, VAI-CR inhibited PKR almost completely, whereas only about 50% inhibition was observed for the WT and VAI-CB RNAs. These results suggest that the perfect base pairing of the duplex regions promotes the inhibitory properties of the VAI RNAs.

Central domain in VAI-CB, VAI-BR, and VAI-CR is intact. Our strategy to construct the VAI mutants with perfectly base-paired duplex regions involved multiple-base substitutions (Fig. 7). This may perturb the native secondary structures of the VAI RNAs. Because the ability of the VAI RNA to block the kinase is strictly dependent on the secondary structures of the RNAs, we determined the secondary structures of these three RNAs as described above. The single-strand-specific RNase cleavage pattern of the RNAs is shown in Fig. 10, and the cleavage map is shown in Fig. 7. Overall, the cleavage pattern of these three RNAs is comparable to that of the WT RNA, indicating that there is no change in the structures of these RNAs. There are a few minor differences, however. In VAI-BR, in which stem I is a perfect duplex structure, the nucleotides in the central domain are less susceptible to RNase attack. For example, the cleavages that are found in WT RNA at nucleotides 131, 136, and 138 are not detected. In addition, a number of strong cleavages in the short stem-loop that are characteristic of the WT RNA are changed to weak cleavages or are absent. In VAI-CB, in which the apical stem-loop is a perfect duplex structure, the loop (loop B) is not cleaved efficiently. Loop A also appears to be less susceptible to RNases. As expected, in VAI-CR, in which both long duplex regions are made of perfect base pairs, the mutational effects of VAI-CB and VAI-BR are combined. In this RNA, most of the cleavages in the central domain are weak and the majority of the cleavages found in stem I and loop B of the WT RNA are absent (Fig. 10). The cleavage map of VAI-CR, which is a combination of VAI-CB and VAI-BR, is not shown. Thus, it seems that when the VAI RNA contains perfectly base-paired stems, the molecule is more compact and the single-stranded regions are probably less exposed. This may also have some effect on the tertiary structures of these
FIG. 8. In vitro inhibition of PKR activity by VAI-CB, VAI-BR, and VAI-CR. WT and mutant VAI genes were transcribed in vitro, purified on native polyacrylamide gels, and assayed for inhibition of autophosphorylation of PKR derived from hexylamine-agarose column fractions with various concentrations of the VAI RNAs. The phosphorylated polypeptides were analyzed by SDS-PAGE. The phosphorylated 68-kDa polypeptide is shown by an arrow. Sample C corresponds to PKR activity in the absence of VAI RNA. Reo, reovirus.

RNAs. These results also suggest that the central domain probably is more stable in VAI-BR and VAI CR mutants. This may explain why these mutants are more efficient in blocking PKR activation.

DISCUSSION

By using whole cells and a series of adenovirus mutants, we recently showed that the critical elements required for function of the VAI RNA are located in the central domain (12) and that these same elements bind to PKR and inactivate it (15). The purpose of this investigation was twofold: (i) to determine whether the correlation that was made previously between binding and function (12, 15) can be extended to the in vitro phenotype of these mutants, and (ii) to determine whether mismatches in the duplex regions are responsible for the lack of activation of PKR by the VAI RNA. The first question is important because VAI RNA-mediated downregulation of ribosome-bound PKR occurs in a complex milieu in which multiple components of the cellular translation apparatus interact. If the in vivo results can be reproduced in vitro with a purified enzyme, it is reasonable to conclude that the prevention of the activation of PKR by VAI RNA may be explained by a simple RNA-protein interaction. Further, if a correlation can be found between in vitro and in vivo phenotypes of the mutants, it would be possible to further study the structure-function correlations of the VAI RNA by introducing additional mutations into the RNA and by rapidly analyzing the mutants in vitro with purified PKR. We repeatedly failed to obtain sufficient quantities of homogeneous PKR protein. We found that when the protein was purified to homogeneity, it was very unstable. Inhibition assays with such an enzyme preparation were not reproducible. Therefore, all assays were carried out with hexylamine-agarose column-purified fractions. The enzyme at this stage contains only about four to five additional polypeptides. We believe that VAI RNA does not require other viral or cellular components for the inhibition of PKR. Although it is possible that the additional proteins present in the PKR preparation influence the binding of VAI RNA to PKR and subsequent inhibition of its activity, we believe that is unlikely. While the PKR band was a major band in all of our preparations, other proteins were not always copurified or not copurified to the same extent. However, the concentrations of the reovirus RNA required for the activation of the enzyme as well as the concentration of VAI RNA required for the inhibition of the enzyme activity remained unchanged regardless of other polypeptides present in different PKR preparations. Recent reports that VAI RNA can bind
FIG. 10. Single-strand-specific RNase cleavage analysis of the WT VAI, VAI-CB, VAI-BR, and VAI-CR RNAs. In vitro-synthesized RNAs were labeled at the 3′ ends (as described in Materials and Methods) and digested with the indicated RNases, T₁, BC, and U2. The products were resolved in 14% DNA-sequencing gels. NE, no RNase; M, ladders generated by partial digestion of the labeled RNA under alkaline conditions (12). Cleavages specific to loops and the short stem-loop of the central domain are bracketed. Sizes are indicated in nucleotides.

to the PKR synthesized in bacteria or in reticulocyte lysates support this conclusion (16, 24, 43).

When lower concentrations of VAI RNA are used in in vitro assays, the inhibitory properties of VAI RNA mutants correlate remarkably well with their in vivo phenotype and the structural integrity of the central domain. However, at high concentrations of RNA, all mutants which contain the long duplex regions can block the PKR activation even though the central domain of these mutants is destroyed, suggesting that this inhibition is nonspecific. These and our previously published VAI RNA binding studies underscore the importance of the central domain in the function of VAI RNA. Pe’ery et al. (45) recently reported a similar study in which a number of central-domain mutants were assayed for the inhibition of PKR in vitro and also for the translational enhancement of a reporter gene in cotransfection assays (53). Some of the mutants failed to function in vivo in transient assays although these mutant RNAs were able to block the PKR activity efficiently in vitro, suggesting that the in vitro inhibition of PKR may not correlate with the in vivo phenotype. At present, direct comparison of our results with their results is difficult. Not only are the mutants used in these two studies different, but there are a number of protocol differences as well (e.g., the preparation of VAI RNA, purity of PKR, in vitro autophosphorylation assays, and assays to determine the biological activity of the mutant VAI RNAs). Perhaps the most important discrepancy lies in the concentration of VAI RNA required for the complete inhibition of PKR activity in vitro. We consistently have found that at RNA concentrations of 0.4 μg/ml or less, PKR activity is inhibited completely. In contrast, in studies reported by others (45), RNA concentrations of about 5 μg/ml were required for the complete inhibition of PKR in vitro. One explanation for the discrepancy is that the PKR that we used in these studies is considerably more purified than that used by others and thus requires less RNA for inhibition (45). It is interesting that when we use a less purified enzyme preparation (Mono-S column-purified fractions), we require about 5 μg of VAI RNA per ml for complete inhibition of the activity. A crude estimation indicates that in our in vitro assays, the ratio of PKR to VAI RNA may be about 1:5. Currently we do not know the number of PKR molecules per cell, and therefore comparison of the in vitro values with in vivo values will not be feasible. Another explanation for the discrepancy between our results and those of Pe’ery et al. (45) may be due to the differences in the type of in vivo assays used for the determination of the biological activity of the mutants.

Our mutants have been characterized in the context of virus chromosome (4, 12), whereas the mutants used by Pe’ery et al. were assayed for the translational enhancement of a reporter gene in transient assays (53). VAI RNA has been shown to stabilize mRNAs in transient assays (54). Therefore, comparison of the in vitro phenotype of the mutants with in vivo results obtained in such assays may not be very accurate. Interestingly, we have observed that at high RNA concentrations, all mutants except sub709 and sub741 inhibited the PKR activity completely. All of these mutants except sub709 and sub741 retain the apical stem-loop. Several explanations are possible for this observation. For example, although long duplex regions of VAI RNA cannot activate the kinase, at high concentrations these duplex regions are capable of binding to the enzyme and thus preventing the binding of dsRNA. Under certain assay conditions, the apical stem-loop has been shown to bind to PKR in vitro (37). Alternatively, the primary contact site for PKR on VAI RNA is the short stem-loop of the central domain, and the apical stem-loop makes a weak contact with PKR. At high concentrations, when the short stem-loop of the central domain is absent, the apical stem-loop is capable of interacting with PKR and inhibiting the activity. A third possibility is that PKR binds to the apical stem-loop site independent of the binding sites of dsRNA and the short stem-loop of the central domain. Binding of the apical stem-loop to this site may somehow affect the activity of the enzyme.

There is a striking correlation between the in vitro inhibition of PKR by VAI mutants and the maintenance of the secondary structure of the central domain of the mutants. As stated above, there are two principal components in this central domain. One is a loop in the 5′ side, and the other is a short stem-loop in the 3′ side. Recent mutational analysis has cast
some doubt on the existence of this short stem-loop. It has been suggested that these sequences may exist as a large loop (28). The structure of the VAI-CB throws some light on this issue. In the revised RNA structure, the conserved sequence ACCC (nucleotides to 119 to 122) has been shown to base pair with GGGU (nucleotides 37 to 40) (28). The mutant VAI-CB contains two copies of this sequence, one between 92 and 97 and the second between 120 and 125. Because the sequences between 92 and 97 are contiguous with the rest of the base-paired RNA sequences of the apical stem, it is very likely that the sequence GGGU (nucleotides 37 to 40) would pair with the sequence ACCC located between 92 and 97 rather than that present between 120 and 125. Therefore, at least in this case, the original secondary structure is consistent with the structure of the mutant. Whether it is a short stem-loop or a large loop in the central domain, our in vitro block assays and the secondary structure analysis suggest that it is this structure that is critical for the function of the VAI RNA. The PKR must recognize a precise structure in this region of the molecule. For example, in mutant sub749, the structure of stem I is disrupted, whereas in mutants sub707 and in708, the size of the loop in the 5' side (loop A) is enlarged by 5 and 10 bases, respectively. Nonetheless, the mutants are able to block the activation of the kinase as efficiently as the WT because the short-stem loop of the central domain is intact. Similarly, the apical stem-loop by itself also may not play an important role in the downregulation of the kinase activity. Several observations support this argument. First, several deletion mutants with large truncations of the apical stem-loop retain full biologic activity as long as the central short stem-loop is preserved in these mutants (4, 12, 38, 39). Second, mutants with WT apical stem-loop or extended apical stem-loop but without the central short stem-loop (sub743, sub745, sub746, sub747, and sub748) cannot function in vivo or in vitro. Third, we have shown that the binding of PKR to VAI RNA correlates with function, and binding requires the elements present in the central domain of the molecule; no binding or negligible binding of PKR was observed when the apical stem-loop alone was present in the molecule (15). Finally, VAI RNA and small RNAs of other viruses do not complement the VAI RNA efficiently in vivo (5, 6) or block PKR activation in vitro as efficiently as VAI RNA, even though these RNAs contain long duplex regions (8, 9, 15a, 17, 18). Thus, the role of the duplex regions such as stems I, II, and III may be to maintain the nucleotide sequences in the short-stem loop in a conformation that is optimal for function. Any large mutations in stem I or stem III in a region proximal to the central domain lead to dramatically altered secondary structures of the central domain and result in the loss of function. Other mutational analyses of the apical stem-loop are also consistent with this suggestion (37).

Recent mutational analysis of PKR indicates that both dsRNA and VAI RNA bind to the N-terminal 171-amino-acid region (16, 24, 36, 43). This region, which is rich in basic amino acid residues contains two functionally nonequivalent motifs, one from residues 11 to 77 and the other from residues 101 to 167. The amino acid sequences of these two motifs display regions of homology with each other and also with sequences of other RNA-binding proteins (16) and may exist as alpha-helical structures. It has been hypothesized that both of these motifs make contacts with the activator, the dsRNA. A unique property of the PKR is that the enzyme is activated and inhibited by the RNA ligands: activation by dsRNA and inhibition by VAI RNA. It has been shown that mutations in PKR that fail to bind to dsRNA also fail to bind to VAI RNA in vitro, suggesting that both dsRNA and VAI RNA bind to the same regions in PKR. We previously showed that PKR binds to the central domain of VAI RNA; most or all of the apical stem-loop probably is not involved in binding (15). Therefore, in this case, the N-terminal region of PKR must recognize the short stem-loop (or a large loop based on the revised secondary structure [28]) of the VAI RNA, and this interaction must prevent the binding of the activator to the enzyme. Thus, the N-terminal RNA binding domain of PKR may display considerable flexibility in its interaction with these two types of RNA ligands, a short stem-loop in the case of VAI RNA and a perfectly base-paired duplex in the case of dsRNA; these two interactions lead to opposing consequences. This also indicates that the interactions of PKR with dsRNA and VAI RNA must be different in nature. Although preliminary mutational analysis of the N-terminal region of PKR indicated that both types of RNAs bind to this region, a fine mutational analysis in which each residue in the critical RNA binding region is mutated will be required to resolve this issue. A simple explanation is that VAI RNA, upon binding to PKR, may induce a conformational change in the protein molecule such that it cannot bind to dsRNA. There is precedence for this. For example, the human immunodeficiency virus Tat protein has been shown to change its structure upon binding to TAR RNA (7, 42). Also relevant here are the tertiary interactions that may influence the VAI RNA-PKR binding. Most RNAs exist as globular structures in solution. It is therefore certain that the tertiary structure of VAI RNA plays a very important role in its binding to PKR. Our current knowledge of the structure of VAI RNA does not allow us to make predictions regarding the tertiary interactions. Control of gene expression by RNA-protein interactions is a field that is rapidly evolving. A number of RNA-protein interactions that modulate the activity of the proteins have been described recently (reviewed in references 25, 34, 35, and 46). These studies suggest that different proteins may employ fundamentally distinct strategies to accomplish sequence-specific RNA binding. The modulation of PKR activity by two different types of RNA ligands cannot be easily explained by using the previously described models of gene regulation based on RNA-protein interaction.

In this study, we have also evaluated the functional significance, if any, of the mismatches in the long duplex regions of VAI RNA. An earlier report suggested that at low concentrations, VAI RNA can activate PKR in vitro (14). This is an attractive hypothesis, for at early times, when the concentration of VAI RNA is very low, PKR could be activated, resulting in a general inhibition of translation. At late times, when VAI RNA accumulates to very high concentrations, selectively, the inhibition of translation of viral mRNA would be relieved. As a result, viral mRNA would be translated more efficiently. Such an effect would contribute to the host shutoff and promote viral replication. Despite repeated efforts, we were unable to detect any activation of PKR in the presence of VAI RNA. We considered it possible that failure of VAI RNA to activate PKR in vitro resulted from mismatches in the base-paired regions. For example, it has been shown that even with a single mismatch, the dsRNA would not activate PKR (40). The mutants with stem I or III, with all of the mismatched sequences corrected from VAI RNA with bulge base pairs, did not activate PKR in vitro. Similarly, VAI CR, in which both stems I and III are perfect duplexes, also did not activate PKR in vitro. These results are consistent with the observation that the minimal length of the dsRNA required for the activation of PKR is about 30 bp (29). The longest duplex region in these mutants consists of 26 bp. The efficient inhibition of the activation of PKR by these mutants also suggests that the bases that are mutated do not play a significant role in the inhibition
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