

# Structural features of adenovirus 2 virus-associated RNA required for binding to the protein kinase DAI

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## ABSTRACT

The double-stranded RNA activated protein kinase DAI contains an RNA binding domain consisting of two copies of a double-stranded RNA binding motif. We have investigated the role of RNA structure in the interaction between DAI and the structured single-stranded RNA, adenovirus VA RNA<sub>1</sub>, which inhibits DAI activation. Mutations in the apical stem, terminal stem, and central domain of the RNA were tested to assess the contribution of these elements to DAI binding *in vitro*. The data demonstrate that over half a turn of intact apical stem is required for the interaction and that there is a correlation between the binding of apical stem mutants and their ability to function both *in vivo* and *in vitro*. There was also evidence of preference for GC-rich sequence in the proximal region of the apical stem. In the central domain the correlation between binding and function of mutant RNAs was poor, suggesting that at least some of this region plays no direct role in binding to DAI, despite its functional importance. Exceptionally, central domain mutations that encroached on the phylogenetically conserved stem 4 of VA RNA disrupted binding, and complementary mutations in this sequence partially restored binding. Measurement of the binding of wild-type VA RNA<sub>1</sub> to DAI and p20, a truncated form of the protein containing the RNA binding domains alone, under various ionic conditions imply that the major interactions are electrostatic and occur via the protein's RNA binding domain. However, differences between full-length DAI and p20 in their binding to mutants in the conserved stem suggest that regions outside the RNA binding domain also participate in the binding. The additional interactions are likely to be non-ionic, and may be important for preventing DAI activation during virus infection.

## INTRODUCTION

Adenovirus type 2 (Ad2) virus-associated (VA) RNA<sub>1</sub> is required for the maintenance of viral protein synthesis at late times

of infection and it can stimulate protein synthesis in uninfected cells (1). VA RNA<sub>1</sub> functions by antagonizing a component of the host cell's anti-viral defense pathway, the interferon-induced, double-stranded RNA (dsRNA)-activated protein kinase, DAI (also known as P1, PK-ds, and PKR) (2–6). This enzyme is activated by autophosphorylation, which occurs in the presence of duplexed dsRNA containing at least 30 base pairs (bp) and optimally at least 85 bp (7–9). Such dsRNAs are apparently produced during viral infection (10). Short RNA duplexes, and specialized effectors such as VA RNA, prevent DAI activation and the consequent phosphorylation of its substrate, the protein synthesis initiation factor eIF-2 (8,9). Phosphorylated eIF-2 traps a second initiation factor, the guanosine nucleotide exchange factor (GEF or eIF-2B), leading to an inhibition of protein synthesis initiation (11–15) and suppressing virus replication (16).

VA RNA<sub>1</sub> is a short (160 nucleotide) RNA which is synthesized by RNA polymerase III. It binds to DAI, but unlike dsRNA it does not activate the enzyme (2,3,17–20). Furthermore, at relatively high concentrations VA RNA<sub>1</sub> prevents the binding of dsRNA and blocks DAI activation (21) although it does not inhibit the activity of the kinase after it has been activated (21,22). VA RNA<sub>1</sub> is formally single-stranded but it is highly structured, consisting of two base-paired stems, the terminal and apical stems, connected by a complex stem-loop structure known as the central domain (23,24). This structure was initially proposed on the basis of ribonuclease sensitivity analysis and mutagenic studies (23–25), but more detailed analysis indicated that the central domain of the original model had to be reconsidered (26). Comparison of VA RNAs from different adenovirus serotypes disclosed the existence of two conserved tetranucleotides, GGGU and ACCC, located in the central domain (27,28). These tetranucleotides are complementary to one another and their pairing results in a secondary structure (Fig. 1) differing slightly in the central domain from that originally proposed as a result of the formation of a new stem (stem 4). This structure is consistent with more recent data obtained with a set of chemical and enzymic probes (29).

The RNA binding site of DAI contains two copies of a dsRNA binding motif that is shared with a number of other RNA-binding

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proteins (30,31). Although VA RNA<sub>1</sub> and dsRNA appear to bind to the same site on DAI (20,30,32), they exert opposing effects on enzyme activation. Since the mode of action of VA RNA<sub>1</sub> is not yet established, two general possibilities can be entertained: either its binding to DAI differs in some subtle way from the binding of dsRNA, resulting in the inhibition of enzyme activation; alternatively, some feature of VA RNA<sub>1</sub> interferes with a subsequent step required for autophosphorylation and activation of DAI. To distinguish between these two possibilities demands a detailed understanding of the structural requirements for the binding and activity of VA RNA<sub>1</sub>. One approach to this objective is to examine the effects of mutations in the RNA on its binding and biological activity, but the findings to date have been equivocal. Our initial study indicated that the activity of VA RNA<sub>1</sub> could be separated from its binding ability, implicating the apical stem as the structure involved in binding DAI, and the central domain in the inhibition of DAI activation (23,25). On the other hand, a study of different mutants concluded that long duplexes, such as the apical stem, are not required for VA RNA<sub>1</sub> binding to DAI and that the central domain is the sole requirement for both activity and binding (33).

Most of the mutants studied in binding assays conducted to this point contain relatively coarse deletions or substitutions of large tracts of nucleotides. Since the structure of VA RNA<sub>1</sub> is very compact, the possibility exists that these mutations might affect RNA structure in ways that are difficult to predict or determine by the methods used. We recently generated a set of mutants containing smaller, more directed, mutations that are specific for certain predicted structural features of the apical stem or central domain, including mutations in the newly discovered conserved stem 4 (26,28,34). To assess the contribution of VA RNA<sub>1</sub> structural features to DAI binding and to resolve the controversial binding results, we have examined the interaction of the kinase with a large number of substitution and deletion mutants in the apical stem and central domain. We conclude that the primary interaction requires an intact apical stem containing at least 8 base pairs. Some central domain mutations affect binding, but generally to a small extent, implying that there are secondary interactions with the central domain. These interactions seem to involve regions of DAI outside its RNA binding domain, suggesting that they may be involved in blocking DAI activation.

## MATERIALS AND METHODS

### Labeling of VA RNAs

Most of the RNAs were uniformly labeled by transcription of DraI linearized plasmid pT7VA and its mutant derivatives using T7 RNA polymerase (19). The *ls1* series of mutant RNAs were uniformly labeled by transcription from plasmid pMHVA and its derivatives using RNA polymerase III (23). The stem 4 mutants were transcribed using T7 polymerase and were labeled at the 3' end with [<sup>32</sup>P]-pCp using T4 RNA ligase (35). Reactions were performed by the addition of master mix containing all the components required for the labeling reaction to ensure identical specific activities (approximately 1 × 10<sup>6</sup> Cerenkov counts/min/μg RNA) and in each case, wild-type Ad2 A RNA<sub>1</sub> was labeled in parallel. The labeled RNAs were purified by electrophoresis through denaturing and native polyacrylamide gels as described previously (19).

**Immobilization of DAI.** Human DAI was obtained from the ribosomal salt wash (RSW) of interferon-treated 293 cells (21) and the RNA binding fragment of DAI (p20) was purified from

*E. coli* overexpressing the protein (9,36). For binding reactions, 10 μl of RSW or 25 ng of p20 were incubated with 0.5 μl of ascites fluid containing a monoclonal antibody directed against DAI (37) in buffer BII (38) containing 20 mM Tris-HCl, pH 7.5, 50 mM KCl, 400 mM NaCl, 1 mM EDTA, 1 mM DTT, 100 units/ml aprotinin, 0.2 mM PMSF, 20% glycerol, and 1% Triton X-100. The immune complexes were isolated by adsorption to an equal volume of a 10% suspension of protein A-Sepharose beads (Pharmacia). In later experiments, protein A-Sepharose was replaced by protein G-Sepharose (Pharmacia) which gave more efficient recovery of immune complexes without otherwise affecting the results. After incubation for 10 min at 4°C, the beads were washed at least six times in buffer BII. The immunosorbent was prepared in bulk and divided prior to the RNA binding reactions.

### Binding of VA RNA<sub>1</sub> to DAI

Sepharose beads carrying immobilized DAI or p20 were equilibrated with RNA binding buffer by washing twice in buffer A (buffer III of Katze *et al.* (38)) or buffer B (buffer III of Mellits *et al.* (25)). Buffer A contains 10 mM Tris-HCl, pH 7.4, 100 mM KCl, 2 mM MgCl<sub>2</sub>, 2 mM MnCl<sub>2</sub>, 10 μM ATP, 7 mM β-mercaptoethanol, 0.1 mM EDTA, 100 units/ml aprotinin and 20% glycerol. Buffer B contains 25 mM Hepes.KOH, pH 7.4, 100 mM KCl, 10 mM MgCl<sub>2</sub>, 0.1 mM EDTA, 1 mM DTT, 100 units/ml aprotinin, 10 μM PMSF and 0.1 mg/ml RNase-free BSA. VA RNA (25,000–50,000 Cerenkov counts/min, giving a final concentration of about 1 μg/ml, approaching that used in DAI phosphorylation assays) and 100 μg/ml calf liver tRNA (Boehringer Mannheim) were mixed with the beads in a final volume of 30 μl for 20 min at 30°C in buffer A, or for 5 min at 30°C followed by 25 min at 4°C in buffer B. Complexes were washed at least four times with 1 ml of binding buffer supplemented with 10 μg/ml calf liver tRNA. Radioactivity bound to the beads was quantified by Cerenkov counting. In each experiment, binding was normalized to the binding of wild-type VA RNA<sub>1</sub> after subtracting the background binding value obtained in the absence of DAI. Most experiments also included the severely disrupted mutant *ls1* as a control for a mutant RNA that binds poorly. In some cases, bound RNA was recovered and examined by electrophoresis through a denaturing polyacrylamide gel: no degradation was detected, and the results were in qualitative agreement with the radioactivity counted.

### Electrophoretic mobility of RNA

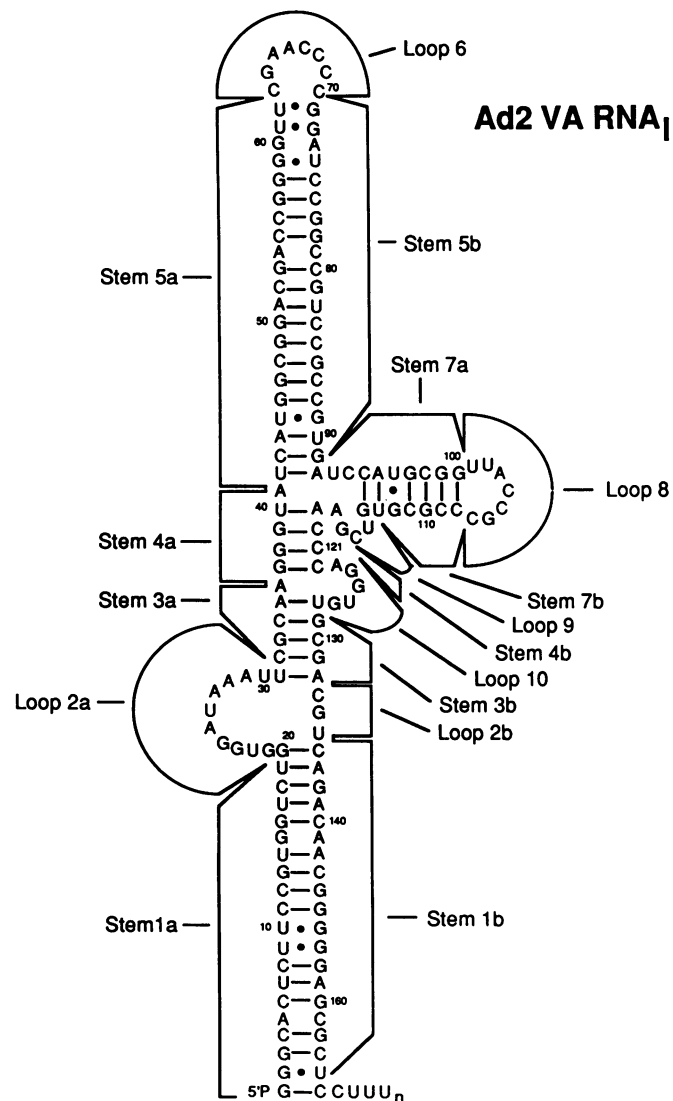
Each mutant RNA was mixed with synthetic marker RNAs (9) and subjected to gel electrophoresis in 8% polyacrylamide gels containing 7 M urea and 0.5 × TBE. When the mobility of the marker RNAs was plotted against chain length, the points fell on a straight line indicating that these transcripts are devoid of secondary structure. The plot was used to determine the mobility for each VA RNA expected for its known chain length. All VA RNAs migrated more slowly than predicted, and a mobility retardation factor was calculated by comparison with the actual mobility measured. The percentage retardation is 100 × (1 – actual mobility/expected mobility).

## RESULTS

### Binding of conserved stem 4 mutants to DAI and p20

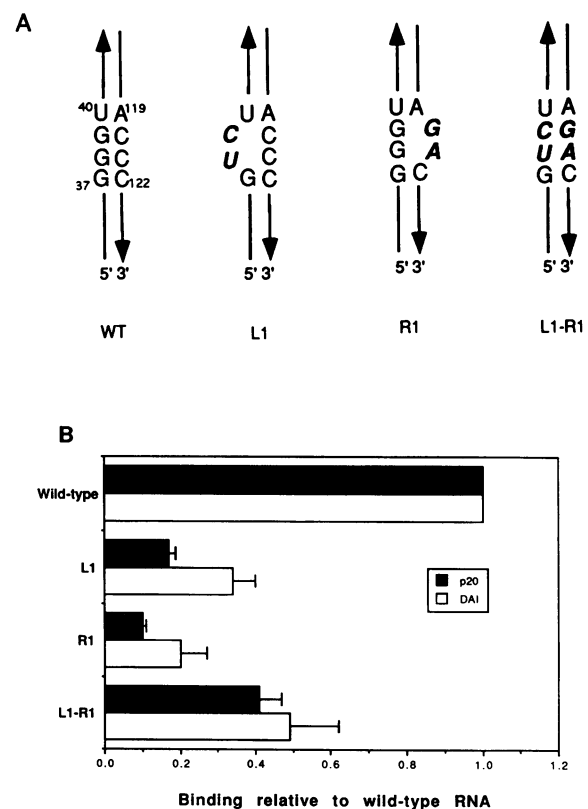
Although it is generally agreed that the central domain of Ad2 VA RNA<sub>1</sub> is essential for the molecule's ability to block DAI

activation (23–25,33), our previous studies (25) indicated that the apical stem rather than the central domain, is critical for binding to DAI. Consistent with this view, DAI apparently binds VA RNA<sub>1</sub> in a similar fashion to dsRNA and at the same or a closely related site (21,30,32). Others, however, have suggested that the central domain is critical for VA RNA<sub>1</sub> binding (33) as well as for its function. Recent work (27,29) led to a revised structural model containing a more compact central domain (Fig. 1), prompting us to reinvestigate the binding of VA RNA<sub>1</sub> to DAI. While the apical stem remains unchanged in the new model, the central domain contains a new stem (stem 4), composed of two highly conserved complementary tetranucleotides (27,28). Stem 4 occupies a key location in the revised structure, forming a link between the apical stem and the terminal stem in the duplex axis of the molecule, as well as an important feature of the central domain.



**Figure 1.** Secondary structure model of Ad2 VA RNA<sub>1</sub>. Stem 1 is the terminal stem, stem 5 and loop 6 comprise the apical stem-loop, and stems 3, 4 and 7 and loops 2, 8, 9 and 10 form the central domain. Stem 4 is phylogenetically conserved.

Mutations that disrupt and restore base-pairing in stem 4 have been constructed, as illustrated in Fig. 2A. The mutations L1 and R1 are two-nucleotide substitutions in stem 4a and stem 4b, respectively, that disrupt base-pairing in stem 4. The combination of these two mutations forms a double mutant, L1-R1, designed to restore base-pairing and reconstitute the stem. The analysis of the structure and function of these mutant RNAs is described elsewhere (28). Here we have examined the effect of the stem 4 mutations on binding to intact DAI and to p20, a truncated version of the protein consisting of its first 184 amino acids and containing its RNA binding domain (18,30,32). Although both the full length kinase and p20 bind VA RNA<sub>1</sub> efficiently (30), the functional importance of the central domain suggested that this region might interact with the catalytic part of the kinase. Since stem 4 is a highly conserved element of the central domain, it also seemed possible that disruption of stem 4 might differentially affect the binding to DAI and p20. As shown in Figure 2B, L1 and R1 both bound to DAI with reduced efficiency (34% and 20% of wild-type, respectively). Instead of being more severely affected than the single mutant as would be expected for independent mutations, the double mutant L1-R1 bound better than either single mutant (46% of wild-type). This indicates that the mutants are structurally compensatory, and supports the existence and importance of stem 4. Since binding was not fully restored, however, it is likely that the sequence in stem 4, as



**Figure 2.** Effects of mutations in conserved stem 4 on binding to DAI and p20. (A) Diagram of mutant sequences, showing the alterations in bold italic type. (B) Binding of 3' end labeled wild-type and mutant RNAs to immobilized DAI or p20 in buffer B, expressed relative to that of wild-type RNA. Error bars show standard errors.

well as its base pairing, plays a role in binding, as implied by its evolutionary conservation.

A similar pattern of binding behavior was observed using p20 in place of DAI, but the consequence of disrupting base pairing in stem 4 was more deleterious than for DAI binding by about two-fold (Fig. 2B). This observation suggests that p20 binding is heavily dependent on duplex structure, while regions of DAI outside the RNA binding domain can partially overcome the effect of disruptive mutations in stem 4. Consistent with this inference, intact DAI gives a slightly more extensive footprint on VA

RNA<sub>1</sub> than p20 does, implying that regions outside the RNA binding domain are also in contact with VA RNA<sub>1</sub> (29).

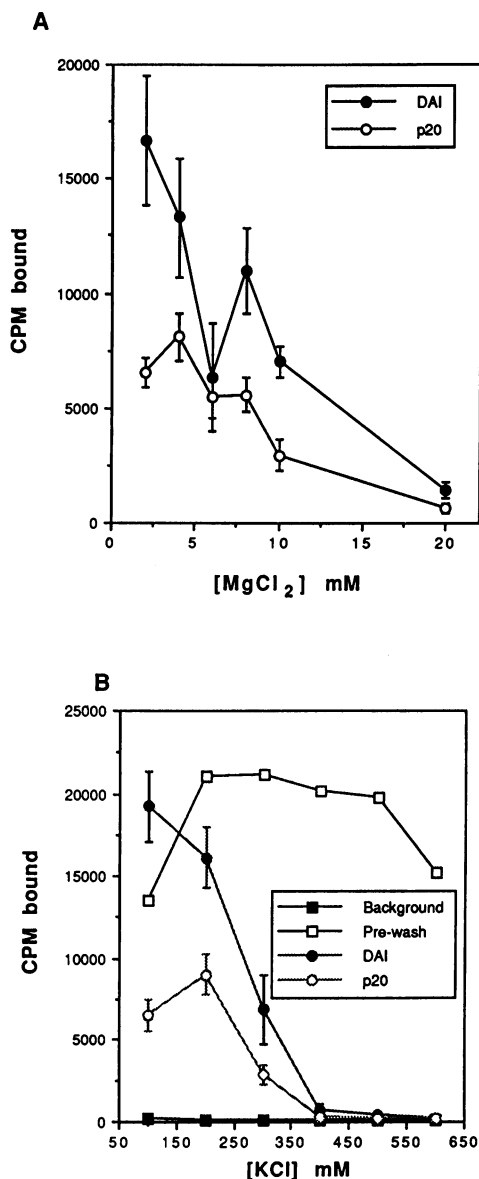
### Influence of binding conditions

The reduced binding of stem 4 mutants to both DAI and p20 indicated that at least part of the central domain is important for binding, and led us to reexamine the contribution of the apical stem and central domain to VA RNA<sub>1</sub> interaction with full-length DAI. Because of the contradictory results in the literature, we first compared the binding of wild-type VA RNA<sub>1</sub> to DAI under the two sets of binding conditions used previously. Binding was about 5-fold higher in buffer A (33) than in buffer B (25) and the background binding ratio between the two buffers, obtained in the absence of monoclonal antibody, DAI or both, was also about 5 (data not shown). The most conspicuous difference between the buffers is that buffer A contains 2mM MgCl<sub>2</sub>, 2mM MnCl<sub>2</sub> and 10mM ATP, while buffer B contains 10mM MgCl<sub>2</sub> and no MnCl<sub>2</sub> or ATP. It has been noted that the binding of VA RNA<sub>1</sub> to DAI is reduced significantly in the absence of Mg<sup>2+</sup> (22), so the difference in the concentration of this ion between buffer A and B could be significant. As shown in Fig. 3A, binding declined with increasing concentrations of MgCl<sub>2</sub> such that it was about 2.5 times less at 10mM than at 2mM MgCl<sub>2</sub>. Similar results were obtained with p20 (Fig. 3A). Since elevated concentrations of this divalent cation reduce the binding of VA RNA<sub>1</sub>, we also examined the effect of monovalent cation concentrations on binding. Fig. 3B shows that increasing concentrations of KCl also reduced the binding of VA RNA<sub>1</sub> to DAI or p20. Pretreatment of immunosorbed DAI with varying KCl concentrations had no significant effect on the binding of VA RNA<sub>1</sub> at 100mM KCl (Fig. 3B, open squares), indicating that the immune complex is not disturbed by high salt concentrations. In view of the strongly basic nature of the RNA binding region of DAI (30), the salt effects are probably due to the shielding of electrostatic interactions between the protein and the RNA. An alternative interpretation, that alterations in VA RNA<sub>1</sub> structure are responsible, is less likely since the nuclease digestion patterns obtained at 2mM and 10mM MgCl<sub>2</sub> are indistinguishable (data not shown).

Prior to testing the full series of VA RNA mutants, we verified the specificity of binding under the same conditions. Calf liver tRNA reduced binding slightly, probably by blocking non-specific interactions, and was therefore routinely included in binding reactions. The binding of wild-type VA RNA<sub>1</sub> was greatly reduced by unlabeled competitor VA RNA<sub>1</sub> or dsRNA (data not shown), confirming that VA RNA<sub>1</sub> and dsRNA interact with DAI at similar sites. Reciprocal experiments demonstrated that VA RNA<sub>1</sub> competes with dsRNA for binding to DAI (21), although Galabru *et al.* (22) did not observe a reduction by dsRNA of VA RNA binding. More recently, however, mutational analysis of DAI has shown that similar or identical elements and amino acids are required for dsRNA binding and VA RNA<sub>1</sub> binding (18,20,30,32), suggesting that the sites are closely related, if not one and the same.

### Apical stem and central domain mutants

To examine the influence of RNA structure on the binding of VA RNA<sub>1</sub> to DAI, we employed two sets of mutants. The first set contains mutations in the apical stem; the second set contains mutations in the central domain, together with a truncated form of the wild-type molecule, AatII, which lacks the 3' part of the



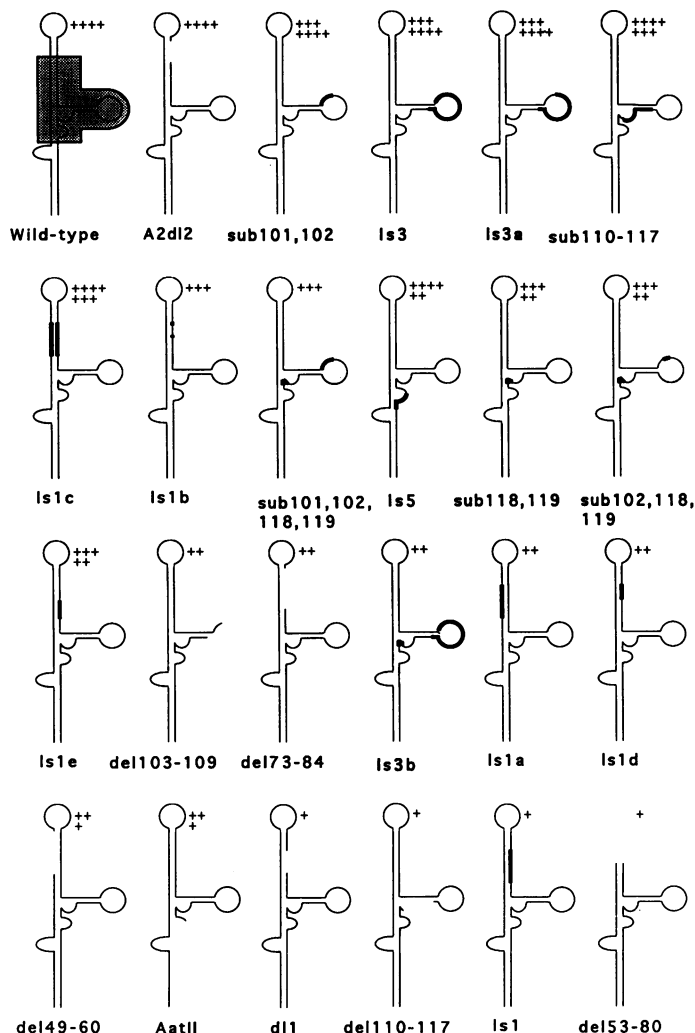
**Figure 3.** Influence of KCl and MgCl<sub>2</sub> concentrations on the binding of VA RNA<sub>1</sub> to DAI and p20. Wild-type VA RNA<sub>1</sub> was 3' end-labeled and reacted with immobilized DAI or p20 under buffer B conditions except that either (A) MgCl<sub>2</sub> or (B) KCl concentrations were varied. For the pre-wash control in (B), the DAI-containing immune complexes were washed at the KCl concentration indicated, then washed twice to bring the KCl concentration back to 100mM KCl prior to the RNA binding reaction and subsequent washing at 100mM KCl. The control background binding reaction was with protein G-Sepharose only. Error bars show standard deviations.

terminal stem. The mutations are specified in Table 1 and their positions on the wild-type secondary structure are illustrated in Fig. 4. The actual secondary structures of the mutant RNAs were determined using ribonuclease sensitivity analysis as described previously (23,25,26,34). The RNAs were all purified by sequential electrophoresis through denaturing and non-denaturing gels to remove dsRNA contaminants (19). During purification it became apparent that some mutations affect the electrophoretic mobility of the RNA in denaturing gels as reported earlier (24,25,39,40). To correlate the gel mobility with the structures of the mutant VA RNA molecules and their binding to DAI *in vitro*, we systematically examined the mobility of the RNAs. The results, which are illustrated in Figure 5 and listed in Table I, separated the VA RNA mutants into three groups: group I, II and III RNAs were retarded about 30%, 15% and 5%, respectively. It is striking that group I contains all the RNAs with mutations in the central domain, plus A2dl2, AatII and wild-type VA RNA<sub>1</sub>, while the less retarded RNAs were all mutated in the apical stem.

Aberrant electrophoretic mobility is usually due to denaturation-resistant secondary structures (41). Since mutations in the central domain do not cause structural changes in the apical stem (26), and group I contains the AatII truncation which lacks a terminal stem, it is likely that the structural feature responsible for the retardation lies in the apical stem. The mutant A2dl2 falls into this group even though it has a deletion of six nucleotides (nts. 73–78) in this stem. Despite the deletion, it is still able to form an extended, stable apical stem (23,25) and, in fact, A2dl2 RNA adopts a more compact apical stem-loop structure than wild-type VA RNA under non-denaturing conditions (25). It is likely that wild-type VA RNA<sub>1</sub> and the central domain mutant RNAs adopt the A2dl2-like structure under the denaturing conditions of the gel. Consistent with this interpretation, deletions in the apical stem that prevent the formation of a stem structure result in RNA mobilities approaching that of a random coil. The two mutants in group III, *del49-60* and *del53-80*, display only 7% and 4% retardation, respectively. Furthermore, mutant RNAs in group II, which display intermediate mobility, retain the ability to form

**Table 1.** Sequence and electrophoretic mobility of VA RNA mutants

A) Apical stem mutations.						
	41	51	61	71	81	Mobility Retardation
Wild-type	AUCAUGCGG	ACGACCGGG	UUCGAACCC	GGAUCCGCC	GUCCCGCUG	33
ls1	-----	-----	-----	-----	CAGAU--U--	17
ls1a	---CA--AUC	UG-----	-----	-----	-----	15
ls1b	-----	-----	-----	-----	---U--U--	14
ls1c	---CA--AUC	UG-----	-----	-----	CAGAU--U--	16
ls1d	-----	-----	-----	-----	CAGA-----	15
ls1e	-----	-----	-----	-----	---AAAC--	15
del49-60	-----xx	xxxxxxxxxx	-----	-----	-----	7
del53-80	-----	--xxxxxxxx	xxxxxxxxxx	xxxxxxxxxx	-----	4
del73-84	-----	-----	-----	-----	-----	14
A2dl2	-----	-----	-----	-----	-----	29
d11	-----	-----	-----	-----	---U-xxx	12
d12	-----	-----	-----	-----	---U-xxx	14
d13	-----	-----	-----	-----	---U-xxx	14
d14	-----	-----	-----	-----	---U-xxx	15
	-----		-----			
	stem 5a		loop 6		stem 5b	
B) Central domain and terminal stem mutations.						
	91	101	111	121	131	
Wild-type	AUCCAUGCGG	UUACCGCCG	CGUGCGAAC	CCAGGUGUGC	GACGUCAGAC	33
d13	xxxxxx---	-----	-----	-----	-----	14
d14	xxxxxxxxxx-	-----	-----	-----	-----	15
ls2	-----GAUCUGG	-----	-----	-----	-----	30
ls3	-----	CAGAUCUGxG	-----	-----	-----	30
ls3a	-----	--GAUCUGxG	-----	-----	-----	30
ls3b	-----	CAGAUCUGxG	-----UG-	-----	-----	30
del103-109	-----	--xxxxxxxx-	-----	xxx-	-----	29
ls4	-----	-----AGA	UCUxxxxxxxx	-----	-----	27
sub110-117	-----	-----U	ACACAUC--	-----	-----	31
del110-117	-----	-----x	xxxxxxxx--	-----	-----	28
sub118-119	-----	-----	xxxxxx-UG-	-----	-----	31
sub101,102	-----	CA-----	-----	-----	-----	30
sub102,118,119	-----	-A-----	-----UG-	-----	-----	30
sub101,102,118,119	-----	CA-----	-----UG-	-----	-----	30
ls5	-----	-----	-----	-----CAGAX	UCU-----	32
AatII	-----	-----	-----	-----	xxxxxxxxxxx	29
	-----		-----			
	stem 7a		stem 7b		stem 4b	
	loop 8		loop 9		stem 3b	
	-----		-----		loop 2b	



**Figure 4.** Schematic representation of the mutations. Superimposed on the wild-type VA RNA<sub>1</sub> structure, deletions are represented by gaps and substitutions by thick lines. The mutants are presented in order of decreasing binding efficiency, ranked as in Table 2. The stippled area outlines the region of wild-type VA RNA<sub>1</sub> that is protected from chemical and nuclease attack by DAI (29).

part of the structure. They all contain disruptions toward the base of the apical stem but they can still form a short hairpin structure by pairing the CCGGG and CCCC GG sequences (nucleotides 55–60 and 67–72), as in A2dl2 RNA. This residual structure presumably accounts for the approximately 15% retardation that characterizes the group. Remarkably, even small changes, such as the two nucleotide mutations in *ls1b* and the compensating mutations of *ls1c* which fully restore the ability to pair in the lower part of the apical stem, result in reduced gel retardation. This observation reinforces the earlier suggestion that the basal part of the apical stem plays an important role in VA RNA structure and function (34), a conclusion that is consistent with footprinting data (29) and is tested below with respect to DAI binding.

#### Binding of apical stem mutants to DAI

Because of the substantial quantitative difference between buffers A and B in wild-type VA RNA<sub>1</sub> binding efficiency, we considered the possibility that there might also be qualitative

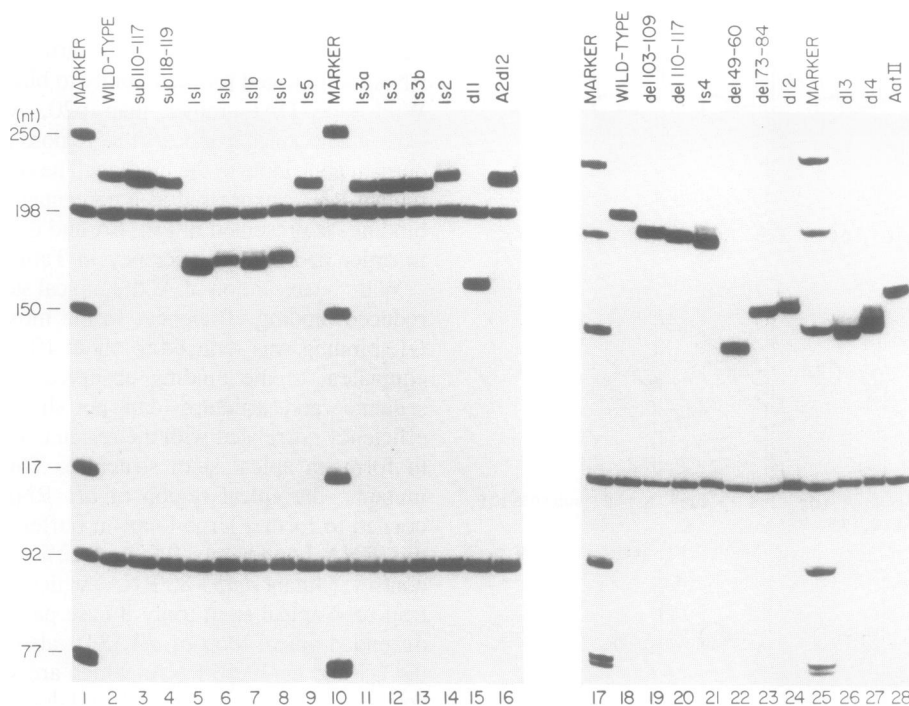
differences between the buffers with respect to the effect on binding of mutations in VA RNA structure. Therefore, all mutant RNAs were tested for their ability to bind to DAI in both buffers. Wild type DAI, rather than p20, was employed in these experiments since it appears that regions outside the RNA binding domain contribute to the binding. The binding efficiency for each mutant RNA, expressed as a percentage of wild-type VA RNA<sub>1</sub> binding, is presented in Fig. 6A and the results are summarized in order of binding efficiency in Table 2a and Fig. 4.

With the exception of A2dl2, apical stem mutants all displayed reduced binding efficiency. In the most severe cases, *dl1* and *ls1*, binding was reduced to about 10% of the wild-type level, equivalent to the binding observed with RNAs of unrelated sequence and structure (data not shown). In general, binding efficiency correlated with the residual ability of the mutant RNA to form an apical stem structure. Thus, among the deletion mutants, the apical region of *dl1* RNA is almost completely opened to form a large loop; in buffers A and B, respectively, this RNA bound only 9% and 12% as well as the wild-type control. Mutant *del53-80* RNA, which also possesses a severely truncated apical stem (only 8 base pairs) but does not have the distended apical loop of *dl1* (34), also bound very weakly, as did *ls1* and *del49-60* RNAs which are severely disrupted in the apical stem as well as in the central domain. The mutant *del73-84* RNA, in which the right side of the apical stem is deleted, rearranges to form a longer apical stem (13 base pairs) with a small bulge on the left side of the stem (34); accordingly, this RNA bound significantly better (36% and 37% of control). Taking these results together, it seems that truncation of the stem reduces binding and that more than 8 base pairs of apical stem are required for the efficient binding of VA RNA<sub>1</sub> to DAI. Not all deletions in the apical stem were deleterious, however; A2dl2 RNA, which exhibits a different, more compact pairing in the apical stem and a central domain structure similar to that of wild-type (23,25), bound to DAI even more efficiently than wild-type RNA (140% and 170%). Therefore, mutant RNA can bind efficiently provided that an extended apical stem is retained.

The series of substitution mutants *ls1a*–*ls1e* introduce more subtle changes within the lower part of the apical stem. These RNAs are less disturbed in the apical stem region and displayed correspondingly less aberrant binding properties. Mutants *ls1b* and *ls1c* RNAs are essentially wild-type in structure according to nuclease sensitivity analysis although their gel behavior revealed some differences. Their binding was reduced only slightly (63 and 82% of control). Mutations *ls1a*, *lsd* and *ls1e*, which introduce interior bulges that interrupt the continuity of the apical stem, reduced RNA binding to a greater extent (26–68% of control). Thus, the stability and continuity of the apical stem make a major contribution to the binding capacity of VA RNA<sub>1</sub> to DAI, corroborating our previous conclusion (25) but not that of others (33).

#### Binding of central domain mutants to DAI

It is generally agreed that mutations within the central domain abrogate the function of VA RNA<sub>1</sub> (23–25,33), but there are contradictory views as to their impact on DAI binding. Our data indicated that central domain mutations only slightly reduced the ability of the RNA to bind to DAI (25), whereas other results (33) suggest that the central domain is the sole determinant of DAI binding. Recently, the functional role of several elements within the central domain was investigated by the construction of a further series of small substitution and deletion mutants (26).



**Figure 5.** Mobility of wild-type and mutant RNAs. Each labeled VA RNA was mixed with marker RNAs and subjected to electrophoresis in 7M urea/polyacrylamide gels. Detection was by autoradiography.

To reassess the role of the central domain, we examined the binding of these and other mutant RNAs to DAI (Fig. 6B, Fig. 4 and Table 2b).

Several of the central domain mutations reduced binding, but in general they had less effect than the apical stem mutations, and none of them abrogated binding. Mutations that substituted sequences between nucleotides 101 and 117 (mutants *ls3*, *ls3a*, *sub101,102*, and *sub110,117*) caused the least reduction in binding and in some cases even increased binding (ranging between 60 and 150% of control). Deletion of the same nucleotides, in *dl103-109* and especially in *dl110-117*, had a greater effect (ranging from 14 to 49% of control) than substitution of the sequence (ranging from 58–102% of control) in *ls3a* and *sub110-117*. Presumably, the removal of a segment of the molecule causes a greater distortion in structure than its substitution. All mutant RNAs containing a substitution at nucleotides 118 and 119 (*sub118,119*, *sub102,118,119*, *sub101,102,118,119*, and *ls3b*), encroaching on conserved stem 4, exhibited modest reductions in binding (23 to 52% of wild-type). Substitution of nucleotides 125–134 (*ls5*) also led to slightly reduced binding (35 and 76% of wild-type). Truncation of the VA RNA<sub>1</sub> molecule to 132 nucleotides in AatII RNA, by deleting stem 1b and loop 2b, generated a molecule with a disrupted terminal stem and a slightly disturbed central domain. These changes also reduced binding (12 and 50% of wild-type), confirming that regions outside the apical stem can influence binding, albeit usually to a lesser extent.

#### Differential effects of binding conditions

The data discussed to this point confirm the importance of the apical stem for binding but modify our previous conclusion in

that several of the new mutants revealed an influence of the central domain on binding. Comparison of RNA binding efficiency in the two buffers, A and B, revealed some further distinctions. It can be seen from Fig. 6A that, with most apical stem mutants, differences between the buffers were minor. Exceptionally, three VA RNAs (*del53-80*, *del49-60* and *ls1e*) bound more than 20% better in buffer A than in buffer B, indicating that in some cases buffer B accentuates the requirement for the apical stem. The binding of certain central domain mutant RNAs was also affected by the buffer composition (Fig. 6B). Large differences were noted with *ls3*, *ls3a* and *sub101,102*, which bound much better in buffer B, while *sub102,118,119* and *ls5*, as well as AatII, bound better in buffer A. These observations suggest that substitutions in the central domain which do not disrupt any stem in the VA RNA<sub>1</sub> molecule have no effect on binding in buffer B, whereas the integrity of the central domain structure is much more important for binding in buffer A. A good example is mutant *ls5* RNA which has a central domain digestion pattern similar to that of wild-type VA RNA, but stem 3 cannot be formed (23,25). In buffer A, *ls5* RNA bound 76% as well as wild-type while in buffer B it bound only 35% as well. Similarly, the binding efficiency of AatII RNA, which is deleted in stem 1 but is structurally relatively normal in the central domain (data not shown), bound 50% as well as wild-type VA RNA in buffer A but only 12% as well in buffer B. Thus, it appears that binding in buffer B is highly dependent on the integrity of the stem structures forming the axis of the RNA molecule (i.e., stems 1, 3, 4 and 5), whereas binding in buffer A is more sensitive to changes in the central domain. Perhaps a small stem-loop structure or a more complicated tertiary structure element is responsible for better binding to DAI in buffer A conditions.

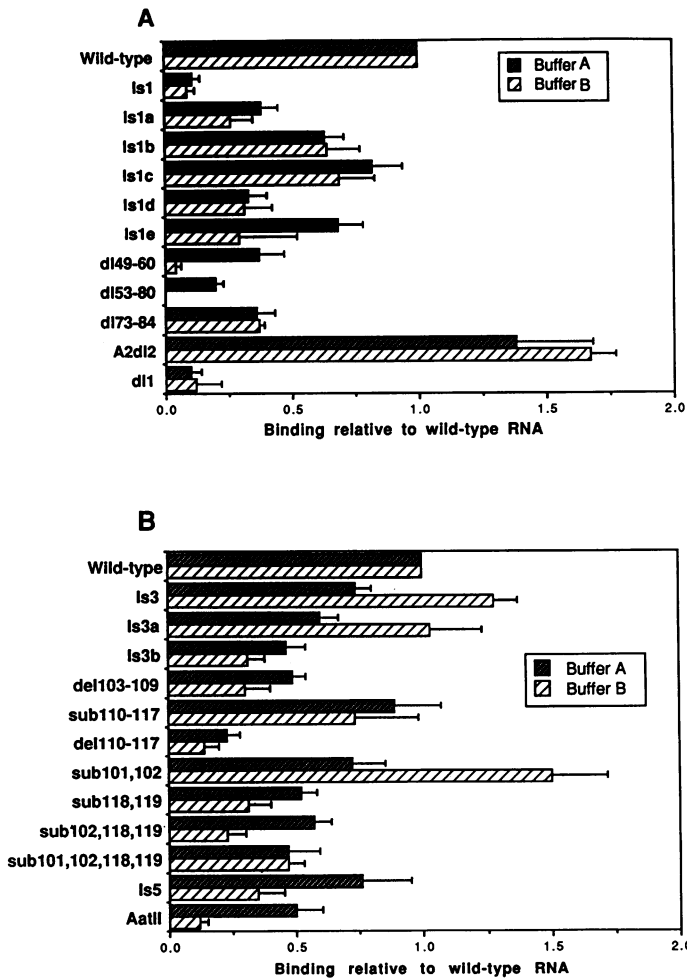
**DISCUSSION**

The protein kinase DAI is regulated by RNA effectors that cause or prevent its activation. The RNA ligands bind to a site in the protein's N-terminal region containing two repeats of a 67 residue motif. This motif, the dsRBM, is found in a number of proteins that bind dsRNA and structured single-stranded RNAs (30,31). Despite growing understanding of the motif and its interaction with RNA, the basis for the differential response of the kinase to activators (such as dsRNA at suitable concentrations) and inhibitors (such as VA RNA<sub>1</sub>) remains unknown. No DAI mutant in the RNA binding domain has been reported to discriminate between dsRNA and VA RNA (18,20,30,32), implying that discrimination takes place subsequent to binding of the RNA ligand. Consistent with this view, our analysis indicates that a duplexed region of VA RNA<sub>1</sub>, its apical stem, plays an important role in binding to DAI while a neighboring structure, the central domain, is required to block enzyme activation (25). On the other hand, studies by Ghadge and co-workers (33,42) concluded that the central domain is responsible

for both binding the enzyme and inhibiting its activation. These contrasting views were derived from examination of different panels of VA RNA mutants. The interpretation of mutational analyses of VA RNA<sub>1</sub>-DAI interactions is complicated by the intricate and compact structure of the RNA inasmuch as mutations may have long distance effects on RNA secondary and tertiary structure. Because of the existence of a revised structural model for VA RNA<sub>1</sub> (26-29) as well as new series of mutants in the apical stem and central domain (26,34), we reexamined the binding of VA RNA<sub>1</sub> to DAI.

**Dependence of binding on ionic conditions**

We observed quantitative and qualitative effects of the ionic milieu on the binding efficiency of VA RNA to DAI. The binding of wild-type VA RNA<sub>1</sub> to DAI decreased as the concentration of KCl or MgCl<sub>2</sub> was increased, probably because cations compete with basic groups of the protein for sites on the nucleic acid backbone (i.e., phosphate groups) (43,44). Very similar salt dependency was observed with p20, implying that the major electrostatic interactions in the RNA-protein complex are mediated by the basic N-terminal region of DAI, as proposed



**Figure 6.** Binding of VA RNA<sub>1</sub> mutants to DAI. Mutant RNAs in the apical stem (A) and the central domain (B) were labeled and reacted with immobilized DAI. Binding was quantified by Cerenkov counting and is expressed relative to the binding of wild-type RNA included as a control in each experiment. Background binding obtained in the absence of DAI was subtracted. Error bars indicate standard errors.

**Table 2.** Binding of VA RNA mutants to DAI

	Binding to DAI <sup>(a)</sup>		Kinase inhibition <sup>(b)</sup>			Mobility Group <sup>(c)</sup>
	in Buffer		at (µg/ml)			
	A	B	50	20	5	
<b>a) Apical stem mutants.</b>						
A2dl2	++++	++++	+++	+++	++	I
wild-type	++++	++++	+++	+++	++	I
Is1c	++++	+++				II
Is1b	+++	+++				II
Is1e	+++	++				II
Is1a	++	++				II
Is1d	++	++				II
del73-84	++	++	++	++	-	II
del49-60	++	+	+	-	-	III
del53-80	+	+	+	-	-	III
Is1	+	+	+	-	-	II
dl1	+	+	-	-	-	II
<b>b) Central domain mutants.</b>						
wild-type	++++	++++	+++	+++	++	I
sub101,102	+++	++++	+++	+++	+++	I
Is3	+++	++++	+	-	-	I
Is3a	+++	++++	+++	++	-	I
sub110-117	++++	+++	+++	++	+	I
Is5	++++	++	+	-	-	I
sub101,102,118,119	+++	+++	+++	+++	+	I
sub118,119	+++	++	+++	++	-	I
sub102,118,119	+++	++	+++	+++	-	I
Is3b	++	++	-	-	-	I
del103-109	++	++	+++	+++	+	I
AatlI	++	+	++	+	-	I
del110-117	+	+	++	++	+	I

<sup>a</sup>Binding data are summarized from Fig. 6. Relative to wild-type VA RNA<sub>1</sub>, + + + + represents >75% binding; + + +, 50-75%; + +, 25-50%; +, <25%.

<sup>b</sup>Kinase inhibition data are from references (25,26): + + +, complete inhibition; + +, partial inhibition; +, slight inhibition; -, no inhibition.

<sup>c</sup>Electrophoretic data are from Table 1: group I, 27-33% retardation; group II, 12-17% retardation; group III, 4-7% retardation.



by Green and Mathews (30). Calculations using the method of Record *et al.* (43) suggest that about 5 monovalent counterions are released upon the binding of VA RNA to either DAI or p20. Although these measurements are not strictly quantitative, they imply that the electrostatic interactions of DAI and p20 with VA RNA<sub>1</sub> are similar.

The influence of ionic conditions was also evident when the binding of certain VA RNA mutants was compared in two different buffers. Wild-type VA RNA<sub>1</sub> bound more efficiently in buffer A than buffer B, consistent with the lower ionic strength of the former. As a rule, the integrity of the central domain was more critical in buffer A whereas the requirement for an intact apical stem was emphasized in buffer B. Buffer A contains less Mg<sup>2+</sup> than buffer B, but this difference is partly compensated for by the presence of Mn<sup>2+</sup> in buffer A, so the different behaviors of the mutant are hard to rationalize at the molecular level. Nevertheless, these effects undoubtedly go some way to explaining the conflicting observations made by Ghadge and coworkers (using buffer A) and Mellits *et al.* (using buffer B).

### Structure and function of VA RNA mutants

Table 2 summarizes the function of apical stem and central domain mutants in terms of their ability to bind DAI and to inhibit its activation, as well as their electrophoretic mobility. It is well known that some nucleic acid structures, such as strong hairpins, persist in sequencing gels despite the high concentration of urea and elevated temperature. This can cause band compression (45) and reflect significant intrinsic features of the molecule. For example, chemical modification of tRNA, which normally migrates more slowly than expected for its size, reduces its secondary structure and thereby increases its mobility (46). Several reports have noted that VA RNA also moves more slowly than expected, and that this retardation is reduced by some mutations (24,25,39,40) especially those located in the apical stem. Data presented here suggest that the region responsible lies in the distal part of this stem and that the effect is due to a structure formed when an alternative pairing scheme is adopted by looping out seven nucleotides (nucleotides 73–79), introducing a bulge in the apical stem. Proximal to this bulge the pairing scheme is unaffected, but distal to it the tip of the apical stem and loop (nucleotides 55–72) is reorganized to form a different stem and loop structure. This pairing scheme is similar to that observed for A2dl2 (25), which lacks these six nucleotides, and is supported by the data of Rohan and Ketner (40) who noted that point mutations in the bulge and apical loop do not affect RNA mobility whereas mutations elsewhere in the apical stem cause increased mobility. The observation that in wild-type VA RNA some of these nucleotides are somewhat sensitive to cutting by single-strand specific nucleases in the presence of Mg<sup>2+</sup> (unpublished data) raises the possibility that this region is in equilibrium between the bulged form and the structure shown in Figure 1 in physiological conditions.

Based on their mobilities, we classified the mutant RNAs into three groups: group I showed the slow mobility of wild-type VA RNA<sub>1</sub>, group III migrated nearly as fast as expected for randomly coiled molecules, and group II occupied an intermediate position. Group I contains the central domain mutants together with AatII RNA (which is deleted in the terminal stem) and A2dl2 (which is deleted in the bulge region). All these RNAs can form the A2dl2-type structure in the apical stem. Group II RNAs have deletions or substitutions in the proximal part of the apical stem

between nucleotides 73 and 99, which affect the region below the bulge but allow the tip of the apical stem-loop to adopt the A2dl2-like structure. This apparently results in the reduced stability of the structure but does not completely prevent it from forming, hence the intermediate electrophoretic mobility. Replacement of GC pairs with AU pairs in the base of the apical stem is sufficient to destabilize the structure in *ls1c* RNA, which is therefore a member of group II. Group III RNAs have deletions encroaching on nucleotides 55–72 which form the tip of the reorganized A2dl2-like apical stem-loop: accordingly, these RNAs retain little or no residual structure under electrophoresis conditions. Inspection of Table 2 shows that most of the mutants which display wild-type function belong to group I, but some group II mutants (such as *ls1c*) were effective in binding assays and were also able to rescue protein synthesis *in vivo* (34). Not all group I mutants were as functional as wild-type RNA, however. Therefore, the ability to form the A2dl2 type of structure in the apical stem is not a good predictor of function (although it correlates well with gel mobility) and it is unlikely that this form of the RNA, if it exists *in vivo*, plays an important role in VA RNA function.

### Roles of the apical stem and central domain in DAI binding

Using an array of mutated VA RNAs we have defined more precisely the contribution of the apical stem and the central domain to the binding interactions of VA RNA with DAI. Our previous results (25) suggested that efficient binding requires an apical stem-loop structure and that efficient binding is not sufficient for function. The present data, summarized diagrammatically in Fig. 4, confirm that the primary DAI binding site is in the VA RNA apical stem and show that the interaction requires at least 8 base pairs of apical stem duplex. This conclusion holds true in both buffers, A and B, and for deletions and substitutions in the apical stem, and it is supported by results obtained using footprinting techniques which show directly that DAI and p20 protect the basal region of the apical stem (29). The interaction seems to be favored by GC-rich sequences which probably ensure stable apical stem base-pairing, since decreasing its GC content or introducing mismatches within the apical stem (e.g., in *ls1c* and *ls1b*) both adversely influence binding. A recent study of the human TAR binding protein, TRBP-1, showed that it also preferentially binds GC-rich stems (47) suggesting that this may be a general feature of the dsRBM which is shared by these two proteins.

Mutations in the central domain influenced DAI binding (Fig. 4), but the effects were not as easy to interpret, probably because the structure of this region is less well understood and the consequences of mutations are less predictable. Some mutations in the central domain had no effect on binding or were even stimulatory (at least in buffer B). Mutations in stem 4 consistently reduced binding, as did mutations involving the substitution of nucleotides 118 and 119 which encroach on the conserved tetranucleotide CCCA. Deletions in the central domain had a greater impact on binding than substitutions (compare *del110-117* with *sub110-117*, for example), but the effects of buffer composition complicate interpretation. Substitution mutants with changes between nucleotides 110 and 133 fared better in buffer B than buffer A, whereas the reverse was true for mutants with changes between nucleotides 101 and 110. Deletion mutants were equally affected in the two buffers, however. These phenomena are probably attributable to subtle alterations in central domain

structure, either at the secondary or tertiary level, due to the presence of manganese ions and ATP in buffer A. We have recently shown that the presence of magnesium alters the conformation of VA RNA<sub>1</sub> (29). This shift is seen at both 2mM Mg<sup>2+</sup> (buffer A) and 10 mM Mg<sup>2+</sup> (buffer B) but the effect of Mn<sup>2+</sup> on VA RNA<sub>1</sub> structure has not been analyzed. The presence of ATP may also affect binding especially if regions of DAI outside the RNA binding domain, such as the catalytic site of DAI, are involved in the interactions with VA RNA<sub>1</sub> as suggested by differences between DAI and the p20 fragment in protection experiments (29).

Overall, we conclude that both the sequence and structure of the central domain influence the binding of VA RNA to DAI, and that the integrity and sequence of stem 4 in particular is important for binding to DAI. Ghadge *et al.* (33) proposed that the central domain was the critical feature responsible for the interaction between VA RNA<sub>1</sub> and DAI. We have used two different binding conditions to examine our mutations and still find that the major interaction between the RNA and protein occurs in the apical stem. The central domain substitution mutants used by Ghadge *et al.* ranged from 9 to 17 nucleotides whereas ours ranged from 2 to 9 nucleotides. Therefore, one possibility is that there is an upper limit for the length of central domain substitutions that can be tolerated by DAI which is not exceeded by our mutations. It is of note that the smallest substitution mutant (*sub746*) tested by Ghadge *et al.* encroaches on stem 4, where even two nucleotide substitutions can disrupt binding. These differences highlight a problem inherent in the use of large mutations, and to some extent small mutations, to analyze highly structured RNAs that interact with proteins. Changes introduced into non-interacting regions may entrain long distance structural alterations of regions involved in the RNA-protein interaction. In this connection, it is significant that two experimental criteria establish that the structural effects of our central domain mutations are probably restricted to this region of the molecule. Ribonuclease sensitivity data evidenced no major differences in the cutting of the apical stem loop, and there were no major changes in gel mobility indicative of disruption of pairing in the apical stem or a gross rearrangement of RNA structure.

### Relationship between binding and function

In general, apical stem mutants that bind well to DAI are also able to block its activation *in vitro* (summarized in Table 2a). This correlation of binding and function *in vitro* is exemplified by deletion mutants *del49-60*, *del53-80* and *dl1*, which are all disrupted in the apical stem and can neither bind efficiently nor function *in vitro*. Mutant *del73-84*, which displays limited binding, exhibited some *in vitro* function at higher concentrations (34), whereas mutant A2dl2 which adopts an alternative pairing scheme in the apical stem is the only deletion mutant that functions well and also binds efficiently. Most of the apical stem substitution mutants have also been tested *in vivo* (34) and show a similar correlation between function *in vivo* and binding *in vitro*. Exceptions are mutants *lslc* and *lslb*, which do not bind as well as wild-type RNA *in vitro* but nevertheless function efficiently *in vivo* (34). Similarly, mutant *dl1* RNA was shown previously to bind poorly *in vitro* (25), consistent with the dependence on the apical stem for binding *in vitro*, although this mutant RNA functions well *in vivo*. Such discrepancies may indicate that the conditions of the *in vitro* assays do not fully reflect those pertaining *in vivo*, either because both buffer A and buffer B

compositions are suboptimal or because other components, such as proteins or polyamines, which influence the interactions are missing.

Central domain mutants did not show a clear correlation between the binding of their RNAs to DAI and their function *in vitro*. Our new data (Table 2b) are consistent with previous observations suggesting that efficient binding is not sufficient to block DAI activation by dsRNA (25). Mutants *ls3*, *ls5* and *ls3a* bound 50–100% as well as wild-type RNA but did not block DAI activation. Other central domain mutants that did not bind as well as wild-type RNA (e.g., *sub101,102,118,119*, *sub102,118,119*, *sub110-117*, and *del103-109*) still inhibited DAI but required higher concentrations to achieve the same degree of inhibition. RNAs mutated in the conserved stem 4 bound DAI with reduced efficiency, consistent with their reduced function (28). They also bound slightly differently to full-length functional DAI than to p20, in that full-length DAI appeared to be somewhat less sensitive to mutations of this region than p20. Furthermore, recent data indicate that these two proteins give slightly different footprints in this region of the RNA (29). These observations suggest that regions outside the RNA binding domain of DAI contained in p20 can interact with the conserved stem. It is likely that this interaction is functionally important and that non-functional central domain mutants, which retain the ability to bind to DAI, cannot correctly form this stem and are thus inactive.

In summary, we conclude that the apical stem of VA RNA<sub>1</sub> is essential for DAI binding and that additional contacts with the central domain also occur. Most of the central domain mutations are less severe so the central domain contacts are probably not required for initial binding of the RNA to the protein. Taken together with information from footprinting analysis (29), it seems that the principal interaction is between the apical stem and the N-terminal RNA binding domain of DAI and that the central domain makes further contacts with regions outside the RNA binding domain. The conserved stem 4 is also important for binding as all mutations within this region affect binding. These findings are consistent with the hypothesis that VA RNA<sub>1</sub> first binds to the RNA binding domain of DAI, then makes additional contacts with other regions of the kinase thereby preventing its activation. Current work is directed toward defining the nature of the RNA-protein interactions involved in the binding of DAI to its RNA effectors, and the mechanism by which they influence the activity of the enzyme.

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