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Activation of Interferon-inducible 2′–5′ Oligoadenylate Synthetase by Adenoviral VAI RNA*

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2′–5′ oligoadenylate (2–5(A)) synthetase and protein kinase, RNA activated (PKR) are the only two known enzymes that bind double-stranded RNA (dsRNA) and get activated by it. We have previously identified their dsRNA binding domains, which do not have any sequence homology. Here, we report a profound difference between the two enzymes with respect to the structural features of the dsRNA that are required for their activation. The adenoviral virus-associated type I (VAI) RNA cannot activate PKR, although it binds to the protein and thereby prevents its activation by authentic dsRNA. In contrast, we observed that VAI RNA can both bind and activate 2–5(A) synthetase. Mutations in VAI RNA, which removed occasional mismatches present in its double-stranded stems, markedly enhanced its 2–5(A) synthetase-activating capacity. These mutants, however, are incapable of activating PKR. Other mutations, which disrupted the structure of the central stem-loop region of the VAI RNA, reduced its ability to activate 2–5(A) synthetase. These debilitated mutants could bind to the synthetase protein, although they fail to bind to PKR.

Interferons (IFNs)[1] induce the synthesis of two enzymes that require double-stranded RNA (dsRNA) as their activators (1). One of these enzymes, a dsRNA-dependent protein kinase (PKR), phosphorylates the α subunit of the translation initiation factor, eIF-2, and thus inhibits the initiation of protein synthesis (2, 3). The other enzyme, 2′–5′ oligoadenylate (2–5(A)) synthetase, polymerizes ATP into 2–5(A), which in turn activates a latent ribonuclease leading to the degradation of mRNAs (4). There are multiple isozymes of 2–5(A) synthetase, all of which are induced by IFN and activated by dsRNA. The 2–5(A) synthetase pathway is responsible for inhibiting the replication of picornaviruses in IFN-treated cells (5–7).

Although both PKR and 2–5(A) synthetase are activated by dsRNA, their activation characteristics are quite different. PKR binds dsRNA with a much higher affinity than 2–5(A) synthetase.[2] This dissimilarity in the dsRNA binding property is reflected in the absence of any structural homology between the dsRNA binding domains of the two enzymes (8, 9). Unlike 2–5(A) synthetase, PKR dose response to dsRNA is biphasic. Low concentrations of dsRNA activate it, and high concentrations inhibit it (2). This phenomenon is probably due to the need for two PKR molecules to bind to the same dsRNA molecule so that intermolecular autophosphorylation is possible.

We are interested in identifying the specific structural features of an RNA molecule that are recognized by PKR and 2–5(A) synthetase for binding and activation. Many RNA molecules, which are not perfectly double stranded, can bind to these enzymes. Several viruses encode RNAs that are partially double-stranded and are known to interact with PKR (10). Most of these viral RNAs inhibit the action of PKR, although reovirus mRNA can activate it (11). It is not known if and how these RNAs affect the action of 2–5(A) synthetase. In the current study, we have addressed this question by studying the effects of adenoviral virus-associated (VA) RNAs on synthetase activity. Adenoviruses, which are relatively insensitive to IFN, encode two RNA polymerase III-directed small RNAs (VAI and VAII RNAs) that accumulate to high levels at late stages of infection. Both RNAs are about 160 nucleotides long and are highly structured. In virus infections, VAI RNA is obligatory for efficient translation of viral and cellular mRNAs at late times. It binds to and blocks the activation of PKR produced by the cell, thereby enabling protein synthesis to proceed at normal levels (12–15). The structure of VAI RNA consists of two long imperfectly base-paired stems of 20–22 base pairs (bp) joined at the center by a domain that is structurally complex and that contains a short stem loop and two adjacent loops. This complex short stem loop and the two adjacent loops together are referred to as the central domain (16, 17). 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 (16, 17).

In this paper, we report an unexpected observation that VAI RNA can bind to and activate 2–5(A) synthetase in a concentration-dependent manner. VAII RNA is less effective in this regard. We also show that the activation potential of the VAI RNA is primarily due to the long duplex regions of the molecule because the activation of 2–5(A) synthetase by VAI RNA increased dramatically and reached to the levels of activation mediated by dsRNA when the imperfectly base-paired

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[1] The abbreviations used are: IFN, interferon; dsRNA, double-stranded RNA; PKR, protein kinase, RNA activated; 2–5(A), 2′–5′ oligoadenylate; DRBD, double-stranded RNA binding domain; VA, virus-associated; EMSA, electrophoretic mobility shift assay; VAI and -II, virus-associated type I and II RNA; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); bp, base pair(s).

[2] S. Y. Desai, R. C. Patel, and G. C. Sen, unpublished observation.

duplex regions of VAI RNA were converted to perfectly base-paired regions.

EXPERIMENTAL PROCEDURES

Materials—Restriction enzymes, poly(I)·poly(C), RNasein, and other fine chemicals were from Boehringer Mannheim. Plasmid pGEM3 was from Promega. Plasmids pET3a and pET15b and the His.Bind resin were from Novagen. Polyethyleneimine cellulose plates were from EM Scientific. [α-^{32}P]ATP (specific activity, 800 Ci/mmol) was from DuPont NEN.

2–5(A) Synthetase Subcloning—The coding region of 2–5(A) synthetase 9–2 cDNA (8) was subcloned into plasmid pET3a at *Nde*I and *Bam*HI sites. The plasmid 2–5(A) synthetase/pGEM3 (8) was digested with *Xho*II and *Bam*HI, and the fragment containing the coding region was purified and ligated with *NdeI*/*Bam*HI-digested pET3a with a *Nde*I-*Xho*II linker adapter. The insert from 2–5(A) synthetase/pET3a was subcloned into the histidine tag vector plasmid pET15b in *Nde*I and *Bam*HI restrictions sites after releasing the 1491-bp insert from pET3a with the same enzymes.

Bacterial Expression and Purification of 2–5(A) Synthetase—Initial transformation and screening was done in *Escherichia coli* (DH5α). For production of the protein, appropriate clones were introduced into *E. coli* BL21 (DE3). Unless specified, all purification steps were carried out at 4 °C. An overnight culture of the colony in LB broth (Difco) containing 100 μg/ml ampicillin was diluted ten times and was grown and induced at 0.6 optical density units with 2 mM isopropyl-1-thio-β-D-galactopyranoside for 2 h. The induced cells (500 ml) were chilled immediately and harvested at 10,000 × *g* for 15 min, washed twice with ice-cold 20 mM Tris-HCl, pH 7.5, resuspended in 20 mM Tris-HCl, pH 8.00, 300 mM NaCl, 5 mM imidazole, 0.5% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride (binding buffer) to give a 20% (w/v) suspension (approximately 12.5 ml), and sonicated on ice in 5-ml portions for a total of 1.8 min with each pulse of 30 s. The resulting suspension was then spun at 40,000 × *g* for 60 min to remove the cell debris, and the supernatant (approximately 12 ml) was then applied to a 1-ml bed volume of His.Bind resin column, which had been successively washed with the binding buffer and 50 mM $NiSO_4$, followed by equilibration with the binding buffer. The column was then washed with 20 volumes of binding buffer followed by 20 volumes of washing buffer, which had the same ingredients as the binding buffer but with 60 mM imidazole. The enzyme was then eluted with two bed volumes of elution buffer containing 20 mM Tris-HCl, pH 8.00, 500 mM NaCl, 500 mM imidazole, and 1 mM phenylmethylsulfonyl fluoride. The eluate was dialyzed against 20 mM Tris-HCl, pH 7.5, and 10% glycerol and was stored at −70 °C till further use.

2–5(A) Synthetase Assay—In-solution synthetase assay was done according to the method of Mory *et al.* (18) with minor modifications. Unless otherwise specified, 10 μl of reaction mixture containing the enzyme, 20 mM Tris-HCl, pH 7.5, 20 mM magnesium acetate, 2.5 mM dithiothreitol, 5 mM ATP, 5 μCi of [α-^{32}P]ATP (specific activity, 800 Ci/mmol), and the activator RNA was incubated for 2 h at 30 °C. The reaction was stopped by boiling the sample for 3 min and was then centrifuged at 14,000 × *g* for 10 min; 8 μl of the supernatant was incubated for 3 h at 37 °C with 3 μl of 1 unit/μl of calf intestine alkaline phosphatase. 2 μl of the sample was then spotted on a polyethyleneimine-cellulose thin layer chromatography plate and was resolved in 750 mM KH_2PO_4, pH 3.5 (19). The 2–5(A) formed was then quantitated by exposing the plate to storage phosphor screen and expressed as arbitrary units.

Preparation of dsRNA—The 82-bp synthetic dsRNA was prepared by hybridizing sense and antisense transcripts generated *in vitro* using the template plasmid pGEM3–9T (9). Complementary strands were generated by digesting the plasmid with appropriate restriction enzymes followed by transcription with SP6 and T7 polymerase. Hybridization was carried out at 50 °C for 6 h in 40 mM PIPES, pH 6.4, 400 mM NaCl, 1 mM EDTA, and 80% deionized formamide. The sample was then diluted with 10 volumes of RNase digestion buffer containing 300 mM NaCl, 10 mM Tris-HCl, pH 7.4, 5 mM EDTA, and 40 μg/ml RNase A, incubated at 20 °C for 1 h, and extracted with phenol-chloroform. The RNA was recovered by precipitation with ethanol.

Preparation of VA RNAs—The VAI genes were transcribed *in vitro* using T7 RNA polymerase and plasmids in which the wild type VAI, VAII, or mutant VAI gene has been cloned behind a T7 promoter. The transcribed RNA samples were purified by electrophoresis on a 6% native polyacrylamide gel before use. None of these RNA preparations could activate PKR either at low or high concentrations. If needed, the RNAs were radiolabeled at the 5′-end using polynucleotide kinase and [γ-^{32}P]ATP or at the 3′-end using ^{32}P as described before (16).

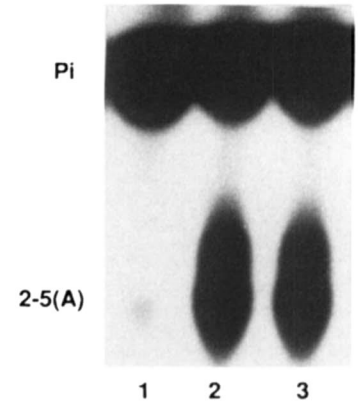

FIG. 1. **Activation of bacterially expressed 2–5(A) synthetase by various dsRNAs.** Synthetase activity was measured as described under "Experimental Procedures." Parts of the autoradiogram are shown. Positions of 2–5(A) molecules and inorganic phosphate (P_i) are indicated. *Lane 1*, no activator; *lane 2*, poly(I)·poly(C); *lane 3*, *in vitro* synthesized 82-bp dsRNA. All RNAs were used at 25 μg/ml.

The mutants 741, 745, 746, and 748 are linkerscan substitution mutants in which *Hin*dIII linker sequences are substituted in the following locations: 741, between nucleotides 76 and 90; 745, between nucleotides 105 and 117; 746, between nucleotides 116 and 126; and 748, between nucleotides 134 and 143 (21). Mutant VAI-CB was constructed by substituting DNA sequences between *Csp*45I (+61) and *Bst*EII (+99) sites with a synthetic double-stranded oligonucleotide in which sequences have been altered to make the stem III of VAI RNA perfectly double stranded. Similarly, VAI BR was constructed by substituting DNA sequence between *Bst*EII (+99) and *Eco*RI (+160) to make stem I perfectly double stranded (20, 21).

Electrophoretic Mobility Shift Analysis (EMSA)—Binding reactions were performed by mixing the end-labeled dsRNA or VAI RNAs and 2–5(A) synthetase in binding buffer (20 mM Tris-HCl, pH 7.5, 50 mM KCl, 2 mM $MgCl_2$, 5% glycerol) and incubating at 25 °C for 10 min. The amount of labeled RNA per lane was about 20,000 cpm. After incubation, a dye-glycerol solution was added, and the samples were loaded immediately on a 4% polyacrylamide gel (acrylamide:bisacrylamide, 29.2:0.8). The gel was cast in 40 mM Tris-glycine buffer and was prerun in the same buffer for 45 min at 150 V. Radioactivity was detected by autoradiography. The same results were obtained by using 0.25 × Tris borate buffer for casting and running the gel. In competition experiments, cold competitor was added 15 min prior to the addition of labeled RNA. Dried gels were analyzed by autoradiography.

2–5(A) Synthetase Antibody—The antipeptide B antibody was a gift from Judith Chebath (22). Antibody against purified bacterially produced histidine-tagged 9–2 synthetase was raised in rabbits using standard procedures. For supershifting EMSA assays, equal quantities of immune or preimmune sera were included in the reaction mixture.

RESULTS

VAI RNA-2–5(A) Synthetase Interactions—VAI RNA is known to inhibit the activation of PKR by authentic dsRNA. This inhibition occurs because VAI RNA competes with dsRNA for binding to PKR, but, unlike dsRNA, it cannot activate the enzyme (15, 20). To study the effect of VAI RNA on 2–5(A) synthetase, we first wanted to determine if it can bind to the protein. The enzyme used for this purpose was the mouse 9–2 isozyme of 2–5(A) synthetase whose cDNA we have previously cloned (8). The enzyme was expressed in *E. coli* as a hexahistidine-tagged protein and purified by affinity chromatography following procedures described under "Experimental Procedures." The purified enzyme was incubated with [α-^{32}P]ATP and activator RNAs, and the resultant 2–5(A) molecules were detected by a thin layer chromatographic assay. As shown in Fig. 1, little 2–5(A) was synthesized in the absence of activator RNA (*lane 1*). Poly(I)·poly(C) of heterogenous length (*lane 2*) and a synthetic dsRNA of 82 bp (*lane 3*) were highly efficient in activating the enzyme. These results demonstrated that the

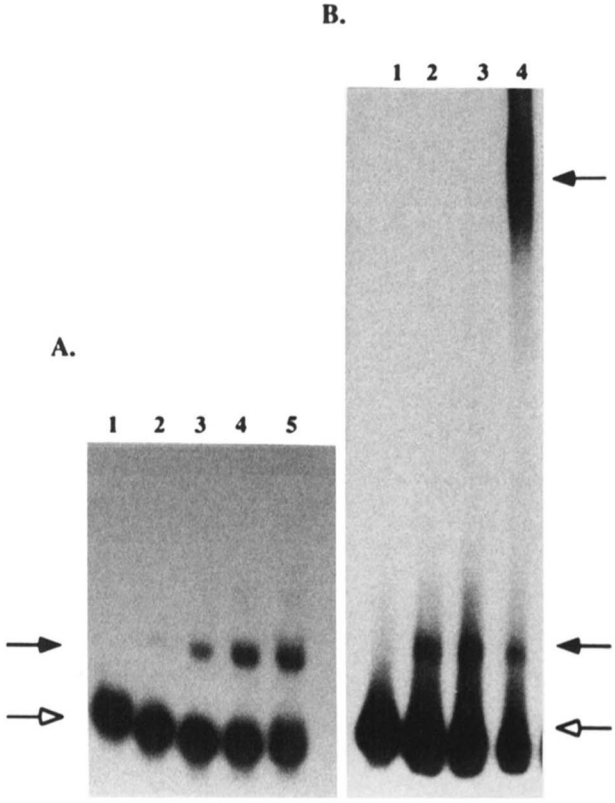

FIG. 2. **Electrophoretic mobility shift assay for measuring dsRNA-2–5(A) synthetase interactions.** All *lanes* had 0.5 ng of labeled 82-bp dsRNA probe. *A*: *lane 1*, no protein; *lanes 2–5*, increasing amounts of 2–5(A) synthetase added. The *open arrow* shows the position of the probe, and the *solid arrow* shows the position of the shifted complex. *B*: *lane 1*, no protein added; *lanes 2–4*, 500 ng of 2–5(A) synthetase; *lane 3*, 5 μl of preimmune serum; *lane 4*, 5 μl of antipeptide serum. The *open arrow* shows the position of the probe, the *lower solid arrow* shows the position of the shifted complex, and the *upper solid arrow* shows the position of supershifted complex.

bacterially produced hexahistidine-tagged 2–5(A) synthetase has enzymatic properties indistinguishable from those of the native enzyme isolated from mammalian cells.

For studying 2–5(A) synthetase-RNA interactions, we developed an electrophoretic mobility shift assay. The synthetic 82-bp dsRNA was radiolabeled and used as the probe. As shown in Fig. 2*A*, increasing amounts of the synthetase protein shifted the mobility of increasing amounts of the probe. The shifted band could be competed out by excess unlabeled probe but not by single-stranded RNA (data not shown). To confirm that the observed shift in the dsRNA mobility was due to its binding to the 2–5(A) synthetase protein and not to any accompanying impurity, an antibody supershift assay was performed (Fig. 2*B*). An antibody raised against a synthetic peptide, whose sequence corresponds to a region of the synthetase protein (22), supershifted a portion of the synthetase-dsRNA complex (*lane 4*). Similar supershifting was observed with an antibody produced against the purified bacterially produced synthetase (data not shown), whereas the preimmune serum did not cause any supershift (*lane 3*). Once the specificity of the mobility shift assay was established, it was used to examine the potential interaction between VAI RNA and 2–5(A) synthetase. In the experiment shown in Fig. 3*A*, unlabeled VAI RNA could effectively compete with the 82-bp dsRNA probe to prevent the complex formation (*lane 4*), although a concentration of VAI RNA 10 times higher than that of poly(I)·poly(C) (*lane 3*) was required for observing the same level of competition. These results suggested that VAI RNA can bind to 2–5(A) synthetase.

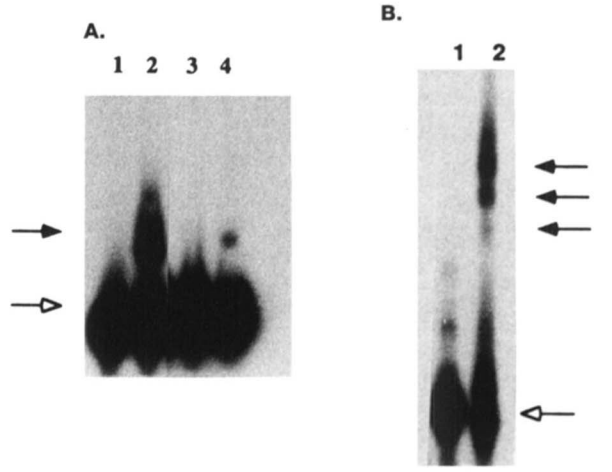

FIG. 3. **VAI RNA-2–5(A) synthetase interactions.** *A*, all lanes had 0.5 ng of labeled 82-bp dsRNA probe. *Lane 1*, no protein; *lanes 2–4*, 500 ng of 2–5(A) synthetase added; *lane 3*, 5 ng of poly(I)·poly(C) added as the competitor; *lane 4*, 500 ng of VAI RNA added as the competitor. *B*, both lanes had 0.5 ng of 5′-labeled VAI RNA. *Lane 1*, no protein; *lane 2*, 500 ng of 2–5(A) synthetase added. The *open arrow* shows the positions of the probes, and the *solid arrows* show the positions of the shifted complexes.

This suggestion was directly tested in the experiment shown in Fig. 3*B*. VAI RNA was radiolabeled at the 5′-end and used as the probe to bind 2–5(A) synthetase. Several shifted complexes were formed (*lane 2*). Although the reason for the appearance of multiple complexes is not known, they may represent alternate conformational states of the VAI RNA. These results demonstrated that VAI RNA can directly bind to the 2–5(A) synthetase protein.

Activation of 2–5(A) Synthetase by VAI RNA—In the next series of experiments, we examined the potential of VAI RNA to activate 2–5(A) synthetase. The VAI RNA preparation used for these studies was free of any dsRNA contamination, as judged by gel electrophoresis, and it could not activate PKR either at low or high concentration (Ref. 20 and data not shown). VAI RNA could, however, effectively activate 2–5(A) synthetase (Fig. 4, *lanes 2* and *3*). At 100 μg/ml it was 5-fold less effective than poly(A)·poly(U) (*lanes 3* and *6*) and 10-fold less effective than poly(I)·poly(C) (data not shown). Two single-stranded RNAs, poly(A) (*lane 4*) and poly(U) (*lane 5*) could not activate the enzyme. These results demonstrated that VAI RNA is an effective activator of 2–5(A) synthetase under conditions at which single-stranded RNAs have no effects.

To further characterize the requirements of activation of 2–5(A) synthetase by VAI RNA, its dose response (Fig. 5) and kinetics of the reaction (Fig. 6) were measured. Increasing concentrations of VAI RNA increasingly catalyzed the enzyme reaction (Fig. 5). The rate of reaction appeared to level off between 50 and 100 μg/ml VAI RNA. The enzyme kinetics was linear for at least 2 h (Fig. 6), irrespective of the activator RNA used. Note that at every time point, the amount of 2–5(A) synthesized was about 10 times higher when an authentic dsRNA was used instead of VAI RNA (Fig. 6, *A* and *B*). The synthetic dsRNA used in this experiment contained 164 nucleotides, whereas VAI RNA contains 157 nucleotides. Thus, on a weight basis, VAI RNA is about 10 times less effective in activating 2–5(A) synthetase than a totally double-stranded RNA.

Role of ds Stems of VAI RNA in Activating 2–5(A) Synthetase—The structure of VAI RNA has been studied extensively (15–17, 20). Computer modeling has suggested several alternative secondary structures that have been experimentally tested by using susceptibility to limited RNase digestion as an

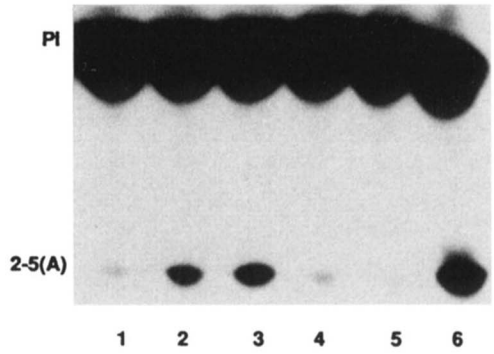

FIG. 4. **Activation of 2–5(A) synthetase by VAI RNA.** *Lane 1*, no RNA activator; *lane 2*, 25 μg/ml VAI RNA; *lane 3*, 100 μg/ml VAI RNA; *lane 4*, 100 μg/ml poly(A); *lane 5*, 100 μg/ml poly(U); *lane 6*, 100 μg/ml poly(A)·poly(U).

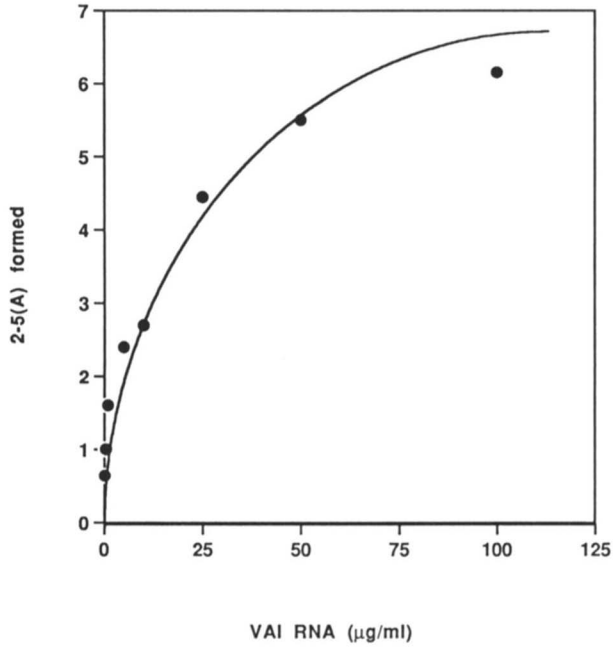

FIG. 5. **Effect of varying concentrations of VAI RNA on synthetase activity.** 2–5(A) synthetase activity was measured in the presence of increasing concentrations of VAI RNA. The amounts of 2–5(A) synthesized were quantitated by phosphorimager analysis of the thin layer chromatogram; they are presented in arbitrary units.

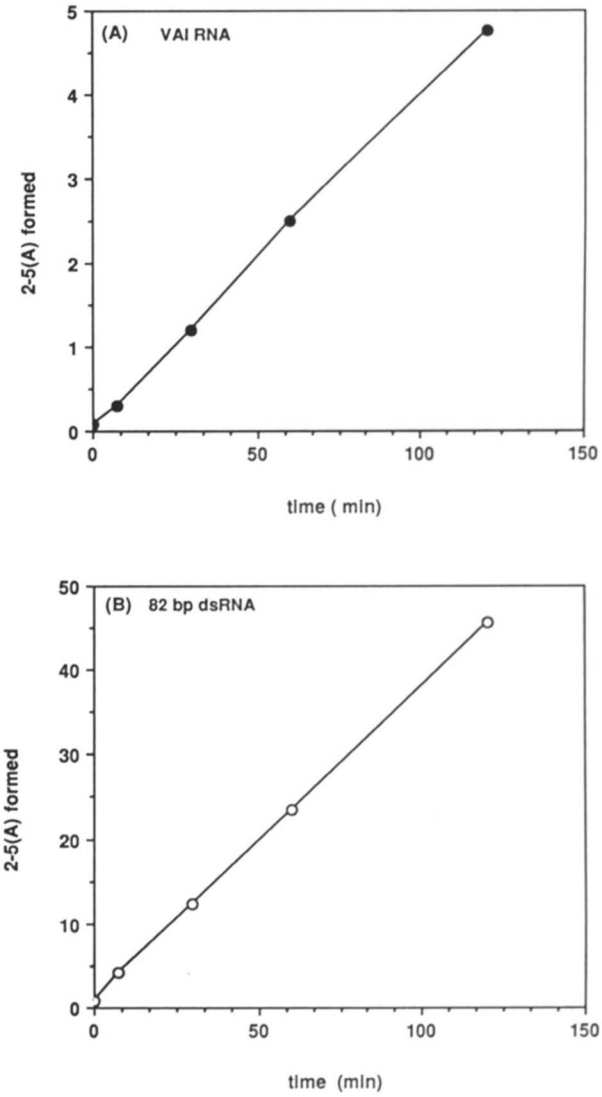

FIG. 6. **Kinetics of 2–5(A) synthesis.** Amounts of 2–5(A) synthesized at various times in response to (*A*) 25 μg/ml VAI RNA and (*B*) 25 μg/ml synthetic dsRNA are shown. The data are presented in arbitrary units after subtracting the corresponding values without any activator RNA at each time point.

assay. The experimentally derived secondary structure of VAI RNA consists of two long duplex regions, stem I and III, connected at the center by a short duplex region, stem II (Fig. 7*A*). Stem I consists of a duplex in which nucleotides 1–22 are base paired to nucleotides 134–155 (numbers here represent the position of the nucleotides from the 5′-end with G start (16)). Stem II consists of a short duplex in which nucleotides 31–35 are base paired to nucleotides 128–132. In stem III, the longest of these, nucleotides 37–62 are base paired with nucleotides 71–94. As a result, nucleotides 63–71 exist as a loop (loop B). The structure in the central part of the molecule that is referred to as the central domain is complex and poorly defined. This part of the molecule contains a small loop in the 5′-side (loop A) and a short stem loop (loop C) and an additional minor loop (loop D) in the 3′-side.

Since stems I and III are the two longest duplex regions of the VAI RNA molecule, they are probably the major determinants of its capacity to activate 2–5(A) synthetase. These two stems, however, are not made of perfect base-paired sequences. Both stems contain five G-U pairs and two mismatches. In addition, stem III contains two stacked bases (nucleotides 42 and 43) (Fig. 7*A*). VAI RNA mutants, in which these pairing defects have been eliminated, were used for evaluating the roles of stems I and III in 2–5(A) synthetase activation. Two such mutants were used. In VAI-BR mutant, stem I is perfectly base paired, whereas in VAI-CB mutant stem III is perfectly base paired (Fig. 7*B*). Neither of these mutants can activate PKR, although they bind to it (20). When tested for 2–5(A) synthetase activation, both were more effective than wild type VAI RNA (Fig. 8). There was, however, a quantitative difference between the efficacies of the two mutants. VAI-BR was about 4 times more potent than wild type VAI RNA, whereas VAI-CB was more than 10 times as effective. VAI-CB was almost as effective as the 82-bp authentic dsRNA or poly(I)·poly (C). These results indicate that stems I and III are the structural elements in VAI RNA, which are important for its ability to activate 2–5(A) synthetase. Between the two stems, stem III, consisting of 26 bp, appears to be more important than stem I, which contains 22 bp.

Role of the Central Domain—The central domain of the VAI RNA molecule (Fig. 7*A*) is thought to be necessary for maintaining the integrity of its structure. In the next experiment, we examined the effects of disrupting this region on VAI RNA

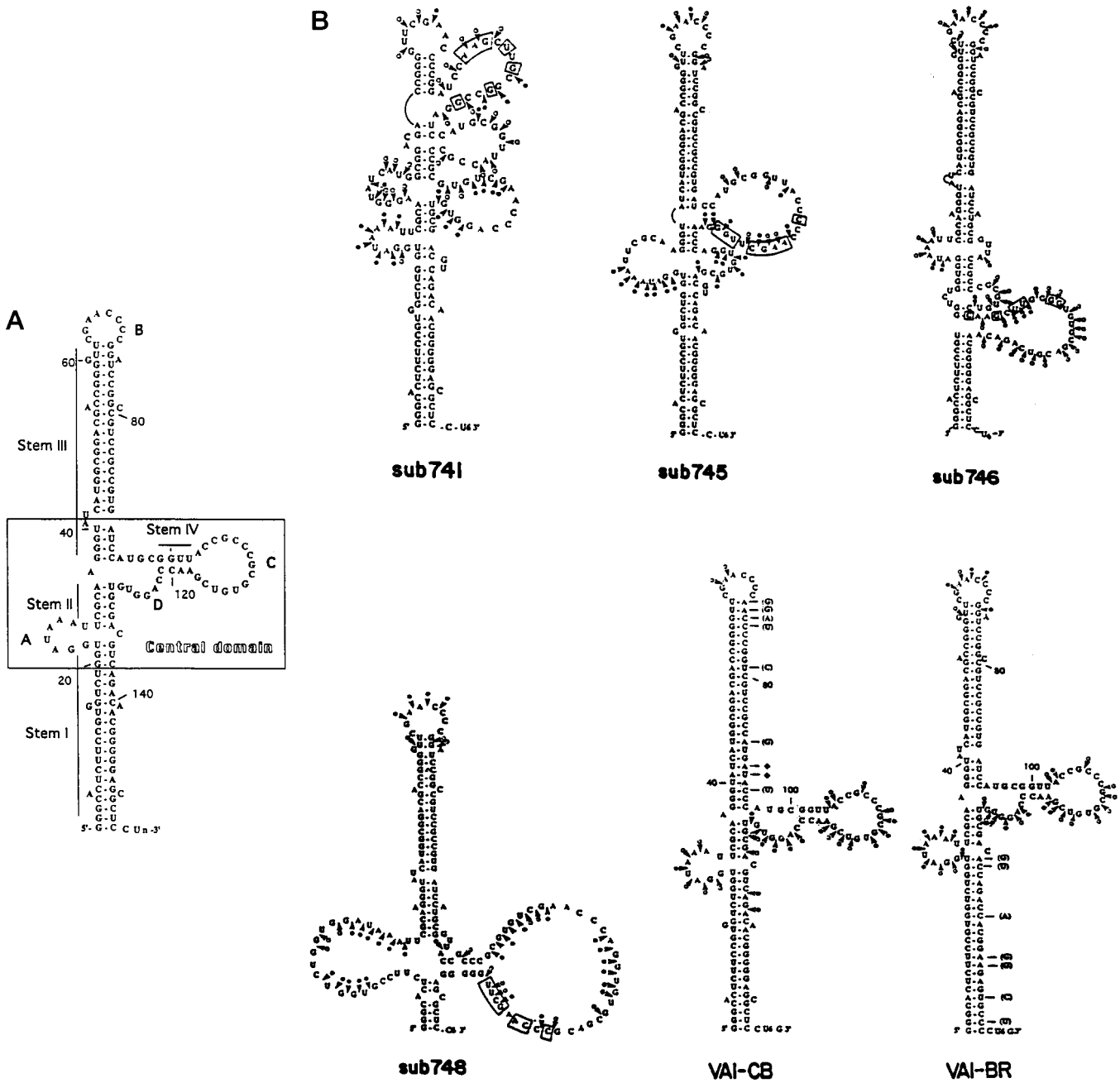

FIG. 7. **Secondary structures of wild type and mutant VAI RNAs.** *A*, experimentally derived secondary structure of the VAI RNA. The structure is based on single-strand-specific ribonuclease sensitivity analysis (16). See text for details. *B*, experimentally derived secondary structure of various mutant VAI RNAs based on single-strand-specific ribonuclease cleavage analysis previously described (20, 21). RNase cleavages are shown by *arrowheads*. Pronounced cleavages are shown by *solid circles* next to the *arrowheads*. Weak cleavages are shown by *open circles* next to the *arrowheads*. The mutated nucleotides are *boxed*. All VAI RNAs derived from T7 constructs contain 6 uridine residues followed by a glycine rather than the 1–4 uridine residues found *in vivo*. As no structural changes are observed for the VAI-CB and VAI-BR RNAs, the cleavage maps of these RNAs are shown on the wild type structure. The nucleotides substituted in the wild type sequence are shown in *parentheses*. In VAI-CB, 2 bases that are inserted to pair with 2 stacked bases at 41 and 42 are shown by *diamonds*.

ability to activate 2–5(A) synthetase. Four central domain mutants, sub741, sub745, sub746, and sub748, were used for this purpose. These mutants contain linker scan substitution mutations, and their secondary structures have been experimentally determined (Fig. 7*B*, Ref. 20). Each of the mutations disrupts the central domain and distorts the secondary structure of the molecule in a distinct and different way. Like wild type VAI RNA, none of these mutants can activate PKR, but unlike the parental molecule, they also fail to bind to PKR (20, 21). All of these mutants were less efficient than wild type VAI RNA in activating 2–5(A) synthetase (Fig. 9). None of them, however, was unable to activate the enzyme. These results indicate that the central domain is not essential, although its disruption leads to a marked reduction in the activity. This reduction may be caused by a concomitant perturbation of the structures of stems I and III. Indeed, among the four mutants, sub745 was the most effective one (Fig. 9). The two-stem structures are preserved in this mutant (Fig. 7*B*), although there is a minor perturbation in the structure of stem III when compared with wild type VAI RNA. Another VA RNA, VAII, was a poor activator of 2–5(A) synthetase (Fig. 9). Since the secondary structure of VAII RNA has not been determined, the un-

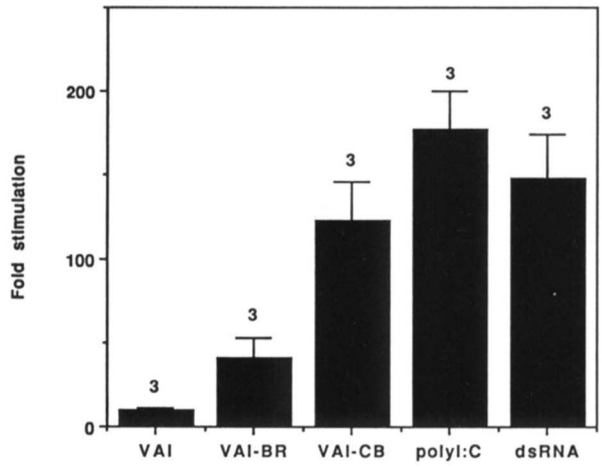

FIG. 8. **Activation of 2–5(A) synthetase by VAI RNA mutants with perfectly base-paired duplex regions.** Synthetase activation capacities of three mutants of VAI RNA were compared with those of VAI RNA, poly(I)·poly(C), and *in vitro* transcribed 82-bp dsRNA. Occasional mismatches in stem I of VAI RNA have been repaired in the mutant VAI-BR; similar restoration has been done in stem III of the mutant VAI-CB. The results are presented as -fold stimulation over the activity without any RNA activator. The *numbers* over each *bar* show the numbers of independent measurements with the specific RNA. The corresponding *error bars* are also shown. The incubations were for 2 h with 25 μg/ml RNA.

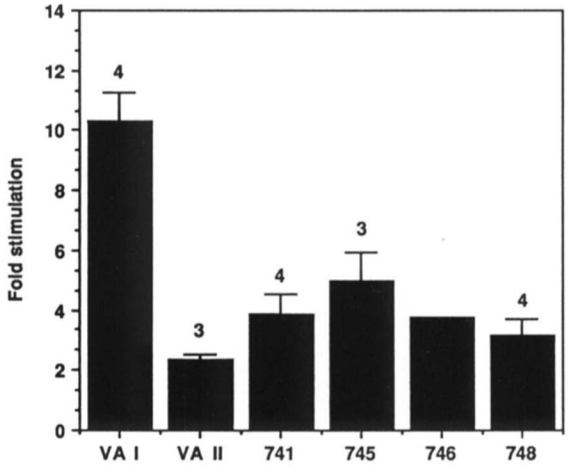

FIG. 9. **Activation of 2–5(A) synthetase by VAII RNA and central domain mutants of VAI RNA.** Four mutants of VAI RNA, in which different central domain regions have been disrupted, and VAII RNA were compared with wild type VAI RNA for their abilities to activate 2–5(A) synthetase. For mutant 746, the mean value of two independent determinations is shown. Other conditions are the same as in Fig. 8.

derlying structural defect cannot be identified at this time.

One reason for the central domain mutant inefficiency in activating the synthetase could be due to their failure to bind to it efficiently. This possibility is a strong one since these mutants cannot bind PKR, although the wild type VAI RNA can (16, 20). We tested this possibility by using the newly developed electrophoretic mobility shift assay (Fig. 2). Three mutant RNAs and the wild type VAI RNA were radiolabeled at the 3′-ends and used as probes for 2–5(A) synthetase binding. Like VAI RNA, all the mutants formed shifted complexes (Fig. 10). Moreover, there was no discernible difference among their efficiencies in complex formation. These results suggest that the central domain mutant inefficiency in activating the synthetase cannot be attributed to a poorer binding of these molecules to the protein.

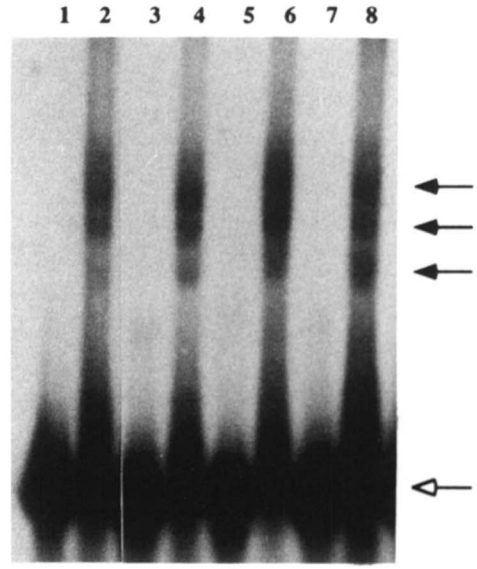

FIG. 10. **Binding of VAI RNA central domain mutants to 2–5(A) synthetase.** The *odd-numbered lanes* contained no protein, and the *even-numbered lanes* contained 2–5(A) synthetase. *Lanes 1* and *2*, VAI RNA; *lanes 3* and *4*, mutant 741 RNA; *lanes 5* and *6*, mutant 745 RNA; *lanes 7* and *8*, mutant 748 RNA. All RNAs were labeled at the 3′-end. The *open arrow* shows the position of the probe, and the *solid arrows* show the positions of shifted complexes.

DISCUSSION

This paper reports development of new tools for studying the mode of activation of 2–5(A) synthetase by dsRNA. A recently cloned murine 2–5(A) synthetase cDNA (8) was expressed in bacteria using an inducible expression vector. The protein was expressed as a fusion protein containing a hexahistidine tag at the amino terminus. This facilitated its purification using affinity chromatography and the subsequent production of an antibody. The histidine tag at the amino terminus did not affect the functioning of the enzyme or its activation by dsRNA. This general procedure can therefore be used for producing and purifying other isozymes of 2–5(A) synthetase and their mutants. We also developed an electrophoretic mobility shift assay for monitoring the physical interaction between 2–5(A) synthetase and dsRNA. A perfectly base-paired synthetic dsRNA was used as the probe for this assay. This assay will be useful for monitoring the relative affinities of different 2–5(A) synthetases and their mutants for dsRNA. It should also be very useful for quick and quantitative measurements of the affinities of different RNA and DNA molecules for this protein by using them as unlabeled competitors for binding.

We are interested in understanding how dsRNA interacts with specific cellular proteins regulating their biochemical activities. There are two complementary aspects of this interest: one is to delineate the physicochemical characteristics of dsRNA-protein interaction, and the other is to evaluate the regulatory roles of such interactions in cell physiology. The current study sheds light mainly on the first aspect and illuminates differences between two IFN-induced dsRNA-activable enzymes with respect to the structural requirements of their activator RNAs. The main observation reported here is that the partially double-stranded adenoviral VAI RNA interacts with 2–5(A) synthetase and activates it. Although this is the first report of VAI RNA interactions with 2–5(A) synthetase, similar interactions with PKR have been extensively studied by us and others (17, 20, 21, 23). Both *in vivo* and *in vitro* studies have demonstrated that VAI RNA blocks the activation of PKR and thereby maintains normal levels of protein synthesis in adenovirus-infected cells. As a result, unlike

the wild type virus, mutants lacking VA RNAs replicate poorly, especially in IFN-treated cells. Analysis of VAI RNA secondary structure (15) showed that the RNA exists in solution as a highly base-paired molecule with two long imperfectly base-paired stems of 23–25 bp joined at the center by a short stem-loop structure (Fig. 7*A*). The base-paired duplex regions were thought to be the crucial determinants of VAI RNA for its interaction with PKR. Extensive mutational analyses, however, revealed that in addition to the ds regions, other structural elements present in the complex stem-loop structure are required for PKR-inhibitory activity of VAI RNA. Our recent study has demonstrated the importance of the short stem-loop structure in the central domain of VAI RNA for its interaction with PKR (20). VAI mutants with mutations in this region, but not elsewhere in the central domain or in the long duplex regions, are defective in binding and inhibiting PKR. These and other results suggest that PKR recognizes a specific secondary or tertiary structure in VAI RNA. This interaction is distinct from its recognition and binding to authentic dsRNA, although the same domain of PKR may be responsible for both interactions (24).

Our studies have identified the dsRNA binding domain (DRBD) of 2–5(A) synthetase (8). The DRBD is located at the amino terminus of the protein between residues 1 and 158. Similar studies with PKR established that its DRBD is also located at the amino terminus (9). Although both DRBDs bind dsRNA, we could not detect any homology in their primary and secondary structures (9). Thus, these two IFN-inducible enzymes apparently belong to two different families of dsRNA binding proteins. Many other members of the PKR family, which bear sequence homology in their DRBDs, have been identified (25). However, the 2–5(A) synthetase family has no other known members. As the noted structural difference would suggest, the characteristics of dsRNA interactions of the two enzymes are also different. PKR binds dsRNA much more strongly than 2–5(A) synthetase; 0.3 M NaCl dissociates the 2–5(A) synthetase-dsRNA complex, whereas the PKR-dsRNA complex is not dissociated even in the presence of 1 M NaCl.[2] Activation of PKR, but not of 2–5(A) synthetase, by dsRNA has a biphasic dose response; low concentrations activate, whereas high concentrations do not. Finally, modified dsRNAs or dsRNAs with periodic mismatches have been shown to activate the two enzymes with different efficiencies (5, 26).

The above differences in the properties of dsRNA interactions of the two enzymes led us to investigate the nature of interactions between 2–5(A) synthetase and adenoviral VA RNAs. Our EMSA results clearly demonstrated that VAI RNA can bind to 2–5(A) synthetase. Moreover, it could compete with authentic dsRNA for binding, thus suggesting that they may be binding to the same site on the protein. These results are consistent with the known properties of VAI RNA interactions with PKR. However, unlike its action on PKR, VAI RNA activated 2–5(A) synthetase. Like authentic dsRNA, this activation was concentration dependent. On a weight basis, VAI RNA was about 10-fold less effective than perfect dsRNA. This is not unexpected, given the fact that VAI RNA is only partially double stranded. Even for perfectly base-paired dsRNAs, there are quantitative differences in their efficacies to activate 2–5(A) synthetase. For example, in the results reported here, we observed that poly(A)·poly(U) was less efficient than poly (I)·poly(C). The VAI RNA preparations used in our studies were free of any contaminating dsRNA which, is sometimes generated as an aberrant product during *in vitro* transcription of RNAs. The strongest evidence for such a conclusion is functional: these preparations of RNAs could not activate PKR either at a low or a high concentration. Moreover, no contaminating RNA could be detected in these preparations when analyzed by denaturing gel electrophoresis.

The results obtained with mutant VAI RNAs are intriguing. Mutants 741, 745, 746, and 748, in which the structure of the central domain of VAI RNA has been disrupted (Fig. 7), do not bind to or inhibit the action of PKR (21). Our results showed that they are also less effective than wild type VAI RNA in activating 2–5(A) synthetase. They are, however, not totally inert. 3–5-fold stimulation was observed with these mutants. This level of activation is not due to nonspecific interaction between 2–5(A) synthetase and any RNA molecule since even a high concentration of perfectly single-stranded RNA, such as poly(A) or poly(U), could not activate the enzyme (Fig. 4). Thus, it is reasonable to speculate that the residual ds structure of these mutants is probably responsible for activating the enzyme. Consistent with the observed, albeit reduced activity of the mutant VAI RNAs, they efficiently bound to 2–5(A) synthetase as monitored by EMSA (Fig. 10). Thus, the observed differences in the ability to activate the enzyme was not reflected at the level of binding to the protein. It is interesting to note that VAII RNA was about 5-fold less efficient than VAI RNA in activating 2–5(A) synthetase. The reason for the inability of VAII RNA to efficiently activate 2–5(A) synthetase is not clear. Currently, the structure of the VAII RNA is not known. Computer-generated structures indicate the presence of ds regions; experimentally determined structure will be necessary to evaluate these results.

The second set of mutants, VAI-BR and VAI-CB, were much better activators than the wild type VAI RNA. In these mutants, the occasional mismatches present in stems I and III have been repaired. Their increased efficacy suggests that these stem structures are important determinants of the ability of VAI RNA to activate 2–5(A) synthetase. Our results also demonstrate that occasional mismatches in the ds region of an RNA can affect its activating potency considerably. Surprisingly, there is a marked difference between the efficacies of VAI-BR and VAI-CB mutants, which indicates that stem III of VAI RNA is a more important element than stem I. The VAI-CB mutant was almost as good as the 82-bp perfect dsRNA and poly(I)·poly(C), thereby suggesting that the 26-bp perfectly paired stem III could be an optimal inducer by itself. The VAI-BR and VAI-CB RNAs, which could activate 2–5(A) synthetase so efficiently, cannot activate PKR, although they can bind to it efficiently and are more active in inhibiting the activity of PKR as compared with the wild type VAI RNA (20). This exemplifies a major difference in the structural requirements of dsRNAs to be effective as activators of the two enzymes.

The underlying mechanism of differential response of the two enzymes to VAI RNA and its mutants remains to be determined. For one set of mutants, those in the central domain, the difference is clearly in their ability to bind to the two proteins. The wild type and the stem mutants, however, can bind to both the proteins. One can speculate that specific conformational changes in the two proteins are required for activating them, and VAI RNA or the BR and CB mutants can promote this change in 2–5(A) synthetase but not in PKR. Alternatively, it is possible that binding of at least two molecules of PKR to the same RNA molecule is necessary for its intermolecular autophosphorylation, and VAI RNA may not be able to bind two PKR molecules. The specific conformational change model is favored by the fact that wild type PKR and mutants of PKR, which do not bind dsRNA, can be activated by heparin (27). Small molecules, such as heparin, are not likely to bind to two large PKR molecules simultaneously, although such a possibility has not been ruled out experimentally.

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