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Interactions between the double-stranded RNA binding motif and RNA: Definition of the binding site for the interferon-induced protein kinase DAI (PKR) on adenovirus VA RNA

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ABSTRACT

The protein kinase DAI, the double-stranded RNA activated inhibitor of translation (also known as PKR), regulates cell growth, virus infection, and other processes. DAI represents a class of proteins containing a recently recognized RNA binding motif, the dsRBM, but little is known about the contacts between these proteins and their RNA ligands. In adenovirus-infected cells, DAI activation is prevented by VA RNA_I, a highly structured RNA that binds to the kinase. VA RNA contains three chief structural features: a terminal stem, an apical stem-loop, and a complex central domain. We used enzymatic and chemical footprinting to identify the interactions between DAI and VA RNA_I. DAI protects the proximal part of the apical stem structure, an adjacent region in the central domain, and a region surrounding a conserved stem in the central domain from nuclease attack. During binding the RNA undergoes a conformational change that is mainly restricted to the central domain. A similar change is induced by magnesium ions alone. Footprinting and interference binding assays using base-specific chemical probes suggest that the protein does not make major contacts with RNA bases. On the other hand, footprinting with probes specific for the RNA backbone shows that DAI engages in a strong interaction with the minor groove of the apical stem and a weaker interaction in the central domain. A truncated form of DAI, p20, containing only the RNA binding domain, gives a similar protection pattern in the apical stem but protects the central domain less effectively. We conclude that the RNA binding domain of DAI interacts directly with the apical stem and central domain of VA RNA, and that other regions of the protein contribute to interactions with the central domain.

Keywords: DAI; dsRBM; footprinting; protein kinase; RNA–protein interactions; VA RNA

INTRODUCTION

Numerous RNA-binding proteins participate in the post-transcriptional regulation of gene expression, and it has emerged that such proteins can be grouped according to the nature of the amino acid sequences that interact with the RNA (Mattaj, 1993; Burd & Dreyfuss, 1994). Several distinct RNA binding motifs have been recognized that interact with a variety of secondary structure elements as well as primary sequence in the RNA. Recently, a new class of RNA-binding motif (dsRBM) has been described in a group of proteins that binds double-stranded or highly structured RNA molecules (Green & Mathews, 1992; St Johnston et al., 1992; Bass et al., 1994). One of the first members of this group is the interferon-induced protein kinase DAI, the dsRNA-activated inhibitor of protein synthesis (also known as PKR, p68, P1, and by other names). Once activated, in a process involving autophosphorylation, DAI can phosphorylate a number of substrates. Most prominent among these is the α subunit of eukaryotic initiation factor 2 (eIF-2; Hunter et al., 1975; Hershey, 1991) but other substrates have also been recognized (Rice et al., 1989; Kumar et al., 1994; Maran et al., 1994). The kinase thereby plays important roles in the cellular anti-viral defense mechanism (Hovanessian, 1989; Sonenberg, 1990; Samuel, 1991; Mathews, 1993), growth regulation (Hovanessian, 1989; Clemens, 1992; Koromilas et al., 1992; Meurs et al., 1993), and differentiation (Judware & Petryshyn, 1991).

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The RNA binding domain of DAI, which contains two tandem copies of the dsRBM, is located in the N-terminal third of the protein, while the conserved kinase domain lies in its C-terminal half (Katze et al., 1991; Feng et al., 1992; Green & Mathews, 1992; McCormack et al., 1992; Patel & Sen, 1992; St Johnston et al., 1992). Separating these two domains is a third basic region that is critical for the enzyme's activation (Lee et al., 1994). The RNA binding motifs are each about 67 amino acids long, are rich in basic residues, and contain a predicted α-helix with the basic residues displayed along one face. Similar motifs, repeated one to five times, are present in a number of cellular and viral proteins (Green & Mathews, 1992; St Johnston et al., 1992). A truncated form of DAI, p20, containing these tandem repeats alone, can bind to as little as one helical turn of dsRNA (Manche et al., 1992; Schmedt et al., 1995). Mutational analysis of DAI has confirmed that both motifs are required for RNA binding, although mutations in the first repeat are more deleterious than those in the second repeat (Green & Mathews, 1992; Green et al., 1995; Romano et al., 1995). To date, little is known about the nature of the contacts made between the motifs and their RNA ligands, which is addressed here using a compact viral RNA ligand.

DAI is subject to both positive and negative regulation via RNA binding. Many viruses activate the kinase during the course of infection, presumably by producing dsRNA in the course of replication or by symmetrical transcription of their genomes; viruses have also evolved means to prevent DAI activation, thereby ensuring their continued replication (Maran & Mathews, 1988; Sonenberg, 1990; Mathews, 1993). One extensively studied mechanism involves the expression of high concentrations of small, structured RNAs that bind to DAI and specifically block its activation. The best characterized of these is the virus-associated (VA) RNA_I of adenovirus type 2 (Ad2; reviewed by Mathews & Shenk, 1991), but a similar function has been attributed to the small Epstein–Barr virus-encoded RNAs (EBERs) (Bhat & Thimmappaya, 1985; Clarke et al., 1990) and human immunodeficiency virus-1 TAR RNA (Gunnery et al., 1990, 1992), as well as to the VA RNAs of other adenovirus types (Larsson et al., 1986a, 1986b; Ma & Mathews, 1993).

The structural requirements for these two types of effector differ. Activation requires a duplex of at least 30 bp, and optimally more than 80 bp, in length (Minks et al., 1979, 1980; Manche et al., 1992). There are no discernible sequence requirements, and paired homopolymers such as poly(I):poly(C) work well, but modifications that disturb or distort the regular duplex structure reduce or eliminate its ability to activate DAI (Minks et al., 1979, 1980). By contrast, VA RNA_I is a single-stranded molecule of 160 nt that binds to DAI and competes with the binding of dsRNA (Kostura & Mathews, 1989), but fails to activate the kinase (Mellits et al., 1990b). Its binding affinity for p20 is about 100-fold lower than that of dsRNA (apparent K_d of 3.1×10^{-7} M and 4×10^{-9} M, respectively [Schmedt et al., 1995]). The ability of VA RNA_I to block DAI depends critically on its higher order structure. A combination of structural, mutational, and phylogenetic analyses has identified a general format for the VA RNAs (Mellits & Mathews, 1988; Furtado et al., 1989; Ma & Mathews, 1993) consisting of three main parts: a base-paired terminal stem connected by a complex central domain to an apical stem-loop (Fig. 1). Within the central domain are two complementary, phylogenetically conserved tetranucleotides (Ma & Mathews, 1993) that are functionally important (Y. Ma & M.B. Mathews, in prep.). Mutagenesis of Ad2 VA RNA_I led to the conclusion that the central domain is critical for function both in vitro and in vivo (Mellits & Mathews, 1988; Furtado et al., 1989; Mellits et al., 1990a, 1992; Ghadge et al., 1991; Pe'ery et al., 1993). Less clear is the importance

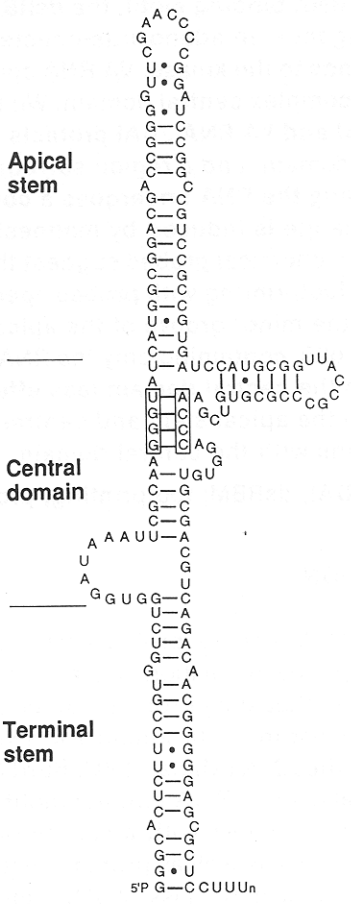


FIGURE 1. Secondary structure model of Ad2 VA RNA_I. This model is based on the cleavage pattern seen in the presence of Mg^{2+} (Fig. 2) as well as on phylogenetic data (Ma & Mathews, 1993) demonstrating conservation of the mutually complementary tetranucleotides (boxed). It probably represents the structure in physiological conditions more accurately than earlier models. In addition to the phylogenetically conserved central domain stem, notable features include the central domain bulge (nt 21–30), the apical loop (nt 63–70), and the central domain loop (nt 101–107).

of the apical stem; whereas our results indicate that it plays an important role in DAI binding (Mellits et al., 1990a; Clarke et al., 1994) others have assigned a lesser, supporting role to this feature (Ghadge et al., 1991, 1994).

The observation that mutations in the RNA binding domain of DAI affect dsRNA and VA RNA binding equally indicates that the protein–RNA interactions follow a stereotyped pattern (Green & Mathews, 1992; Green et al., 1995). To explore the nature of the complex formed between the dsRBM and its RNA ligands, we have characterized the interactions between DAI and VA RNA_I directly. The results demonstrate that DAI protects parts of the apical stem and the central domain from ribonuclease cleavage. Footprinting and interference binding assays using chemical probes specific for RNA bases revealed no discernible protection, suggesting that the protein does not make contacts with the bases that would be necessary for a sequence-specific interaction. Footprinting with probes specific to the RNA backbone revealed strong interactions between DAI and the apical stem as well as weaker interactions within the central domain stem. We also observed a localized conformational change within the central domain that is stabilized by DAI binding. These findings suggest that the inhibitory effect of VA RNA binding depends on specific interactions of its apical stem and central domain with the dsRBMs and other regions of DAI. The interaction of the RNA binding domain of DAI with the minor groove of the base-paired apical stem of VA RNA_I is likely to represent a general feature of this class of RNA binding proteins.

RESULTS

The RNA binding region of DAI contains two dsRBMs. Its ligands, VA RNA and dsRNA, differ in their structure and in the consequences of their binding for enzyme activation. Nevertheless, they appear to interact with the protein in similar ways (Green & Mathews, 1992; Green et al., 1995). To understand how proteins containing this motif interact with RNA, and to address the mechanism whereby VA RNA blocks the kinase, we studied its contacts with DAI.

Effect of Mg^{2+} on VA RNA structure

Because they have been extensively employed in studies of VA RNA secondary structure, we first used RNases to identify cleavage sites that are occluded by the binding of DAI. End-labeled RNA was digested under limiting conditions, such that <10% of the chains were cleaved, and protection was detected by comparison of the digests of free and DAI-bound RNA by gel electrophoresis (Fig. 2). Both single-strand specific enzymes (RNases T_1 and T_2, cutting after guanine or any base, respectively) and nuclease V_1 (cutting in double-stranded and structured regions) were used, providing some redundancy in cleavage site detection and allowing most of the VA RNA_I structure to be examined. The experiments presented here were performed with 3′ labeled RNA to allow good resolution of the central domain, but similar cleavage patterns were obtained with 5′ labeled RNA, as well as with the pyrimidine-specific *Bacillus cereus* RNase (data not shown).

It is well known that RNA structure can be stabilized by magnesium ions. Because VA RNA_I binding studies have been conducted in the presence of this ion whereas VA RNA structure has generally been analyzed in its absence, we first examined the influence of Mg^{2+} on the sensitivity of the RNA to single-strand specific nucleases. Figure 2A shows that Mg^{2+} altered the pattern of cleavage of unbound VA RNA_I; these effects are summarized in Figure 2B, which maps on a secondary structure model the single-strand specific cleavage sites that remain unchanged, are reduced in intensity by Mg^{2+}, or are enhanced by the ion. In the case of RNase T_1 (Fig. 2A, lanes 4–6), the presence of Mg^{2+} caused consistent reductions and enhancements of specific cleavages in the central domain in the absence of DAI. With RNase T_2, several alterations in the central domain cleavage pattern were also evident in the absence of DAI as well as small enhancements of cleavage of the apical loop and central domain bulge (Fig. 2A, lanes 10–12, and Fig. 2B). It is unlikely that these changes are due to effects of Mg^{2+} on RNase activity because the changes vary from site to site in the VA RNA_I molecule; more likely they reflect localized structural rearrangements of the RNA brought about by the divalent cation.

Protection by DAI from nuclease cleavage

Surprisingly, Mg^{2+} is not required for the binding of VA RNA_I to DAI (data not shown), so we were able to examine the nuclease sensitivity of bound VA RNA_I in the presence and absence of this ion, as well as in an intermediate condition in which the RNA was bound in the presence of $MgCl_2$ and then cleaved in its absence. Even in the absence of Mg^{2+}, binding to DAI elicited changes in the RNase cutting pattern similar to those caused by Mg^{2+} on the free RNA (Fig. 2A, compare lanes 4–6 to lanes 7 and 8, and lanes 10–12 to lanes 13–15). DAI also caused several further reductions in RNase cleavage in the conserved central domain stem and in the region immediately surrounding it. The effects of DAI binding are summarized in Figure 2B. The similarity between the changes induced by Mg^{2+} and by DAI implies that these agents elicit similar rearrangements in the VA RNA_I molecule, and the exis-

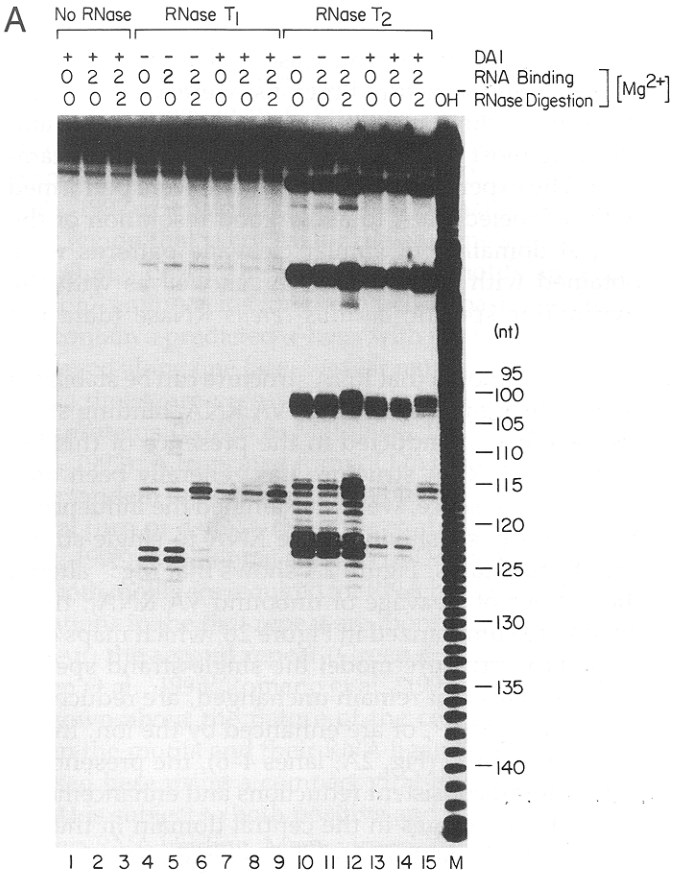
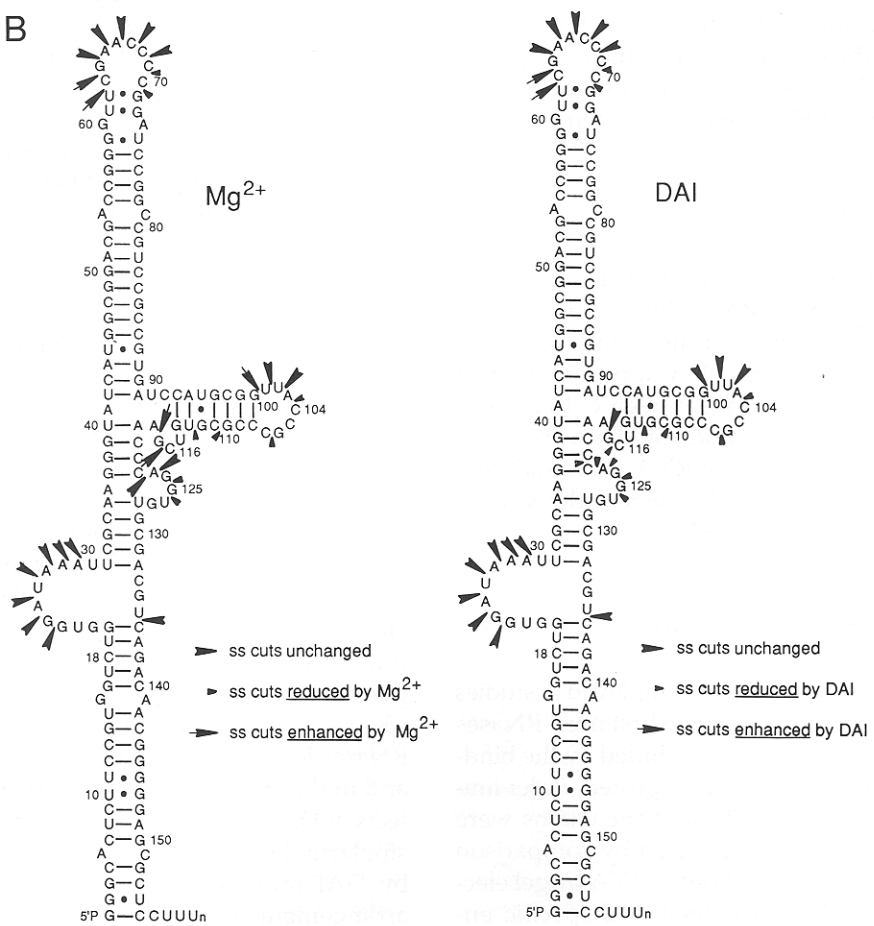


FIGURE 2. Footprinting of the VA RNA–DAI complex with single-strand specific RNases. **A:** VA RNA_I labeled at its 3′ end was bound to immobilized DAI in the presence or absence of $MgCl_2$, then digested with RNase T_1 or T_2 in the presence or absence of $MgCl_2$ as indicated. The RNA was displayed in a denaturing polyacrylamide gel and detected by autoradiography. Digests of DAI-bound VA RNA and of unbound VA RNA are denoted by + and −, respectively; the presence of 2 mM $MgCl_2$ and its absence are denoted by 2 and 0, respectively; the lane headed OH^- is a marker track containing VA RNA_I partially cleaved with alkali. Nucleotide positions are noted. **B:** Summary of the effect of $MgCl_2$ on the cleavage of VA RNA_I by single-strand specific RNases, and of the effects of DAI binding on the cleavage of VA RNA_I (compared to unbound VA RNA_I in the absence of $MgCl_2$). Arrowheads indicate sites of cleavage and changes in cleavage efficiency.



tence of DAI-specific changes suggests that at least the lower part of the central domain is protected by DAI. Strikingly, none of the other regions containing single-stranded sites, namely, the apical loop, central domain bulge, and central domain loop, was protected from RNase cleavage by DAI (Fig. 2B).

Protection of duplexed regions of VA RNA was examined using the structure-specific nuclease V_1 (Fig. 3A). In this case the RNA–protein complex could not be probed in the absence of Mg^{2+} as this ion is required for nuclease V_1 activity. DAI-bound VA RNA_I displayed reduced cleavage of apical stem and central domain nucleotides, signifying protection of the proximal region of the apical stem and an adjacent region of the central domain stem (Fig. 3B). An additional protection was also observed at G-77, approximately one turn of the helix from the protections at C-86 to G-88. Protection was also observed in the conserved central domain stem, confirming results obtained with single-strand specific RNases. Cleavage of some central domain nucleotides remained unaffected by DAI binding, indicating that DAI does not interact with the whole region.

Although DAI (and Mg^{2+}) caused a reduction of single-strand RNase cleavage at nucleotides 110 and 112, DAI did not affect nuclease V_1 cleavage of this region. This difference implies that the reduced cleavage at these sites is due to structural changes caused by Mg^{2+} or DAI, rather than to protection. Again, no protection was seen in the terminal stem, but DAI enhanced cleavage in the distal region of the apical stem (Fig. 3B), implying that binding imparts a conformational change to this stem.

This analysis demonstrates that DAI protects the proximal region of the apical stem, the adjacent part of the central domain, and the region surrounding the phylogenetically conserved stem (boxed in Fig. 1). The binding of DAI also causes a localized conformational change in the central domain of the RNA that is independent of the presence of Mg^{2+}, although this change is similar to that caused by Mg^{2+} alone. Therefore, even in the absence of magnesium, DAI can stabilize the central domain of VA RNA_I in a conformation similar to that occurring under physiological conditions where magnesium is present.

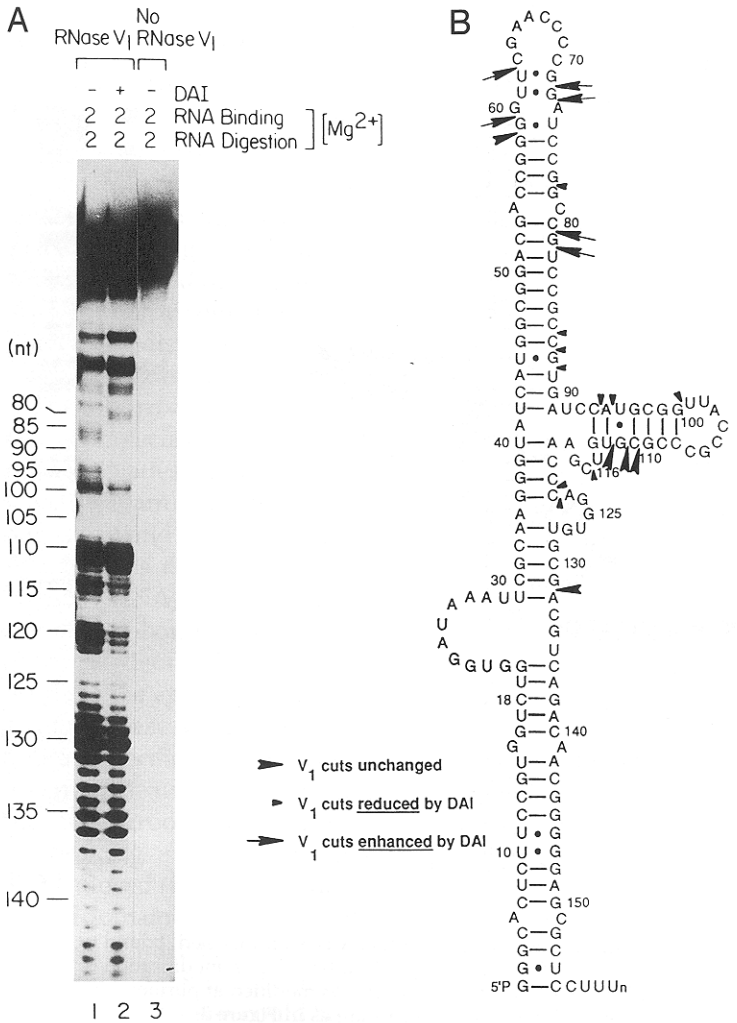


FIGURE 3. Footprinting of the VA RNA–DAI complex with RNase V_1. End-labeled VA RNA_I was bound to DAI and digested with V_1 nuclease in the presence of $MgCl_2$. Details are as in Figure 2. **A:** RNase V_1 footprinting of the complex. **B:** Summary of the effects of DAI binding on RNase V_1 cleavage of VA RNA_I. Note that DAI reduced cleavage by nuclease V_1 but not by single-strand specific nuclease at nucleotide 100. This could be due to reduced base pairing, resulting from increased breathing at the end of a helix, or to differences between the two enzymes in RNA recognition.

Probing with base-specific reagents

Nucleases provide a relatively low-resolution map of the DAI binding site as they can be sterically hindered by the bound protein, while chemical probes that approach solvent size should allow a higher resolution. Having localized regions at the base of the apical stem and in the central domain that were protected from cleavage by DAI binding, we next used diethyl pyrocarbonate (DEPC) in an attempt to detail the interactions between DAI and VA RNA_I. Under mild conditions, DEPC preferentially modifies the N7 atom of single-strand adenosine residues, a reaction that is inhibited by base-stacking and by coordination of divalent ions (Peattie & Gilbert, 1980). Modification was performed either in the presence or absence of Mg^{2+}, as well as in the intermediate condition where VA RNA was bound to DAI in the presence of Mg^{2+} but cleaved in its absence, to distinguish between protections and cation-dependent conformational changes.

As shown in Figure 4A and summarized in Figure 4B, strong cleavage was detected at adenosine residues located in single-stranded regions of the central domain bulge, apical loop, and central domain loops. Additional cleavages were apparent at other central domain nucleotides that cannot participate in base-pairing or are not stacked and at the bifurcation between the central domain stems. Regardless of DAI binding, the modification pattern was influenced by the presence of Mg^{2+} (Fig. 4A, compare lanes 5 and 8 with lanes 3, 4, 6, and 7). Mg^{2+} reduced cleavage at nucleotides 41 and 123 and accentuated cleavage at nucleotides 103 and 118. Although these changes are not identical to those observed with nucleases (Fig. 2), they are in the same region of the molecule, confirming that the conformation of the central domain is influenced by Mg^{2+}.

Binding to DAI did not alter the DEPC modification and cleavage pattern (Fig. 4A, compare lanes 3–5 and 6–8), suggesting that there are no direct interactions between DAI and the N7 atom of susceptible adenosine

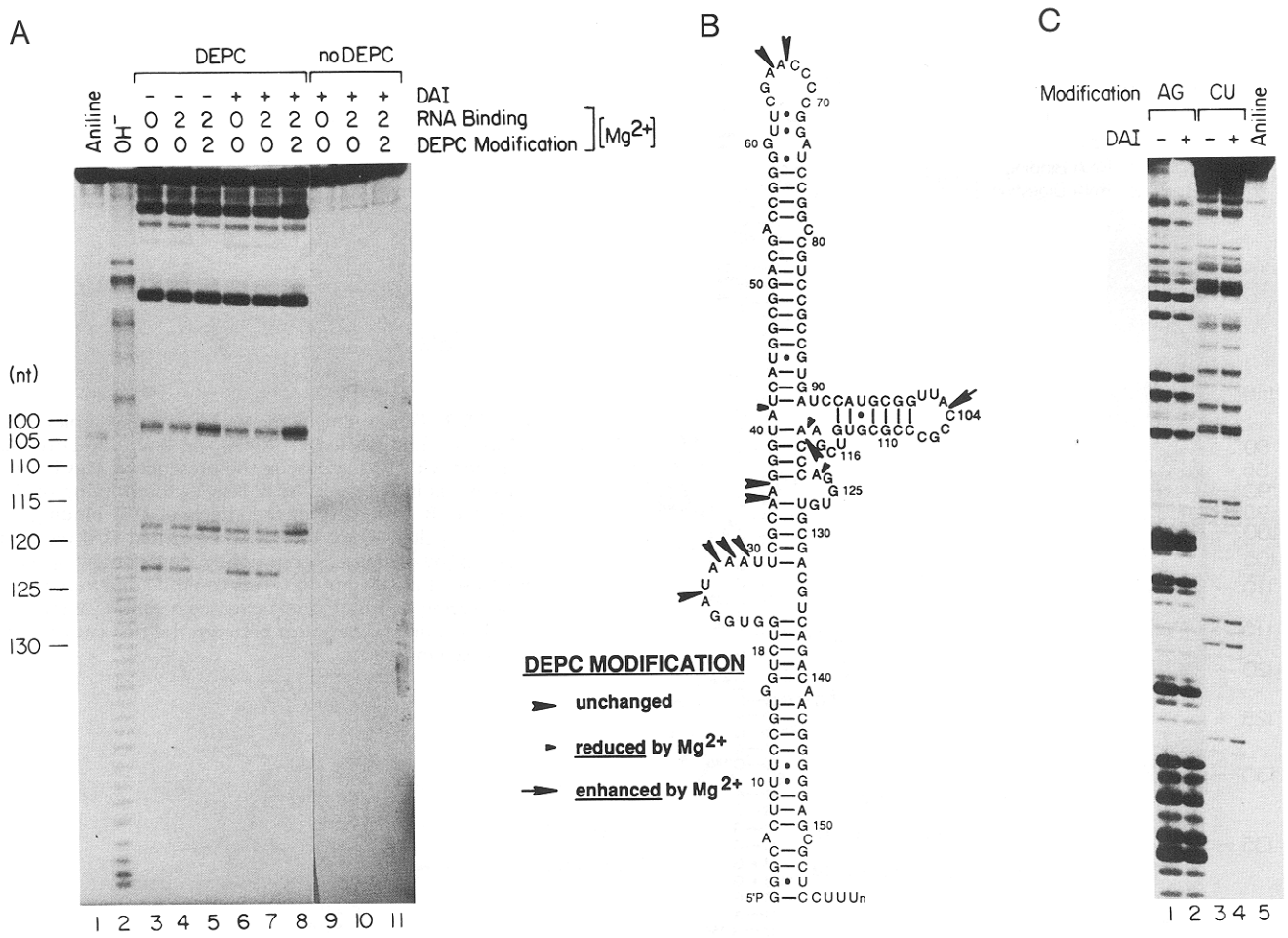


FIGURE 4. Base-specific chemical modification of the VA RNA–DAI complex. **A:** VA RNA_I was 3′ end-labeled, bound to immobilized DAI, and footprinted using DEPC. **B:** Summary of the effects of $MgCl_2$ or DAI binding on modification of VA RNA_I by DEPC. **C:** Interference binding analysis of the VA RNA_I–DAI complex. RNA was modified at purines with DEPC (AG), at pyrimidines with hydrazine (CU), or unmodified (aniline). Other details are as in Figure 2.

residues. To determine whether any such base-specific interactions are important for binding, we used the modification–interference method. Prior to binding, VA RNA_I was reacted under denaturing conditions with DEPC (to carboxyethylate purines at N7) or with hydrazine (to remove pyrimidine bases). After binding to DAI, the RNA was recovered, cleaved at the modification sites using aniline, and then compared by gel electrophoresis to free RNA treated in a similar way. A modification that precluded binding would be absent from the DAI-bound RNA products but present in the free RNA products. No difference between the DAI-bound VA RNA_I and the free VA RNA_I could be consistently distinguished with either reagent (Fig. 4C), suggesting that DAI does not interact directly with any base moiety, or else that no single base-specific interaction between the protein and the RNA is critical for binding. The former interpretation is consistent with the view that the higher order structure of VA RNA is more important for its function than its primary sequence.

Protection by DAI of the VA RNA backbone

Because no base-specific interactions could be seen, we turned to chemical probes specific for the RNA backbone. For this purpose we employed hydroxyl radical cleavage of the ribose group (Tullius, 1987; Wang & Padgett, 1989) and iodine-induced cleavage of phosphorothioate groups incorporated into the RNA chain by in vitro transcription (Schatz et al., 1991; Rudinger et al., 1992). The cleavage reactions were performed in the presence of Mg^{2+} as both probes react equally well with single- and double-stranded RNA, although they are affected by some types of tertiary interactions and RNA chain distortion (Tullius, 1987; Celander & Cech, 1990; Schatz et al., 1991; Rudinger et al., 1992).

The hydroxyl radical is a small, short-lived, uncharged species that cleaves at the 1′ or 4′ position of ribose moieties, resulting in scission of the RNA backbone (Latham & Cech, 1982; Tullius, 1987). The reaction is fast and occurs under mild conditions, ensuring that there is no significant release of RNA from the DAI–VA RNA_I complex (data not shown). Comparison of DAI-bound and free VA RNA_I revealed protection of nucleotides 85, 86, and 87 (Fig. 5). This region overlaps the apical stem sites that are shielded by DAI from nuclease V_1 cleavage (Fig. 3). Because hydroxyl radicals specifically react with the ribose sugar, protection from cleavage indicates an interaction of DAI with the minor groove of the RNA duplex. As in the RNase footprinting experiments, there was evidence for a DAI-induced (but Mg^{2+}-independent) alteration in the central domain conformation. Binding caused enhanced cleavage at nucleotide C-94 located between the apical stem and the central domain (Fig. 5A,B). The increased reactivity at this site could be caused by a realignment of the apical and central domain stems with respect to each other, resulting in a local distortion of the RNA backbone in the protein-bound molecule.

Protection from iodine-mediated cleavage of RNA containing phosphorothioate groups complements hydroxyl radical footprinting experiments, as the phosphate groups of an A-type RNA duplex line its minor groove. Separate transcription reactions were performed with each sulfur-substituted nucleotide so that on average each RNA molecule contained a single substitution randomly located in its backbone. Each RNA was bound to DAI, cleaved at the thiophosphate group by incubation with a low concentration of iodine, recovered, and examined by gel electrophoresis in comparison with free RNA that had been treated in the same way. Iodine is a small uncharged probe that reacts rapidly with the modified phosphate group, so RNA release is also insignificant in this protocol (data not shown). Strong protections indicate regions of solvent exclusion, including regions of direct interaction (Schatz et al., 1991; Rudinger et al., 1992).

Consistent with the protection of nucleotides 85–87 from hydroxyl radical cleavage, DAI protected nucleotides 83–87 in the apical stem (Fig. 6A,B) from iodine-mediated cleavage. In addition, nucleotides 49–52 were protected; this appears to represent an extension of the protection to include an adjacent region of the apical stem, as these sites should lie in the same minor groove and on the same face of the helix as nucleotides 83–87. Weaker protection of nucleotides 107–109 in the central domain was also detected. Thus, the results obtained

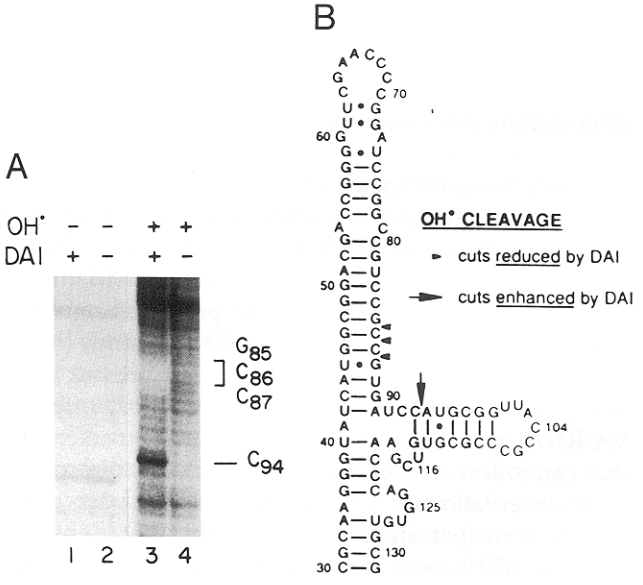


FIGURE 5. Hydroxyl radical footprinting of the VA RNA–DAI complex. **A:** VA RNA was 3′ end-labeled, bound to immobilized DAI, and cleaved with hydroxyl radicals. Only the relevant portion of the gel is shown. **B:** Summary of the hydroxyl radical cleavages protected by DAI. Protection in the region of nt 40–50 was also observed in some experiments but was not obtained consistently.

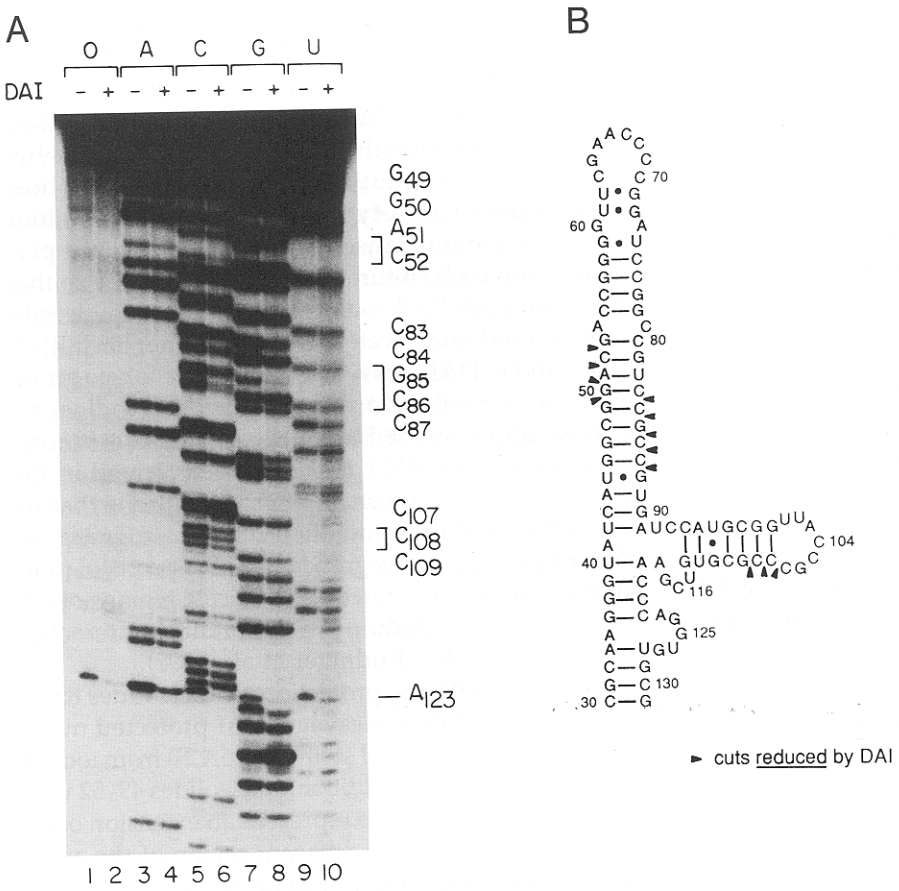


FIGURE 6. Footprinting of phosphorothioate-containing VA RNA_I bound to DAI. **A:** The four 3′ end-labeled phosphorothioate-containing VA RNA_I transcripts were bound to DAI (+) or not bound (−). Letters above pairs of lanes refer to RNA containing the particular [αS]-NTP specified, and 0 represents RNA that does not contain any thio groups. Control reactions omitting the I_2 cleavage step were performed for both the bound and free RNA; no significant cleavages (other than the nonspecific cleavage at A-123) were detected, and these lanes are not shown. Other details are as in Figure 2. Note the anomalous protection from nonspecific cleavage at A-123, although the sequence-specific iodine-induced cleavage at this position was not dramatically affected by DAI binding. **B:** Summary of changes due to DAI binding.

with these probes demonstrate that DAI interacts with the VA RNA backbone and reinforce the conclusion that it protects certain nucleotides in the central domain and the neighboring region of the apical stem.

Interactions with the p20 fragment of DAI

The first 184 amino acids of DAI, the p20 fragment of the protein, contains its RNA binding domain and binds VA RNA and dsRNA with approximately the same affinity as the intact molecule (Green & Mathews, 1992). No mutation isolated to this point distinguishes between the binding of these two RNA ligands (Katze et al., 1991; Green & Mathews, 1992; McCormack et al., 1992; Green et al., 1995), but the inhibitory effect of VA RNA_I binding differs sharply from the activation that can follow dsRNA binding. One way to reconcile these observations is to postulate that other parts of the protein participate in RNA recognition. Consistent with the idea, p20 is more sensitive than the intact DAI molecule toward central domain mutations that disrupt conserved stem 4 and disturb binding (Clarke et al., 1994). In an attempt to detect differences between p20 and intact DAI, the protection analysis of Figure 6 was repeated with p20 instead of DAI. As with intact DAI, phosphorothioate groups at nucleotides 83–87 and 107–109 were protected by p20, but protection at nucleotides 49–52 was not observed as reproducibly as with full length DAI (Fig. 7A). The results are consistent with a strong interaction at the base of the apical stem and a weaker interaction in the central domain.

To explore this observation further, the ability of full-length DAI and p20 to protect VA RNA_I from cleavage by RNases T_1 and T_2 was compared in the presence and absence of Mg^{2+} (Fig. 7B). Full-length DAI gave a pattern similar to that observed in Figure 2A (lanes 1–4 and 7–10), although some additional nonspecific background cleavage products were also apparent (at nucleotides 103, 104, 111, and 122). In the absence of Mg^{2+}, p20 gave less efficient protection than intact DAI and the cleavage pattern more closely resembled that of free VA RNA_I than that of DAI-bound VA RNA (Fig. 7B, odd-numbered lanes). With the addition of Mg^{2+}, the protection pattern reverted to that of the full-length protein (Fig. 7B, even-numbered lanes). Thus, the C-terminal part of the protein extends protection to central domain sequences that are not protected by the RNA binding domain, an effect that may be due to localized conformational change within the central domain that influences the protection pattern indirectly.

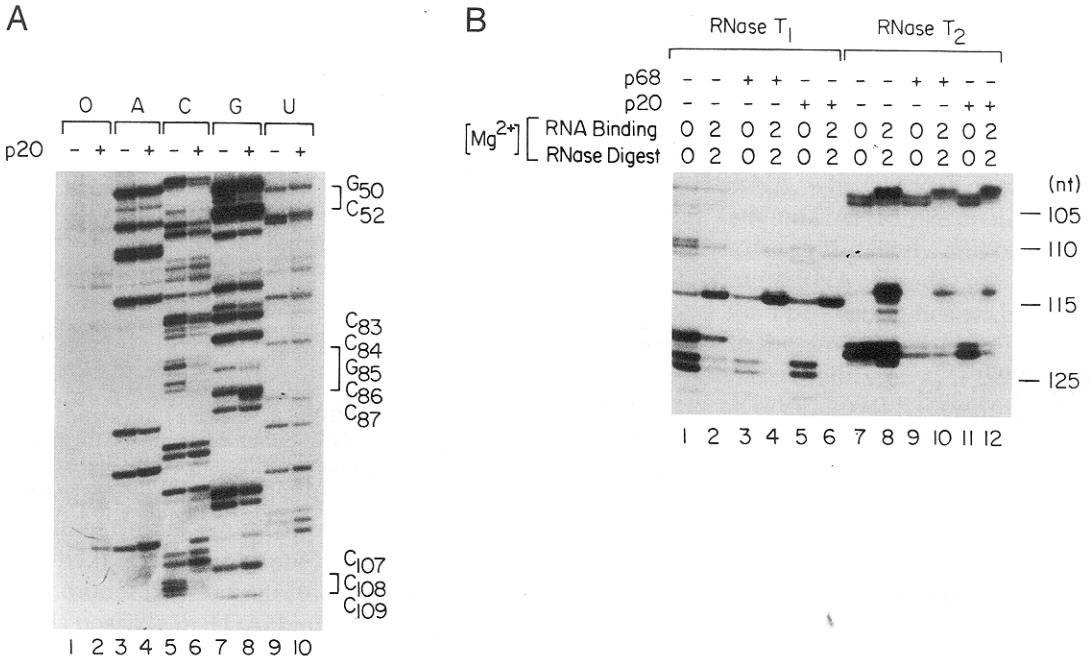


FIGURE 7. Protection by the p20 fragment of DAI. **A:** Footprinting reactions were conducted as in Figure 6A, using iodine to cleave the four phosphorothioate-containing RNAs (A, C, G, and U; lanes 2–10) or regular VA RNA (0; lanes 1 and 2). Free and p20-bound RNA reactions are indicated by − and + symbols, respectively. **B:** Footprinting reactions were conducted with end-labeled VA RNA_I bound to immobilized full-length DAI (p68) or p20 and digested with RNase T_1 or T_2 as shown. The RNA binding and RNase digestion reactions were done in the presence of 2 mM $MgCl_2$ or in its absence. Unbound RNA was digested in a similar fashion. Other details are as in Figure 2. Only the relevant portions of the gels are shown, and nucleotide positions are marked.

DISCUSSION

The protein kinase DAI belongs to a family of proteins containing a newly recognized dsRNA binding motif, the dsRBM. Its ability to bind VA RNA, a small, highly structured single-stranded RNA, as well as long dsRNAs, provided the opportunity to characterize the interactions in detail. We have used chemical and enzymatic probes to examine the binding site for DAI on VA RNA_I directly. This study also revealed a magnesium-dependent change in VA RNA_I conformation and gave support to a revised model structure for VA RNA_I as well as insights into its function.

Magnesium stabilizes an alternative VA RNA_I secondary structure

Recent mutational studies (Pe'ery et al., 1993) indicated that our earlier VA RNA_I model (Mellits & Mathews, 1988) needed some refinement in the region of the central domain, and phylogenetic comparisons (Ma & Mathews, 1993, and in prep.) identified conserved sequences in this region that appear to pair with one another. These findings were not entirely compatible with the existing nuclease sensitivity information, and it was difficult to reconcile all the data in a single model. In the course of footprinting the VA RNA_I–DAI complex we observed that the cleavage pattern of VA RNA_I was influenced by the presence of Mg^{2+}: cleavage by single-strand specific RNAses was enhanced at some sites and reduced at others. Neighboring sites that are cut by different nucleases generally displayed a consistent change when Mg^{2+} was added, suggestive of a localized structural rearrangement. These cleavage data allow for a more compact central domain, yielding the secondary structure model shown in Figures 1 and 8A. This model satisfactorily accommodates the results of mutagenesis and phylogenetic analysis (Ma & Mathews, 1993; Pe'ery et al., 1993) and fits the nuclease V_1 cleavage pattern rather better than the earlier model. Several V_1 sensitive nucleotides previously placed in the central domain loop now reside in a base-paired stem, while only one nuclease V_1 cut is now placed in a single-stranded region of the molecule. Some potential single-strand specific cleavage sites are still shown in unpaired regions, but as they cannot be paired in any other obvious way, we assume that these nucleotides are shielded by tertiary interactions.

The model is also compatible with results obtained using DEPC as a sequence-specific chemical probe for RNA secondary structure. Several adenosine residues that were not cleaved by single-strand specific nucleases were modified by DEPC, presumably because it is subject to less steric hindrance, and all cleavages were at residues predicted to be accessible to the probe. The addition of $MgCl_2$ changed the DEPC cleavage pattern

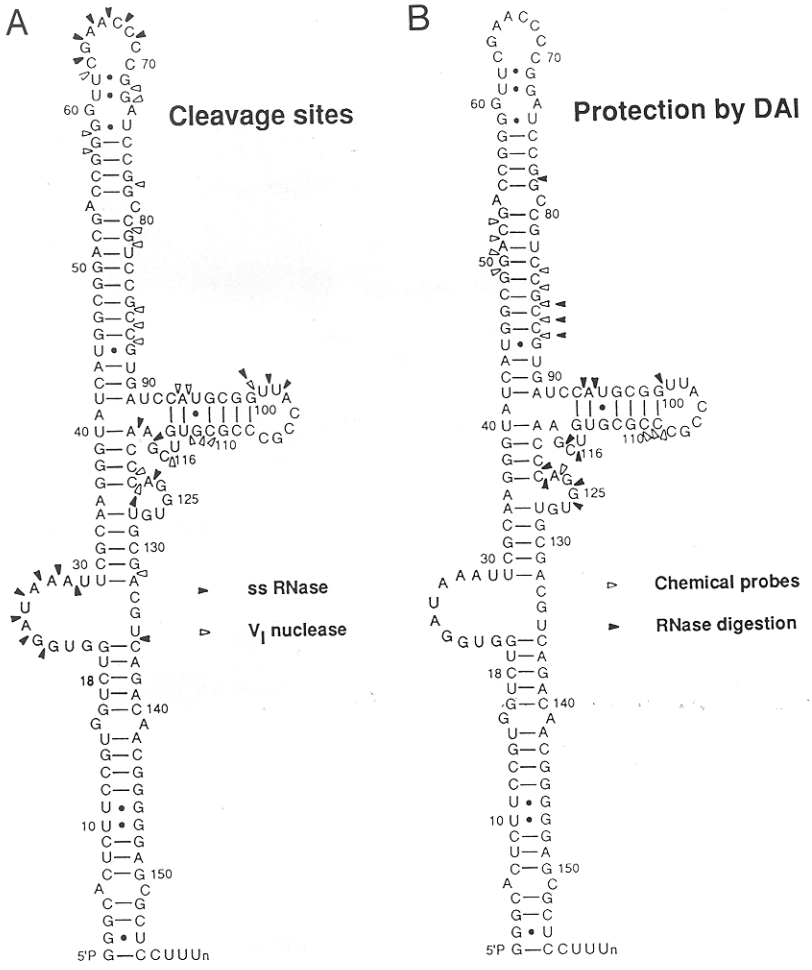


FIGURE 8. Cleavage sites and protection of VA RNA_I. Summarized are (**A**) the single- and double-strand specific cleavage sites in the presence of 2 mM Mg^{2+} and (**B**) the sites that are protected from cleavage by binding to DAI.

slightly, and not always in the same way as seen with RNase T_2. Of the three DEPC sites that are influenced by Mg^{2+}, two (A-41 and A-118) were resistant to RNase T_2, whereas sensitivity of the other two sites (A-103 and A-123) changed in opposite directions. The site at A-118 is adjacent to a site (G-117) that displays enhanced cleavage by RNase T_1 in the presence of magnesium ions. These sites are in regions that appear to participate in higher order interactions (Pe'ery et al., 1993) and as no obvious pairing partners exist, the altered reactivity is most likely due to altered stacking or ion coordination (Peattie & Gilbert, 1980). Coordination of a hydrated magnesium ion plays an important role in maintaining tertiary structure in tRNA (Holbrook et al., 1977; Jack et al., 1977) and hammerhead ribozymes (Fu & McLaughlin, 1992), via a network of interactions that include the N7 group of purines. In VA RNA, the coordination of a magnesium ion could maintain tertiary structure by bridging two unpaired regions, such as the loop at nt 123–127 with the central domain bulge at nt 21–30, as suggested previously (Pe'ery et al., 1993), an explanation that would also account for the reduced RNase cleavage in the central domain loop.

Binding and function of VA RNA_I

Results of footprinting experiments, summarized in Figure 8B, indicate that a major DAI binding site is in the proximal region of the VA RNA_I apical stem, where nuclease, hydroxyl radical, and phosphate cleavages were all strongly protected by the protein. There were additional interactions with the central domain, where RNase and phosphate cleavages were shielded by DAI. The apical stem interaction was also observed with the isolated RNA binding domain of DAI (p20), although central domain nucleotides were shielded only weakly if at all by p20. DAI, unlike p20, also caused a conformational change in the central domain which resembles that induced by Mg^{2+} but is independent of the presence of this ion. Localized conformational changes of RNA structure induced by protein binding have been reported for 7SL RNA (Andreazzoli & Gerbi, 1991), tRNA (Wikman et al., 1982; Rould et al., 1989; Matsumoto et al., 1990), HIV-1 RRE (Kjems et al., 1991), and HIV-1 TAR RNA (Puglisi et al., 1992). In the case of VA RNA_I, it appears that such changes are due to interactions with regions of DAI outside its recognized RNA binding domain, whereas the strong

protection of the apical stem and central domain are due to interactions with the protein's RNA binding domain.

Protection of the apical stem is consistent with the conclusions, derived from mutational analysis, that DAI binds to the stem and that the base of the stem is important for function (Mellits et al., 1990a, 1992; Clarke et al., 1994). On the other hand, despite its functional importance, not all of the central domain was protected by DAI, and only limited protection against minor groove-specific probes was seen. Mutational analysis indicates that the interaction of VA RNA_I with DAI requires only 8 bp in the apical stem and 5 bp in the central domain (Mellits et al., 1992; Pe'ery et al., 1993; Clarke et al., 1994). Assuming these stems to be co-linear in the three-dimensional structure of the molecule, the resultant duplex would approximate a single helical turn, which is the minimum length of dsRNA that can accommodate a p20 molecule (Manche et al., 1992). Thus, it is possible that DAI binds to such a structure in VA RNA in much the same way as it binds to dsRNA, but fails to become activated because some subsequent step (such as autophosphorylation or dimerization) is inhibited, perhaps because of hindrance by the central domain. Alternatively, it is possible that the two stems do not lie on a single helical axis, but interact with DAI in such a way as to prevent enzyme activation. This could occur if the two RNA binding motifs were to associate with the two helical stretches in a fashion that is incompatible with activation. Finally, the increased susceptibility of C-94 to hydroxyl radicals may indicate that binding of DAI causes a distortion in RNA structure that is inimical to activation.

Interaction of the dsRBM with RNA

The RNA binding domain of DAI contains two copies of a motif, the dsRBM, the most highly conserved feature of which is a predicted α-helical stretch (Green & Mathews, 1992; St Johnston et al., 1992). Because dsRNA forms an A-type helix, in which the minor groove is wide and easily accessible while the major groove is narrow and relatively inaccessible (Steitz, 1990), it has been proposed that the RNA binding domain of DAI interacts with duplex RNA by inserting the α-helices of the conserved motifs into the minor groove of the RNA duplex (Green & Mathews, 1992). Direct evidence, from protection by DAI against hydroxyl radical cleavage and I_2/phosphorothioate cleavage of VA RNA_I, supports the existence of a minor groove interaction. Because VA RNA_I and dsRNA seem to interact with the same elements of DAI (Kostura & Mathews, 1989; Green & Mathews, 1992; Katze et al., 1991; McCormack et al., 1992; Green et al., 1995), and p20 gives apical stem protection similar to DAI, the same minor groove interaction probably takes place between DAI and dsRNA. Prominent features of the minor groove include the phosphate groups that line the edge of the groove, the RNA specific 2′-OH groups, and the N-2 groups of guanines that allow discrimination between G-C and A-T base pairs (Steitz, 1990). The fact that ssRNA, DNA, DNA:RNA hybrids, and 2′-*O*-methylated dsRNA, which lack all or some of these features, fail to activate DAI (Minks et al., 1980) points to the minor groove as the likely site of interaction between DAI and its RNA ligands.

The apical stem protection lies within a GC-rich region adjacent to the central domain (Fig. 7B). Both mutational analysis (Mellits et al., 1992) and binding studies (Clarke et al., 1994) have shown some preference for a GC-rich duplex. This sequence specificity may be related to the local helical geometry of A-type RNA and DNA helices. In an RNA duplex, 5′ pyrimidine to 3′ purine steps exhibit a phenomenon known as Calladine's clash (Dickerson, 1983). This is especially severe at 5′-GC-3′ steps and is caused by the N-2 and N-3 groups coming into close proximity. To relieve the clash there is a localized opening or widening of the minor groove (Dickerson, 1983; Dock-Bregeon et al., 1989; Sponer & Kypr, 1991) that may render the apical stem of VA RNA more accessible to the RNA binding elements of DAI. It is also interesting to note that the dsRBM of TRBP-1 (Gatignol et al., 1993) preferentially binds RNA with an alternating G-C base paired stem, suggesting that these may be general features of the interactions between duplex RNA and the dsRBM. More distally, DAI binding caused enhanced cleavage at certain nuclease V_1 sites in the apical stem. These sites are approximately one-half and one and a half turns from the protected apical stem nucleotides. Similar enhanced cleavage has been observed during footprinting of the U1 RNA/U1A complex (Jessen et al., 1991), 5S rRNA/TFIIIa complex (Theunissen et al., 1992), and $tRNA^{Ser}$/seryl-tRNA-synthetase complex (Dock-Bregeon et al., 1990). In these examples, the enhanced cuts either bordered the protein-binding site or were on the opposite side of the RNA duplex from the site of interaction. Possibly such enhanced apical stem cuts are caused by a localized distal tightening of the helix that could result from a proximal widening to accommodate the protein. The pattern of protection on one side of the duplex and enhanced cleavage on the other would suggest that DAI binds to one face of the apical stem.

In summary, the interaction between DAI and VA RNA involves the GC-rich proximal part of the apical stem and an adjacent stem in the central domain of the RNA, which are protected by the protein's N-terminal RNA binding domain. These interactions take place in the minor groove, and are probably typical of interactions between RNA and proteins sharing the dsRNA binding motif found in DAI. In addition, C-terminal regions of the protein, lying outside its RNA binding domain, interact with the central domain of the RNA, including the phylogenetically conserved stem, and

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