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Two Functionally Distinct RNA-Binding Motifs in the Regulatory Domain of the Protein Kinase DAI

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The RNA-binding domain of the protein kinase DAI, the double-stranded RNA inhibitor of translation, contains two repeats of a motif that is also found in a number of other RNA-binding proteins. This motif consists of 67 amino acid residues and is predicted to contain a positively charged α helix at its C terminus. We have analyzed the effects of equivalent single amino acid changes in three conserved residues distributed over each copy of the motif. Mutants in the C-terminal portion of either repeat were severely defective, indicating that both copies of the motif are essential for RNA binding. Changes in the N-terminal and central parts of the motif were more debilitating if they were made in the first motif than in the second, suggesting that the first motif is the more important for RNA binding and that the second motif is structurally more flexible. When the second motif was replaced by a duplicate of the first motif, the ectopic copy retained its greater sensitivity to mutation, implying that the two motifs have distinct functions with respect to the process of RNA binding. Furthermore, the mutations have the same effect on the binding of double-stranded RNA and VA RNA, consistent with the existence of a single RNA-binding domain for both activating and inhibitory RNAs.

The double-stranded RNA (dsRNA)-activated inhibitor of translation, DAI (also termed PKR, p68, DsI, and P1/eIF-2 kinase), is a cellular protein kinase that plays important roles in growth control (18, 31), the induction of apoptosis (21), and the interferon-induced host antiviral response pathway (reviewed in reference 24). Activation of DAI unmasks its ability to phosphorylate the α subunit of eukaryotic initiation factor 2 (eIF-2), leading to the inhibition of translation (reviewed in references 13, 34, and 35). During the initiation of protein synthesis, eIF-2 is responsible for bringing the initiator tRNA (Met-tRNA$_i$) to the 40S ribosomal subunit, forming a 43S Met-tRNA$_i$ complex. The transport of Met-tRNA$_i$ to the ribosome occurs by the formation of a ternary complex with eIF-2 and GTP. At the end of the initiation process, when the mRNA and 60S ribosomal subunit have joined, eIF-2 is released in a binary complex with GDP. For eIF-2 to reenter the initiation pathway, its associated GDP moiety must be exchanged for GTP. This recycling event is catalyzed by the guanosine nucleotide exchange factor (also called eIF-2B). When the α subunit of eIF-2 is phosphorylated on serine-51 (5), recycling fails because of the formation of a nondissociable complex between the guanosine nucleotide exchange factor and eIF-2(α)P (37). The failure of recycling results in a shortage of eIF-2 · GTP and consequently the cessation of translation.

DAI is activated by a process that involves autophosphorylation and requires critical concentrations of dsRNA (6, 8, 19, 40). Two models have been proposed to explain the interactions between DAI and dsRNA. The first model proposes that DAI has one RNA-binding site and activation results from intermolecular phosphorylation within a DAI dimer (19). At limited RNA concentrations, two DAI molecules interact with the same dsRNA molecule and intermolecular autophosphorylation occurs; at higher RNA levels, however, the DAI molecules interact with the RNA individually and therefore are unable to activate through intermolecular autophosphorylation. A second model proposes that activation of DAI involves intramolecular phosphorylation and that there are two binding sites for RNA on DAI, one for activation and one for inhibition (9). Under this model, RNA binds preferentially to the activation site, leading to autophosphorylation; at higher RNA concentrations, the RNA binds to both sites, preventing intramolecular autophosphorylation.

DAI is fastidious about the dsRNA that can cause its activation. The RNA is usually perfectly duplexed, and if the overall topology of the RNA is altered (by base modifications or the intercalation of ethidium bromide), the ability of DAI to bind RNA is lowered (1, 14, 32, 33). The affinity of DAI for dsRNA increases as the length of the RNA duplex increases, an optimum being reached at around 85 bp (22, 32). This binding affinity correlates well with the ability of these dsRNAs to activate DAI and also with a mobility shift change (22). Shorter dsRNAs, though unable to activate DAI, can still interact with the enzyme and inhibit DAI function at high dsRNA concentrations (22, 32). A recently discovered exception is the RNA of hepatitis delta virus (38). This closed circular single-stranded RNA activates DAI even though it lacks duplex regions longer than 20 bp.

The host antiviral defense mechanism serves to limit viral proliferation. To thwart this defense mechanism, viruses have evolved a number of different strategies that target DAI activity. One such strategy entails the synthesis of short, highly structured, single-stranded RNAs which bind to DAI but do not activate the enzyme. Such short RNAs, exemplified by adenovirus-associated (VA) RNA, inhibit activation of DAI by dsRNA that is apparently generated by symmetrical transcription of the viral genome during infection (23). VA RNA is produced throughout the viral life cycle, but it accumulates to very high levels within the cell at the late stages of viral infection. It is these high levels of VA RNA that prevent DAI activation (reviewed in reference 25). Other examples of viruses that produce small RNAs to inhibit DAI activation include human immunodeficiency virus type 1 (TAR RNA) and Epstein-Barr virus (EBER RNA) (4, 11, 12). A wide variety of

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other techniques have been evolved by viruses to evade DAI activation (reviewed in references 24 and 41).

Many proteins can bind RNA, and within these proteins, at least nine types of RNA-binding motif have been identified (26). Though the different motifs vary significantly, some common elements include basic and/or aromatic amino acid residues (17, 20). These residues are involved in electrostatic interactions with the phosphate backbone of RNA and intercalation between the nucleotides of RNA. The RNA-binding domain of DAI (Fig. 1A) is located in the first 171 amino acids at the protein's N terminus (7, 10, 16, 28, 36). This region contains two repeats of a motif that is also found in a number of other RNA-binding proteins, including the mouse DAI homolog TIK, the *Drosophila* protein staufen, TAR RNA-binding protein 1, vaccinia virus E3L protein, *Escherichia coli* RNase III, and the *Xenopus* proteins Xrlbpa and 4F (2, 10, 28, 42). Like DAI, many of the proteins that contain this motif have multiple copies, up to five in staufen (42). Computer analysis of published sequences for this motif has resulted in the generation of a consensus sequence (42). Each copy of the motif extends over about 67 residues (Fig. 1B) and contains a positively charged putative α-helical region in the C-terminal third of the motif (10, 28, 42). The motif is very well conserved in the potentially α-helical region but exhibits lower levels of conservation over the first two-thirds of its length (10, 42). Furthermore, some copies of the motif maintain high levels of homology within the helical region but display only limited homology outside this region, raising the possibility that the first two-thirds of the motif are less important for RNA binding or perhaps are not part of the motif at all. As a result, it has been proposed that there are two distinguishable kinds of motif, a full-length motif and a short motif (42).

The two motifs in DAI represent a full-length motif and a short motif (motifs 1 and 2, respectively). Deletion of either copy of the motif from DAI greatly reduces binding, but simply exchanging their locations in the molecule does not (mutant Sub 2:1 [10]), indicating that the two copies are both required but that their relative positions are less important. They do not appear to be identical in function, however: whereas the second motif can be replaced by a duplicate copy of the first motif, a reciprocal construct containing tandem repeats of the second motif displays only weak binding activity (10). Moreover, small deletions and clustered point mutations in the first motif are generally more debilitating than mutations in the second motif (7, 10). These findings imply that the first copy of the motif is more important for binding than the second, but it is difficult to draw firm conclusions from these data since none of the mutations tested were in equivalent residues in both copies of the motif.

To define the motifs more precisely and to discern their roles in RNA binding, we have mutated individual conserved residues located in the left third, middle, and right third of the motif, making identical mutations in both copies of the motif. The results establish the significance of the motif as a whole for binding both VA RNA and dsRNA and demonstrate that although both motifs are required, the first motif appears to make the more important interactions with the RNA. Furthermore, the mutations that we have introduced affect dsRNA and VA RNA binding equally, supporting the hypothesis that there is only one effective site for RNA binding rather than two (one activating and one inhibitory).

MATERIALS AND METHODS

Construction of DAI mutants. Mutants SRG2ΔLS14 to -LS19 were prepared by oligonucleotide-directed mutagenesis of the vector pUC118D and were transferred into SRG2ΔL (10) by exchange of *Hin*dIII-*Msc*I DNA fragments. To generate Sub 2:2*, which contains a deletion of motif 1 and a complete duplication of motif 2, pSRG2ΔL was digested with *Hin*dIII and *Nco*I to delete motif 1, and the resultant 3′ recessed ends were filled by using T4 DNA polymerase (New England Biolabs). Ligated between these sites was a 264-bp fragment that encodes motif 2 in its entirety. This fragment was obtained by digestion of SRG2ΔL with *Nco*I and *Bsa*AI; its termini were also filled by using T4 DNA polymerase. The translational start site of this construct corresponds to the methionine at residue 98 of the original sequence. Construction of Sub 1:1 has been described previously (10). The 1:1m, 1m:1, and 1m:1m series were prepared in a similar manner after site-directed mutagenesis using the LS15 oligonucleotide primer. All constructs were confirmed by DNA sequencing.

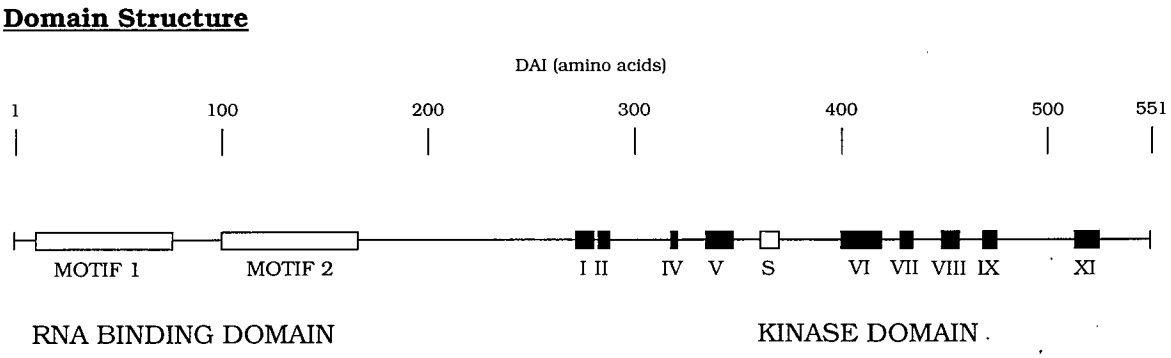
RNA synthesis and purification. Uncapped RNA was generated as previously described (29) and used as a template for synthesis of truncated DAI. RNA was purified by extraction with phenol and with chloroform-isoamyl alcohol (24:1). The RNA was precipitated with ethanol, dissolved in TE (10 mM Tris-HCl [pH 7.4], 1 mM EDTA) and passed twice through Sephadex G-50 medium (Pharmacia) spin columns. The RNA was recovered by precipitation with ethanol and was dissolved in TE.

Cell-free translation and RNA binding assays. The reactions were carried out exactly as previously described (10). Briefly, proteins were synthesized in a wheat germ translation system, using [^{35}S]Met to label the proteins generated in vitro. The proteins were then assayed in two kinds of RNA binding assay. The first involved the immobilization of DAI as an immune complex on protein A-Sepharose beads carrying polyclonal antibody to DAI. The ability of the mutant proteins to retain synthetic, ^{32}P-labeled dsRNA was measured by direct counting and sodium dodecyl sulfate (SDS)-polyacrylamide gel analysis. In the second assay, adenovirus type 2 VA RNA_I was attached to CNBr-activated Sepharose beads, and the ability of the ^{35}S-labeled mutant DAI proteins to bind to this matrix was determined by SDS-polyacrylamide gel analysis.

RESULTS

Equivalent mutations in the two motifs have differential effects on RNA binding. The RNA-binding domain of DAI is located in the N-terminal third of the protein, while the 11 kinase subdomains occupy the protein's C-terminal half (7, 10, 16, 28, 30, 36, 44). The RNA-binding domain contains two copies of a 67-amino-acid residue motif which is also present in a number of other RNA-binding proteins (10, 28, 42). Limited mutational data suggested that the first (N-terminal) copy of this motif is more important than the second for RNA binding (7, 10, 28). To test this conclusion rigorously, and to discover whether both copies of the motif in their entirety are involved in RNA binding, we constructed three pairs of mutants, making identical changes in the two motifs (Fig. 1). The three amino acids changed were chosen for their location and conservation. They are distributed across the entire motif, that is, in its N terminus, center, and C terminus. Moreover, the mutations are in highly conserved residues that are presumably important for some functional aspect of the motif. Previous experiments had shown that a truncated form of DAI, containing just the first 184 residues from the protein's N terminus, binds RNA as efficiently as does the full-length protein. Therefore, the mutations were prepared in this truncated form of the protein by cell-free transcription and translation, using T7 RNA polymerase and a wheat germ translation system. To assay RNA binding, wild-type and mutant proteins were immobilized by adsorption on protein A-Sepharose beads carrying polyclonal antibody to DAI. The resultant complexes were then exposed to ^{32}P-labeled dsRNA, and the amount of bound dsRNA was determined by direct estimation of radioactivity (Fig. 2) and by gel electrophoresis (data not shown).

All of the mutations impaired RNA binding, but the magnitude of the reduction varied dramatically depending both on the position of the mutation in the RNA-binding motif and on the motif which harbors the mutation. Changes in the highly conserved α-helical region (LS16 and LS19) virtually abolished binding whether the mutation was located in the first or second copy of the motif. Changes in the central and N-terminal sites reduced binding by an extent that ranged from 10 to 90%, but changes in the second motif (LS17 and LS18) had less effect



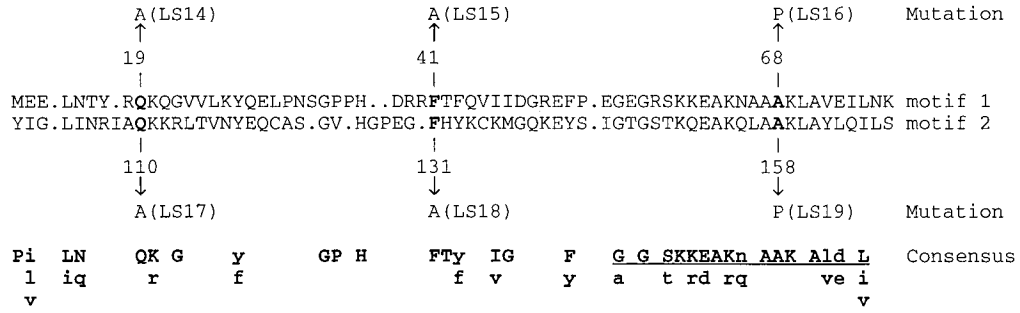


FIG. 1. Structures of DAI and mutants. (A) Schematic representation of the linear wild-type DAI protein. The repeat of the RNA-binding domain is portrayed by the large open boxes in the N terminus of the protein. The kinase catalytic domains are represented by the black boxes. The open box (S) indicates the putative substrate-binding domain. (B) The amino acid sequence of each copy of the RNA-binding motif and the consensus sequence for this motif. The locations of the single amino acid mutations used in this study are diagrammed. In the consensus sequence, identical or similar amino acids are shown in uppercase or lowercase, respectively. The predicted α-helical region is underlined.

than the equivalent mutations in the first motif (LS14 and LS15). Interestingly, the relative susceptibilities of the two motifs to equivalent mutations were nonuniform. For example, changing Ala to Gln at positions 19 and 110 in motifs 1 and 2 (LS14 and LS17) reduced binding to 20 and 45% of control levels, respectively. On the other hand changing Phe to Ala at residue 41 in motif 1 (LS15) was a severe mutation (10% of the control level), but the equivalent change at residue 131 in the second motif (LS18) was only slightly deleterious (90% of the control level).

The same residues are involved in binding both dsRNA and VA RNA. The experiments described above were conducted with dsRNA of 85 bp, which is long enough to activate the kinase optimally (22). To determine whether the binding of an inhibitory RNA, VA RNA_I, was affected to the same extent by these mutations, we used an alternative assay. The immobilized protein assay could not be used to examine the ability of the mutant DAIs to bind VA RNA because of a nonspecific VA RNA-binding protein that contaminates the immune complex even when derived from an unprogrammed wheat germ lysate (data not shown). Therefore, we measured the ability of the ^{35}S-labeled mutant proteins to bind to VA RNA covalently attached to Sepharose beads (10). Complexes were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. When the three pairs of mutants were assayed for their VA RNA-binding capacities (Fig. 3), the results were similar to

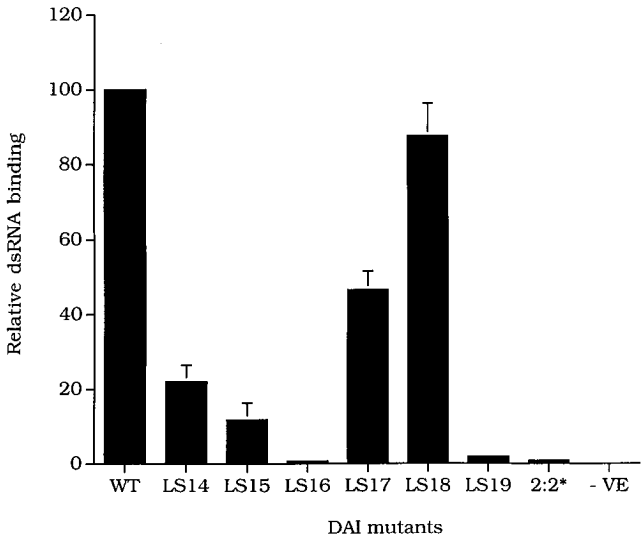


FIG. 2. Binding of dsRNA to DAI and DAI mutants. ^{32}P-labeled dsRNA (85 bp) was adsorbed to ^{35}S-labeled proteins immobilized on antibody-Sepharose beads. The resultant RNA-protein complexes were analyzed by direct counting of radioactivity: error bars represent the variation from seven different experiments. WT, wild type; −VE, negative control (unprogrammed wheat germ translation).

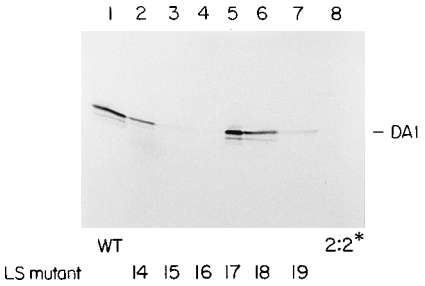


FIG. 3. Binding of VA RNA by DAI. Equal radioactive counts of [^{35}S]Met-labeled translation products for wild-type (WT) DAI and the mutants were incubated with VA RNA-Sepharose beads. The resultant protein-RNA complexes were analyzed by 20% polyacrylamide gel electrophoresis.

those obtained for dsRNA binding: mutations in the first motif had a greater effect on VA RNA binding than equivalent mutations in the second motif. Furthermore, each individual mutation appeared to influence both VA RNA and dsRNA binding to approximately the same extent, emphasizing the importance of the predicted α helix and the different roles played by the two copies of the motif in RNA binding. Thus, no difference was apparent between an activating and an inhibitory RNA.

Motif 2 cannot substitute for motif 1. These data suggest that the second motif is less important for RNA binding than the first. We had previously shown that a mutant DAI, Sub 2:2, containing a deletion of motif 1 and a duplication of motif 2, was severely debilitated (10). In this construct, however, the spacing between the duplicated motifs was reduced by nine amino acids, and the first three residues of the second repeat were missing. Small deletions from the N terminus of motifs from Xlrbp and staufen have been shown to reduce RNA binding (42). Therefore, to determine if either of these two features adversely influenced the binding properties of the motif 2 repeat mutant, we constructed a further mutant, Sub 2:2*. This mutant contains two complete copies of motif 2 with the alanine doublet in the middle of each motif (Fig. 1) separated by 88 residues, instead of 90 residues as in wild-type DAI; this slight decrease should be acceptable since a reduction of four residues in the spacer region between motifs 1 and 2 had no effect on RNA binding (mutant Δ5 [10]). The new mutant, Sub 2:2*, was tested in both types of RNA binding assay together with the other mutants (Fig. 2 and Fig. 3, lane 8). The truncated form of this protein is 208 residues, compared with the 184 residues of the other proteins, which accounts for its slower migration during electrophoresis. Like the original duplication of motif 2, Sub 2:2* bound dsRNA and VA RNA very poorly, demonstrating that the second copy of the motif cannot substitute for the first copy in DAI.

Motif 1 and motif 2 do not function analogously. From these data and our previous work (10), it appears that there are distinct functional differences between the two motifs contained in the RNA-binding domain of DAI. The first motif is more important for RNA binding, and it is less tolerant of changes in its sequence. Given that motif 2, as a representative of the so-called short domains (42), is homologous mainly at its C terminus, it could be argued that a duplicate copy of motif 1 would be as permissive as motif 2 toward sequence changes. To test this inference, we took advantage of the sharply different responses of the motifs to the central mutation (Phe→Ala at positions 41 and 131). As shown in Fig. 2, this mutation reduces binding to 10% of wild-type levels when located in the motif 1 (LS15) but has very limited effect on RNA binding when located in motif 2 (LS18). Since the replacement of the second motif with a duplicate copy of the first motif (Sub 1:1) also has little effect on RNA binding (10), we examined the effect of introducing the Phe→Ala mutation into the different copies of motif 1 in Sub 1:1. The three constructs generated for this experiment are designated Sub 1m:1, Sub 1:1m, and Sub 1m:1m to indicate the introduction of the Phe→Ala mutation within the first copy, second copy, or both copies of motif 1. Figure 4A illustrates the constructs prepared for this experiment in comparison with the wild-type molecule (1:2) and the mutants, LS15 (1m:2) and LS18 (1:2m).

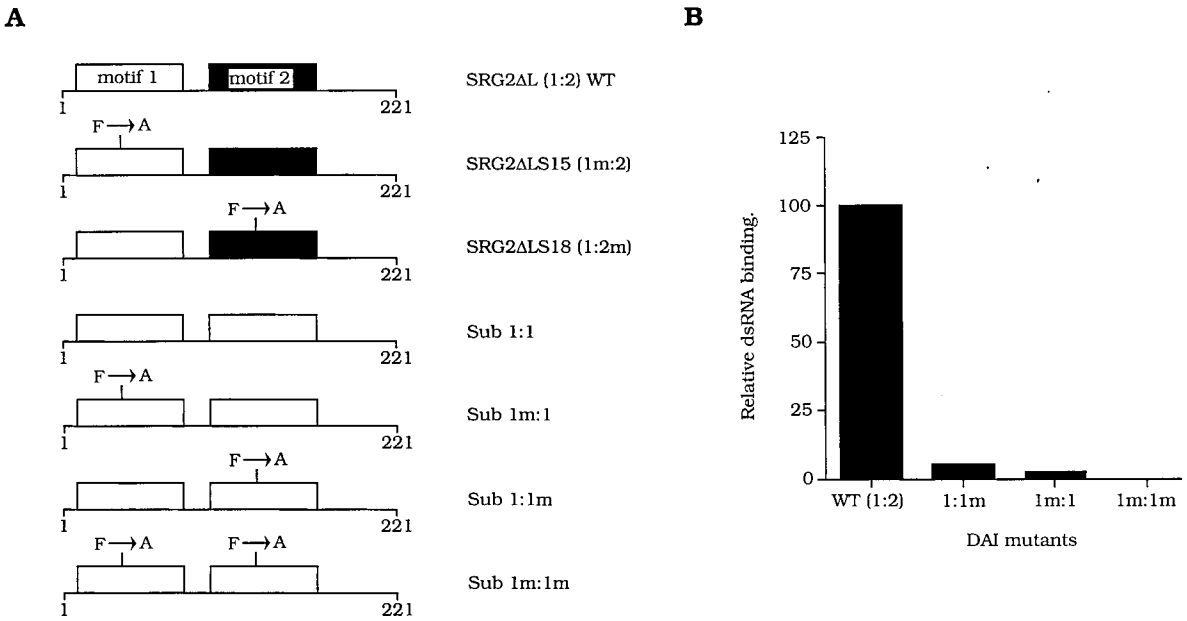


FIG. 4. Comparison of function between the two motifs. (A) Schematic representation of the constructs used in this experiment. (B) The RNA-binding assay was carried out as described for Fig. 2; the results shown are for the mutants in panel A not described previously. WT, wild type.

The abilities of these mutants to bind dsRNA are compared in Fig. 4B. From these data, it is apparent that the conversion of Phe to Ala in either the first or second copy of motif 1, or in both copies, was severely detrimental to RNA binding. Thus, motif 2 is not merely a degenerate copy of motif 1, in which only the presumptive α-helical region is sufficient, nor is the differential sensitivity of motif 1 and motif 2 simply a matter of their relative positions in the molecule because Sub 1:1m is much more severely affected by the mutations than LS18 (i.e., 1:2m). Instead we are led to the conclusion that although motif 1 can functionally substitute for motif 2, the two motifs do not bind RNA in the same manner since equivalent mutations introduced into conserved residues in the two motifs exert dramatically different effects on RNA binding even when they occupy the same position in the protein molecule.

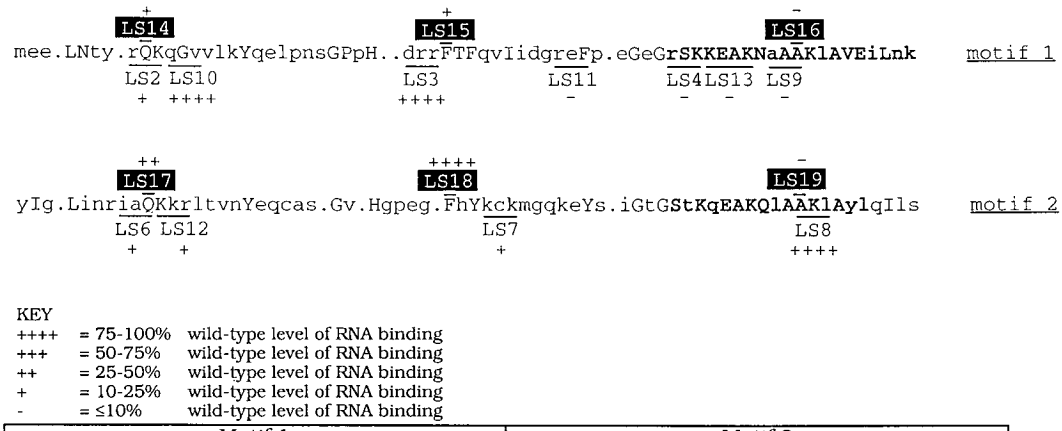
DISCUSSION

The RNA-binding domain of DAI comprises a 67-amino-acid motif repeated twice (10, 28, 42). Although there is homology with a number of RNA-binding proteins across the entire motif, most of the conserved residues are located in the C terminus in a region that is predicted to form a positively charged α-helix (10, 28, 42). This region represents a core motif within the extended motif. In this study, the substitution of proline for a highly conserved alanine residue within the helix, thereby potentially disrupting the helical structure, prevented RNA binding when the mutation was placed in either copy of the motif. The significance of this Ala→Pro mutation is twofold: first, it supports the view that the structure of the helical region is essential for RNA binding; second, it establishes that two copies of the motif are indispensable for efficient RNA binding since a single point mutation in either one drastically reduces the RNA-binding property of the mutant proteins. This result is in agreement with conclusions drawn from previous work in which analysis of larger mutations had indicated that both the charge and the structure of the helical region were important for RNA binding (10).

While the Ala→Pro mutation in the C-terminal part of the consensus has the most profound effect on RNA binding, giving over 95% inhibition, the other mutational sites studied, distributed throughout the motif, also influence RNA binding. This finding indicates that the entire motif is involved in RNA binding, consistent with earlier results obtained with N- and C-terminal deletions (42) and linker-scanning mutants (10). However, the two motifs are not equally important for binding. Changes at the two sites outside the C-terminal core had a greater influence on RNA binding when placed in the first motif than equivalent changes in the second motif, giving inhibitions of 80 to 90% compared with 10 to 50%. Furthermore, a mutant DAI protein containing a deletion of motif 1 and a duplication of motif 2 was severely debilitated, whereas the reciprocal mutant containing a duplication of motif 1 binds RNA nearly as efficiently as wild-type DAI (10). Not only do the two motifs differ quantitatively in their relative importance for binding, but there also appears to be a qualitative difference in their interactions with RNA. This functional difference is illustrated by the contrasting effect of the substitution of alanine for phenylalanine in the middle of the motif. Although this residue is conserved in many copies of the motif (2, 10, 42), the mutation had little effect when located in motif 2 (mutant LS18, i.e., 1:2m) but reduced binding by at least 10-fold when located in motif 1. The Phe→Ala change was severely detrimental in the wild-type molecule (LS15, i.e., 1m:2) and in the context of the motif 1 duplication (1:1m, 1m:1, and 1m:1m). This finding implies that the binding of RNA requires an intact motif 1 but makes lesser demands on motif 2. The required features of motif 2 appear to include its C-terminal potential α helix but not all of the conserved residues located elsewhere in the consensus sequence. However, the effects of substitutions, especially combinations of substitutions, in the remaining two-thirds of motif 2 show that this region does play some role in RNA binding (10).

Results similar to those presented here were obtained when the mutations that we have generated were studied for their effects on DAI activity in yeast cells (39). This study demonstrated a high correlation between the ability of mutations in the RNA-binding domain to inhibit RNA binding in vitro and to prevent the growth suppression phenotype caused by DAI in yeast cells (39). Thus, some mutations in motif 1 permitted yeast growth under conditions of DAI overexpression, but none of the mutations in motif 2 had this effect, implying that motif 1 is more important for RNA binding and consequently DAI activity in vivo. The yeast system is extremely sensitive and clearly revealed differences in activity between two Ala→Pro mutations (LS16 and LS19), which were barely distinguishable in binding assays (39). Data presented here and previously (10), summarized in Fig. 5, call attention to structural and functional distinctions between motifs 1 and 2. Motif 1 plays the more significant role in RNA binding, has the higher level of homology to the RNA-binding motif consensus sequence, and displays regions of functional importance that are absent in motif 2. Although the greater importance of motif 1 was postulated from previous studies (7, 10, 28), no firm conclusions could be drawn because the mutations were not made in identical residues in the two motifs. The functional nonequivalence of the two motifs is consistent with the finding that DAI mutants Δ1 and Δ2, containing deletions of motifs 1 and 2, respectively, complemented one another in yeast cells (39). The requirement for both motifs seen here and in the yeast experiments (39) are in disagreement with the results of McCormack et al. (27). This group demonstrated the importance of the first motif by mutating one of the conserved lysine residues in the α-helical region, but they reported that only motif 1 is required for RNA binding (27). Since the constructs used in these experiments were based on TrpE-DAI fusion proteins, presumably the TrpE component of these fusion proteins compensated in some way for the lack of motif 2.

How do we explain the differences between the first and second motifs? One possible interpretation is that the two similar motifs have evolved different functions within the RNA-binding role. The first motif may make direct interactions with RNA, while the second motif might be responsible for presenting the first motif to the RNA. This possibility is analogous to the function of two α helices in helix-turn-helix motifs. Helix-turn-helix motifs are present in many DNA-binding proteins, such as the lambda repressor (15), and bind DNA by inserting one helix (the recognition helix) into the major groove of DNA. The second helix and the surrounding amino acid residues are responsible for interactions with the DNA backbone that hold the recognition motif in the correct orientation (15). In the A-form configuration of dsRNA, the minor groove is wide and easily accessible whereas the major groove is too narrow to permit protein-RNA interactions (43). DAI has been shown to interact with phosphate and hydroxyl groups, both of which are located in the minor groove of RNA (3). Possibly the first motif of DAI is responsible for direct interactions with the minor groove of the RNA, while the second motif stabilizes the first motif in the correct position within the minor groove. It would follow that interactions between the first motif and RNA are the more critical and therefore that mutations in the first motif are more damaging than



Motif 1				Motif 2			
Mutation	Original residues	Mutated residues	RNA binding	Mutation	Original residues	Mutated residues	RNA binding
LS2	^{18}RQK	GAL	-	LS12	^{108}IAQ	GAL	+
LS14	^{19}Q	A	+	LS17	^{109}Q	A	++
LS10	^{21}QGV	GAL	++++	LS6	^{111}KKR	GAL	+
LS3	^{38}DRR	GAL	++++				
LS15	^{41}F	A	+	LS18	^{131}F	A	++++
LS11	^{51}REF	GAL	-	LS7	^{134}KCK	GAL	+
LS4	^{58}RSK	GAL	-				
LS13	^{61}KEAK	AEAA	-				
LS9	^{66}AAA	GAL	-	LS8	^{158}AKL	GAL	++++
LS16	^{68}A	P	-	LS19	^{158}A	P	-

FIG. 5. Summary of RNA-binding abilities of the DAI mutants. The RNA-binding motifs of DAI are represented in the standard single-letter code. Residues that are conserved in relation to the consensus sequence in Fig. 1 are shown in uppercase. Previously described mutations are shown below the motifs, and current mutations are printed in reverse type above each motif. The effect of each mutation on RNA binding is indicated. The position of each mutation and the residues changed are also shown.

identical ones in the second motif. Since mutant 1:1, containing a duplicate copy of motif 1 instead of motif 2, binds nearly as well as the undisturbed molecule, it would be necessary to suppose that motif 1 has retained the functions of motif 2 as well as its own specific functions in RNA binding. Furthermore, more efficient binding of the domain swap mutant 2:1 than the wild-type molecule would imply considerable geometrical plasticity in the cooperation between the two motifs.

An alternative and more attractive explanation for the differences between the two motifs is that both motifs bind RNA, but motif 2 performs this function less efficiently since it has diverged further from the consensus sequence. Motif 1 may form a compact, highly organized structure that makes a number of critical interactions along its entire length, so that mutations in many sites within the motif affect RNA binding adversely. Motif 2, on the other hand, may be a more flexible structure interacting with RNA chiefly through its helical region, so that mutations elsewhere have a lesser or variable effect. Presumably, the motifs function cooperatively in binding RNA, and it is specific sequences in the N-terminal two-thirds of the motif that prevent motif 1 from interacting with RNA exclusively through its helical region, thereby giving rise to the contrasting results seen between mutants 1:1m and 1:2m (LS18). By the same token, the less important role of motif 2 in RNA binding may have led to its deviation from the consensus sequence, especially in its N-terminal region. This model satisfactorily accommodates the data obtained with domain swap and point mutants, both those in equivalent residues, presented here, and the clustered point mutants distributed less systematically through the motifs (10). In addition, this view that both motifs interact with RNA is easier to reconcile with their structural similarity and with the genetic evidence of their ability to complement (39).

Notably, all seven mutations tested here altered the ability of DAI to bind VA RNA and dsRNA to similar extents. In fact, in all studies of the RNA-binding domain of DAI so far published, no difference in VA RNA binding and dsRNA binding has been detected. This suggests that the binding sites for the two RNAs are identical or only subtly different. More extensive mutagenesis is required to settle this point, but if, as it currently appears, the two RNAs do bind to the same region, then the differentiation between RNAs which activate or inhibit DAI must occur at a stage after RNA binding. A possible interpretation of this finding is that the binding of DAI to dsRNA results in a conformational change, such as the removal of a pseudosubstrate site from the active site of the kinase, permitting autophosphorylation and the activation of eIF-2 kinase. Presumably, the compact highly structured form of VA RNA does not permit the conformational change that occurs upon binding to linear dsRNA (22), possibly because of interactions between the enzyme and the complex stem-loop structure that forms the central domain of the RNA and is critical to its function (3, 25). Thus, VA RNA binds to DAI in a nonproductive manner, thereby preventing dsRNA binding and consequently inhibiting DAI activation.

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REFERENCES

1. **Baglioni, C., and P. A. Maroney.** 1981. Inhibition of double-stranded ribonucleic acid activated protein kinase and 2′,5′-oligo(adenylic acid) polymerase by ethidium bromide. Biochemistry **20:**758–762.
2. **Bass, B. L., S. R. Hurst, and J. D. Singer.** 1994. Binding properties of newly identified *Xenopus* proteins containing dsRNA-binding motifs. Curr. Biol. **4:**301–314.
3. **Clarke, P. A., and M. B. Mathews.** Interactions between the double-stranded RNA-binding motif and RNA: definition of the binding site for the interferon-induced protein kinase DAI on adenovirus VA RNA. RNA J., in press.
4. **Clarke, P. A., N. A. Sharp, and M. J. Clemens.** 1990. Translation control by Epstein-Barr virus small RNA EBER-1. Reversal of the double stranded RNA induced inhibition of protein synthesis in reticulocyte lysates. Eur. J. Biochem. **193:**635–641.
5. **Colthurst, D. R., D. G. Campbell, and C. G. Proud.** 1987. Structure and regulation of eukaryotic initiation factor eIF-2: sequence of the site in the α subunit phosphorylated by the haem-controlled repressor and by the double-stranded RNA activated inhibitor. Eur. J. Biochem. **166:**357–363.
6. **Farrell, P. J., K. Balkow, T. Hunt, R. J. Jackson, and H. Trachsel.** 1977. Phosphorylation of initiation factor eIF-2 and the control of reticulocyte protein synthesis. Cell **11:**187–200.
7. **Feng, G. S., K. Chong, A. Kumar, and B. R. G. Williams.** 1992. Identification of double-stranded RNA-binding domains in the interferon-induced double-stranded RNA-activated p68 kinase. Proc. Natl. Acad. Sci. USA **89:**5447–5451.
8. **Galabru, J., and A. Hovanessian.** 1987. Autophosphorylation of the protein kinase dependent on double-stranded RNA. J. Biol. Chem. **262:**15538–15544.
9. **Galabru, J., M. G. Katze, N. Robert, and A. G. Hovanessian.** 1989. The binding of double-stranded RNA and adenovirus VA RNA to the interferon-induced protein kinase. Eur. J. Biochem. **178:**581–589.
10. **Green, S. R., and M. B. Mathews.** 1992. Two RNA-binding motifs in the double-stranded RNA-activated protein kinase, DAI. Genes Dev. **6:**2478–2490.
11. **Gunnery, S., S. R. Green, and M. B. Mathews.** 1992. Tat-responsive region RNA of human immunodeficiency virus type 1 stimulates protein synthesis *in vivo* and *in vitro*: relationship between structure and function. Proc. Natl. Acad. Sci. USA **89:**11557–11561.
12. **Gunnery, S., A. P. Rice, H. D. Robertson, and M. B. Mathews.** 1990. Tat-responsive region RNA of human immunodeficiency virus 1 can prevent activation of the double-stranded-RNA-activated protein kinase. Proc. Natl. Acad. Sci. USA **87:**8687–8691.
13. **Hershey, J. W. B.** 1991. Translational control in mammalian cells. Annu. Rev. Biochem. **60:**717–755.
14. **Hunter, T., T. Hunt, R. J. Jackson, and H. D. Robertson.** 1975. The characteristics of inhibition of protein synthesis by double-stranded ribonucleic acid in reticulocytes lysates. J. Biol. Chem. **250:**409–417.
15. **Jordan, S. R., and C. O. Pabo.** 1988. Structure of the lambda complex at 2.5 Å resolution: details of the repressor-operator interactions. Science **242:**893–899.
16. **Katze, M. G., M. Wambach, M. L. Wong, M. Garfinkel, E. Meurs, K. Chong, B. R. G. Williams, A. G. Hovanessian, and G. N. Barber.** 1991. Functional expression and RNA binding analysis of the interferon-induced, double-stranded RNA-activated, 68,000-M_r protein kinase in a cell-free system. Mol. Cell. Biol. **11:**5497–5505.
17. **Kenan, D. J., C. C. Query, and J. D. Keene.** 1991. RNA recognition: towards identifying determinants of specificity. Trends Biochem. Sci. **16:**214–220.
18. **Koromilas, A. E., S. Roy, G. N. Barber, M. G. Katze, and N. Sonenberg.** 1992. Malignant transformation by a mutant of the IFN-inducible dsRNA-dependent protein kinase. Science **257:**1685–1689.
19. **Kostura, M., and M. B. Mathews.** 1989. Purification and activation of the double-stranded RNA-dependent eIF-2 kinase DAI. J. Biol. Chem. **9:**1576–1586.
20. **Lazinski, D., E. Grzadzielska, and A. Das.** 1989. Sequence-specific recognition of RNA hairpins by bacteriophage antiterminators requires a conserved arginine-rich motif. Cell **59:**207–218.
21. **Lee, S. B., and M. Esteban.** 1994. The interferon-induced double-stranded RNA-activated protein kinase induces apoptosis. Virology **199:**491–496.
22. **Manche, L., S. R. Green, C. Schmedt, and M. B. Mathews.** 1992. Interactions between double-stranded RNA regulators and the protein kinase DAI. Mol. Cell. Biol. **12:**5238–5248.
23. **Maran, A., and M. B. Mathews.** 1988. Characterization of the double-stranded RNA implicated in the inhibition of protein synthesis in cells infected with a mutant adenovirus defective for VA RNAI. Virology **164:**106–113.
24. **Mathews, M. B.** 1993. Viral evasion of cellular defense mechanisms: regulation of the protein kinase DAI by RNA effectors. Semin. Virol. **4:**247–257.
25. **Mathews, M. B., and T. Shenk.** 1991. Adenovirus virus-associated RNA and translation control. J. Virol. **65:**5657–5662.
26. **Mattaj, I. W.** 1993. RNA recognition: a family matter? Cell **73:**837–840.
27. **McCormack, S. J., L. G. Ortega, J. P. Dohan, and C. E. Samuel.** 1994. Mechanism of interferon action: motif 1 of the interferon-induced, RNA-dependent protein kinase (PKR) is sufficient to mediate RNA-binding activity. Virology **198:**92–99.
28. **McCormack, S. J., D. C. Thomis, and C. E. Samuel.** 1992. Mechanism of interferon action: identification of a RNA binding domain within the N-terminal region of the human RNA-dependent P1/eIF-2α protein kinase. Virology **188:**47–56.
29. **Mellits, K. H., T. Pe'ery, L. Manche, H. D. Robertson, and M. B. Mathews.** 1990. Removal of double-stranded contaminants from RNA transcripts: synthesis of adenovirus VA RNA from a T7 vector. Nucleic Acids Res. **18:**5401–5406.
30. **Meurs, E., K. Chong, J. Galabru, N. S. B. Thomas, I. M. Kerr, B. R. G. Williams, and A. G. Hovanessian.** 1990. Molecular cloning and characterization of the human double-stranded RNA-activated protein kinase induced by interferon. Cell **62:**379–390.
31. **Meurs, E. F., J. Galabru, G. N. Barber, M. G. Katze, and A. G. Hovanessian.** 1993. Tumor suppressor function of the interferon-induced double-stranded RNA-activated protein kinase. Proc. Natl. Acad. Sci. USA **90:**232–236.
32. **Minks, M. A., D. K. West, S. Benvin, and C. Baglioni.** 1979. Structural requirements of double-stranded RNA for the activation of 2′,5′-oligo(A) polymerase and protein kinase of interferon-treated HeLa cells. J. Biol. Chem. **254:**10180–10183.
33. **Minks, M. A., D. K. West, S. Benvin, J. J. Greene, P. O. P. Ts'o, and C. Baglioni.** 1980. Activation of 2′,5′-oligo(A) polymerase and protein kinase of interferon-treated HeLa cells by 2′-O-methylated poly(inosinic acid) poly(cytidylic acid). J. Biol. Chem. **255:**6403–6407.
34. **Moldave, K.** 1985. Eukaryotic protein synthesis. Annu. Rev. Biochem. **54:**1109–1149.
35. **Pain, V. M.** 1986. Initiation of protein synthesis in mammalian cells. Biochem. J. **235:**625–637.
36. **Patel, R. C., and G. C. Sen.** 1992. Identification of the double-stranded RNA binding domains of the human interferon-inducible protein kinase. J. Biol. Chem. **267:**7671–7676.
37. **Proud, C. G.** 1986. Guanine nucleotides, protein phosphorylation and the control of protein synthesis. Trends Biochem. Sci. **11:**73–77.
38. **Robertson, H. D., L. Manche, and M. B. Mathews.** Submitted for publication.
39. **Romano, P. R., S. R. Green, M. B. Mathews, and A. G. Hinnebusch.** 1995. Structural requirements for double-stranded RNA binding, dimerization, and activation of the human eIF-2α kinase DAI in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **15:**365–378.
40. **Sen, G. C., H. Taira, and P. Lengyel.** 1978. Interferon, double-stranded RNA, and protein phosphorylation. J. Biol. Chem. **253:**5915–5921.
41. **Sonenberg, N.** 1990. Measures and countermeasures in the modulation of initiation factor activities by viruses. New Biol. **2:**402–409.
42. **St Johnston, D., N. H. Brown, J. G. Gall, and M. Jantsch.** 1992. A conserved double-stranded RNA-binding domain. Proc. Natl. Acad. Sci. USA **89:**10979–10983.
43. **Steitz, T. A.** 1990. Structural studies of protein-nucleic acid interaction: the source of sequence-specific binding. Q. Rev. Biophys. **23:**205–280.
44. **Thomis, D. C., J. P. Doohan, and C. E. Samuel.** 1992. Mechanism of interferon action: cDNA structure, expression, and regulation of the interferon-induced, RNA-dependent P1/eIF-2α protein kinase from human cells. Virology **188:**33–46.