Two RNA-binding motifs in the double-stranded RNA-activated protein kinase, DAI

Simon R. Green and Michael B. Mathews[1]

Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724 USA.

The protein kinase DAI, the double-stranded RNA-activated inhibitor of translation, is an essential component of the interferon-induced cellular antiviral response. The enzyme is regulated by the binding of activator and inhibitor RNAs. We synthesized DAI in vitro and located its RNA-binding domain within the amino-terminal 171 residues. This domain contains two copies of an RNA-binding motif characterized by a high density of basic amino acids, by the presence of conserved residues, and by a probable α-helical structure. Deletion of either of the two motifs prevents the binding of dsRNA, but their relative positions can be exchanged, suggesting that they cooperate to interact with dsRNA. Clustered point mutations within the RNA-binding motifs and duplications of the individual motifs indicate that the first copy of the motif plays the more important role. Mutations that impair binding have similar effects on the binding of double-stranded RNAs of various lengths and of adenovirus VA RNA_I, implying that discrimination between activator and inhibitory RNAs takes place subsequent to RNA binding.

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Phosphorylation plays an important role in regulating the activities of many of the protein components of the translational machinery (Hershey 1989, 1990, 1991). One of the most intensively studied of these regulatory events involves the phosphorylation of eukaryotic initiation factor 2 (eIF-2). This factor is comprised of three different subunits (α, β, and γ) of which both the α and β subunits are phosphorylated (Hershey 1990, 1991). Although the physiological significance of eIF-2β phosphorylation remains to be determined, it is well established that phosphorylation of the α-subunit can lead to the rapid cessation of protein synthesis (Hershey 1990, 1991). In one of the first steps of translational initiation, eIF-2 transports the initiator tRNA (Met-$tRNA_i$) in a ternary complex with GTP to the 40S ribosomal subunit (for review, see Moldave 1985; Pain 1986; Hershey 1991). The α- and β-subunits of eIF-2 are also involved in translational start site selection in yeast (Donahue et al. 1988; Cigan et al. 1989). Before the association of the 40S complex with the 60S subunit to form the 80S ribosomal complex, eIF-2 is released in a binary complex with GDP that must be replaced by GTP to permit the binding of a fresh molecule of Met-$tRNA_i$ and reentry of the factor into the initiation process (Moldave 1985; Pain 1986; Hershey 1991). This regeneration is catalyzed by the guanosine nucleotide exchange factor (GEF or eIF-2B) (Konieczny and Safer 1983; Panniers and Henshaw 1983). If the α-subunit of eIF-2 is phosphorylated at Ser^{51}, the exchange reaction is blocked by the formation of a nondissociable complex between GEF and eIF-2 · GDP (Proud 1986; Colthurst et al. 1987). Initiation ceases in the absence of free GEF, and because GEF is present in cells at a lower concentration than eIF-2 (Safer 1983), the recycling factor may be completely sequestered when only a fraction (20–50%) of the eIF-2α in the cell is phosphorylated.

Three known protein kinases are capable of phosphorylating eIF-2α on Ser^{51} and they mediate a variety of control processes. In yeast, GCN2 kinase causes a gene-specific translational derepression rather than a general shutdown of polypeptide synthesis (Dever et al. 1992). Under starvation conditions, this kinase is responsible for the translational induction of the transcriptional activator GCN4 by phosphorylating eIF-2α, thereby allowing ribosomes to bypass the regulatory open reading frames in the 5′ leader sequence of GCN4 mRNA (Hinnebusch 1990; Dever et al. 1992). GCN2 kinase is probably activated by the presence of uncharged tRNAs (Wek et al. 1990). The other two eIF-2α kinases are characteristic of higher eukaryotes (for review, see Ochoa 1983; Mathews et al. 1990; Hershey 1991). The heme controlled repressor (HCR or HRI) is found mainly in reticulocytes. It is activated by a number of stimuli, most notably by the absence of heme or Fe^{2+}, and it serves to prevent globin synthesis in the absence of its prosthetic group, heme (Jackson 1991). The third kinase, the double-stranded RNA (dsRNA) activated inhibitor (DAI, also termed DsI, p68, and P1 kinase), is present in most cell

[1]Corresponding author.

types. DAI plays an important role in the interferon-induced antiviral response, and it has also been associated with cellular differentiation (Petryshyn et al. 1984; Judware and Petryshyn 1991), the inhibition of cell proliferation (Chong et al. 1992), the heat shock response (Dubois et al. 1989; Edery et al. 1989), and possibly transcriptional activation (Zinn et al. 1988).

The regulation of DAI is poorly understood. The enzyme is usually present in cells at a low level and in an inactive state. Its cellular concentration is increased by interferon at the transcriptional level (Hovanessian 1989; Samuel 1991), whereas its activity is regulated by both activator and inhibitor RNAs (Mathews and Shenk 1991). Activation of DAI is accompanied by autophosphorylation of the latent enzyme, an event that apparently unmasks the ability of the enzyme to phosphorylate eIF-2α (Farrell et al. 1977; Levin and London 1978; Sen et al. 1978; Berry et al. 1985; Galabru and Hovanessian 1987; Kostura and Mathews 1989). Optimal activation of DAI requires dsRNA that is perfectly duplexed and greater than ~85 bp in length, but there is no RNA sequence dependence (Hunter et al. 1975; Minks et al. 1979, 1980; Manche et al. 1992). At relatively high concentrations, activation is inhibited by short dsRNAs (less than ~30 bp), which are not capable of activating DAI (Minks et al. 1979, 1980; Manche et al. 1992). Furthermore, small, highly structured, single-stranded RNAs such as adenovirus virus-associated (VA) RNA_I, the EBERs of Epstein–Barr virus, and TAR RNA encoded by human immunodeficiency virus-1 (HIV-1) are specialized effectors: They block DAI activation at relatively high concentrations but are incapable of activating the enzyme (for review, see Mathews and Shenk 1991). Paradoxically, at similar high concentrations, long dsRNA also becomes inhibitory (Hunter et al. 1975; Farrell et al. 1977; Lenz and Baglioni 1978).

To understand the complex molecular interactions between DAI and RNA effectors, we undertook a mutational analysis of the protein, examining the ability of a variety of deletion, truncation, and substitution mutants to interact with RNA. The RNA-binding domain comprises two basic regions located in the first 171 amino acids of the DAI sequence. Each of these regions contains a consensus RNA-binding motif and a putative α-helix. Mutations in the second motif are not as debilitating as similar mutations in motif 1, and the duplication of the second motif does not compensate for deletion of the first motif. Positional interchange of the two regions is not deleterious and their spacing can be varied, within limits, indicating that they cooperate to form the RNA-binding site. Clustered point mutations in these two regions affect the binding of long and short dsRNAs and of adenovirus VA RNA_I in the same way, implying that inhibitory RNAs bind to the same site as activators.

Results

Localization of the RNA-binding domain

DAI is a protein kinase that binds to dsRNA, leading to autophosphorylation and activation of the enzyme's ability to phosphorylate eIF-2. Inspection of its predicted amino acid sequence, derived from a cDNA clone (Meurs et al. 1990; Thomis et al. 1992), suggested that the protein can be divided into two regions of roughly equal size. The 270 amino acids of the carboxyl terminus contain the 11 subdomains that are essential for kinase activity and evidently comprise the catalytic domain of a protein kinase (Fig. 1A; Hanks et al. 1988). Because the first 280 amino acids of the protein are free of such catalytic domains, we hypothesized that this region is involved in regulating kinase activity and contains the RNA-binding domain(s) of the enzyme. The amino-terminal half of the protein possesses no RNA-binding motifs typical of small nuclear ribonucleoproteins (snRNPs) (Kenan et al. 1989), but it contains a high density of lysine and arginine residues (Fig. 1B), which have been implicated in both DNA helix and RNA hairpin binding (Lazinski et al. 1989; Steitz 1990). These basic residues are concentrated in three distinct regions within the amino terminus of DAI (Fig. 1A). To examine their role in RNA binding, we excised the basic regions individually and assessed the ability of the deleted proteins to interact with RNA ligands.

Templates encoding the three deletion mutants, Δ1, Δ2, and Δ3 (Fig. 1A), were transcribed in vitro with bacteriophage T7 RNA polymerase, and the resultant RNAs were translated in a wheat germ cell-free system to generate ^{35}S-labeled mutant proteins. These proteins were then compared to the full-length protein for their ability to bind to either dsRNA or adenovirus VA RNA_I coupled to Sepharose beads. Equal volumes of the translation products (Fig. 2A) were exposed to the immobilized ligands, and the resultant complexes containing radiolabeled protein were analyzed by gel electrophoresis and autoradiography (Fig. 2B,C). Neither Δ1 nor Δ2 (deletions of amino acids 1–97 and 104–157, respectively) was able to bind to dsRNA or VA RNA, but Δ3 (a deletion of amino acids 234–272) retained its ability to bind both types of RNA (Fig. 2B,C; lanes 3–5). Furthermore, a deletion mutant lacking sequences near the carboxyl terminus (Δ4, a deletion of residues 482–523) and a point mutant in kinase domain II (Lys 296 → Arg) also bound to both RNA matrices (Fig. 2B,C; lanes 6 and 2). Background binding to beads lacking an RNA ligand was negligible (Fig. 2B, lane 8). These data suggested that the first two regions of basic amino acids are essential for RNA binding, whereas the third basic region is not required.

To vary the spacing between the first two basic regions, we also made two internal deletion mutants, Δ5 and Δ6. Decreasing the distance between basic regions 1 and 2 by four amino acids (Δ5) had very little effect on dsRNA binding, whereas removing 19 amino acids from this area (Δ6) abrogated dsRNA-binding ability (see Fig. 5, lanes 6,7 below). Therefore, the spacing is not absolutely critical, but some of the residues between the two basic regions appear to be indispensable.

In addition to the full-length wild-type protein, many shorter polypeptides also bound to the dsRNA–Sepharose matrix (Fig. 2B, lane 1). The smallest of these polypeptides, which are probably carboxy-terminal trunca-

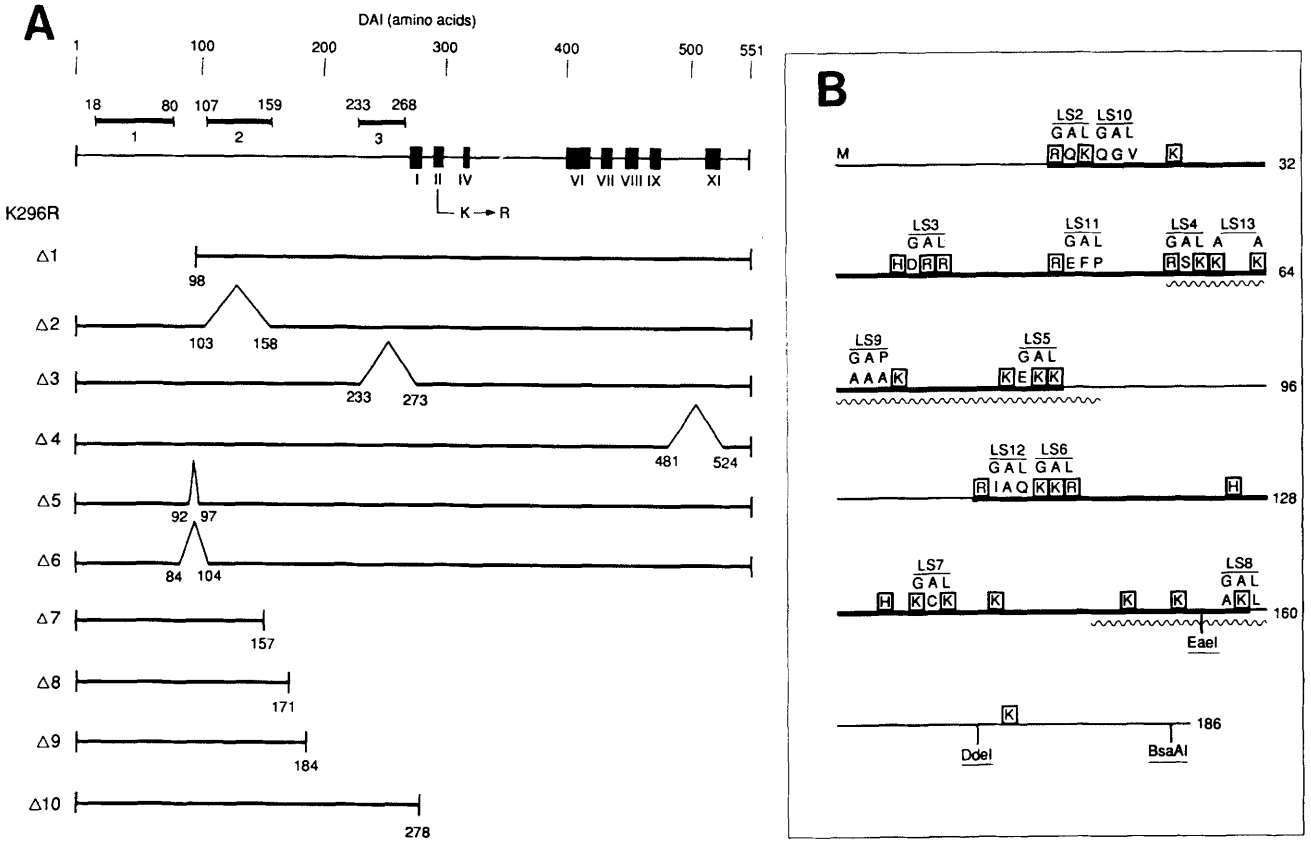

Figure 1. Structure of DAI and mutants. (*A*) Schematic representation of full-length DAI and mutants. The *top* line represents the linear DAI protein sequence indicating the highly conserved kinase domains in the carboxy-terminal half of the protein (boxes I–XI) and the three basic regions within its amino-terminal half (thick lines 1, 2, and 3). The position of the single amino acid substitution in mutant K296R is marked. The lower lines depict the deletion mutants (Δ1–Δ6) and the sites of truncation (Δ7–Δ10). The numbers below each line denote the amino acid residues present in the deleted proteins. (*B*) LS mutants. The first 186 amino acids of the DAI sequence are represented, with the location of basic regions 1 and 2 indicated by thickening of the line. All the basic amino acids are boxed, and the residues changed by site-directed mutagenesis are shown with the mutant residues positioned above the mutated wild-type residues. Restriction enzyme sites used for the generation of truncated DNA (Δ7–Δ9) are also shown.

tions resulting from premature termination events, had apparent molecular weights of <25,000. To define the minimum length of the amino-terminal segment of DAI required for RNA binding more precisely, truncated proteins were prepared (Δ7–Δ10, Fig. 1A) and tested in a similar manner. The truncated protein Δ9 (amino acids 1–184) bound to both types of RNA affinity matrix (Fig. 3A, lanes 6,9), whereas Δ7 (residues 1–155) did not bind efficiently (lanes 5,8), indicating that the carboxy-terminal boundary of the RNA-binding domain lies between residues 157 and 184. This assignment was confirmed in a second type of binding assay. DAI was immobilized on protein A–Sepharose beads by polyclonal antibody and was then exposed to synthetic ^{32}P-labeled dsRNA of 85 bp. The bound RNA was quantitated by direct radioactive counting and visualized by electrophoresis and autoradiography. Figure 3B shows that full-length DAI and the Δ9 truncated protein (residues 1–184) bound the labeled dsRNA with similar efficiencies. Further experiments demonstrated that a reduction in length to 171 amino acids (Δ8) had little or no effect on the ability of DAI to bind dsRNA, whereas truncation at residue 157 (Δ7) effectively eliminated binding as in the dsRNA–Sepharose binding assay (see Fig. 5, lanes 2,3, below). Thus, we conclude that the RNA-binding domain lies within the first 171 amino acids of DAI, in agreement with the results of Katze et al. (1991) and Patel and Sen (1992). This domain contains both of the basic regions that appear to be essential.

Mutational analysis of the RNA-binding domain

To examine the two regions more closely, we made a series of clustered point mutations that changed basic amino acids to residues with uncharged side chains (Fig. 1B; Table 1). The mutations were made in such a way that three adjacent amino acids were exchanged in each mutant by linker scanning mutagenesis. Initially, seven such mutants were generated, LS2–LS8, distributed through the two basic regions in Δ9 (residues 1–184). In addition, we constructed a number of double mutants by combining two LS mutations (e.g., LS3,5, which contains

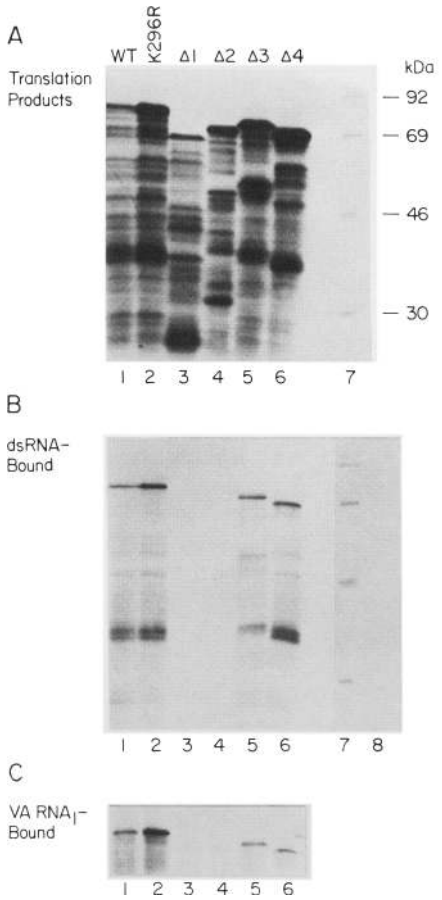

Figure 2. DAI translation and location of the RNA-binding domain. (*A*) Total translation products. Capped RNA transcripts synthesized by T7 polymerase in vitro, were used to program the wheat germ cell-free translation system. The resultant translation products were analyzed by electrophoresis in 12.5% polyacrylamide–SDS gels and autoradiography. Lanes *1–6* contain products from the wild-type clone (SRG2ΔL) and the mutants K296R, Δ1, Δ2, Δ3, and Δ4, respectively. Lane 7 contains ^{14}C-labeled molecular weight markers. (*B*) Binding to dsRNA–Sepharose. The translation products (5 μl) were incubated with dsRNA–Sepharose beads. After washing, the adsorbed proteins were analyzed as in *A* (lanes *1–7*). (Lane *8*) Control using Δ4 protein and beads lacking RNA ligand. (*C*) Binding to VA RNA–Sepharose. As in *B*, except that the translation products were incubated with VA RNA–Sepharose beads instead of dsRNA–Sepharose.

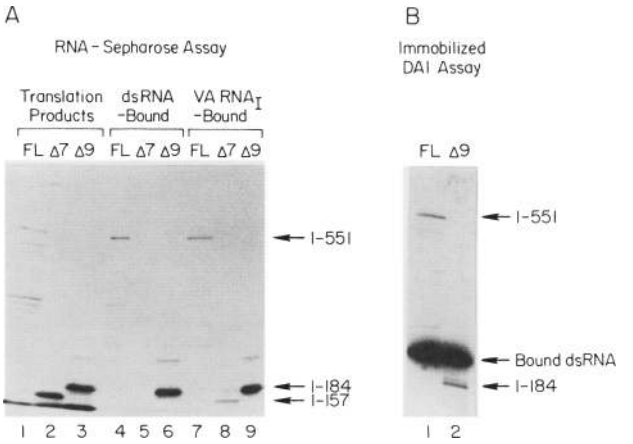

Figure 3. RNA-binding activity of truncated DAI. (*A*) RNA–Sepharose assay. RNA from full-length wild-type construct and the truncations Δ7 and Δ9 was translated. Equal volumes of the translation products (5 μl) were tested in both dsRNA–Sepharose and VA RNA–Sepharose-binding assays. Translation products and adsorbed proteins were analyzed by electophoresis in 15% polyacrylamide–SDS gels and autoradiography. (*B*) Immobilized DAI assay. Equal amounts of radioactive protein of wild-type full-length and Δ9 DAI were adsorbed to polyclonal antibody and immobilized on protein A–Sepharose. After incubation with ^{32}P-labeled dsRNA (85 bp), the beads were washed and the resultant RNA–protein complexes were analyzed as in *A*.

both the LS3 and LS5 mutations). The mutant proteins were labeled by in vitro transcription and translation and were subjected to binding analysis using the dsRNA–Sepharose assay.

Essentially equal amounts of protein were used in each assay (Fig. 4A). Strikingly, the introduction of the LS4 mutation resulted in a dramatic shift in gel mobility. DAI migrates anomalously during electrophoresis in SDS gels, with an apparent molecular weight of 68,000 compared with 62,000 predicted from the cDNA sequence (Meurs et al. 1990). The wild-type 184 residue Δ9 protein migrated with the expected mobility (equivalent to ~20 kD), but the LS4 substitution reduced its apparent molecular mass by ~4 kD. This effect was not observed with any of the other LS mutations, suggesting that LS4 may disturb a structurally important region of the DAI molecule. Two different algorithms (Chou and Fasman 1974; Garnier et al. 1978) predict that the original LS4 residues represent the amino terminus of an α-helix lying between residues 58 and 82 in region 1 (see below and Fig. 8B). Although the LS4 mutation, like all of the mutations described here, had been verified by DNA sequence analysis, we confirmed that the aberrant mobility was not the result of the presence of an unde-

Table 1. *LS mutants*

Mutant	Position	Original residues	Mutated residues
LS2	18–20	Arg Gln Lys	Gly Ala Leu
LS3	38–40	Asp Arg Arg	Gly Ala Leu
LS4	58–60	Arg Ser Lys	Gly Ala Leu
LS5	78–80	Glu Lys Lys	Gly Ala Leu
LS6	111–113	Lys Lys Arg	Gly Ala Leu
LS7	134–136	Lys Cys Lys	Gly Ala Leu
LS8	158–160	Ala Lys Leu	Gly Ala Leu
LS9	66–68	Ala Ala Ala	Gly Ala Pro
LS10	21–23	Glu Gly Val	Gly Ala Leu
LS11	51–53	Glu Phe Pro	Gly Ala Leu
LS12	108–110	Ile Ala Gln	Gly Ala Leu
LS13	61–64	Lys Glu Ala Lys	Ala Glu Ala Ala

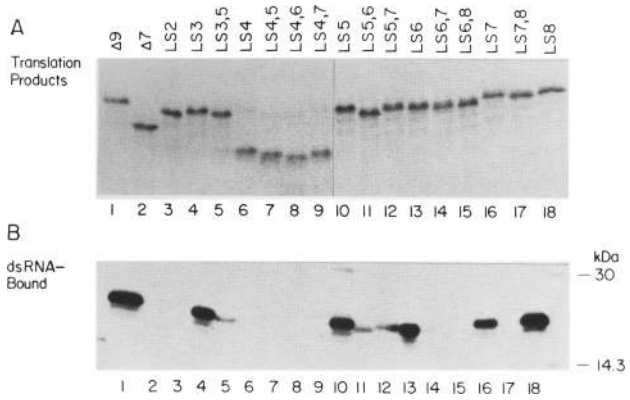

Figure 4. Binding of dsRNA to DAI mutants using the dsRNA–Sepharose assay. (*A*) Total translation products. Uncapped RNA was synthesized from a number of mutant templates truncated as for Δ9. Equal amounts of radioactive proteins were analyzed by electrophoresis in 20% polyacrylamide–SDS gels and autoradiography. (*B*) Binding of DAI mutants to dsRNA. Equal amounts of labeled translated proteins were reacted with dsRNA–Sepharose beads and analyzed as in *A*.

tected stop codon by truncating the template so as to yield a longer polypeptide of 278 residues (Δ10; Fig. 1A). Relative to the LS4 Δ9 polypeptide, the Δ10 product exhibited the expected increase in apparent size (data not shown), thereby excluding the existence of undetected stop codons in the LS4 construct. However, this extended LS4 protein still ran anomalously slowly in relation to the wild-type Δ10 protein (data not shown), consistent with a structural peculiarity determined by the residues located in the region of the LS4 mutation.

The ability of the mutant polypeptides to bind dsRNA varied dramatically (Fig. 4B). In the dsRNA–Sepharose assay, mutants LS3, LS5, and LS8 all appeared to bind dsRNA as efficiently as the wild-type Δ9 protein; LS6 and LS7 bound at a reduced level; and LS2 and LS4 did not appear to bind at all. Thus, two of the single mutations in the first basic region (LS2 and LS4) completely abrogated dsRNA binding, whereas two others (LS3 and LS5) had no effect. None of the single mutations in the second basic region (LS6, LS7 and LS8) had a dramatic effect on dsRNA binding, despite the observation that a deletion of this region eliminates RNA binding (Fig. 2A). However, when the mutations were combined to form double mutants, all nine of those tested displayed a significantly reduced ability to bind dsRNA (Fig. 4B). This was the case even with mutations in region 2 that had little effect on dsRNA binding individually (LS6,7, LS6,8 and LS7,8; Fig. 4B, lanes 14,15,17), suggesting that they act synergistically to impair dsRNA binding.

To extend and quantitate these results, we turned to the alternative binding assay using immobilized DAI. This assay permits estimation of the dsRNA bound by direct radioactive counting (Fig. 5A), as well as visualization of dsRNA and DAI by autoradiography (Fig. 5B). The Δ9 truncated form of DAI (residues 1–184) and its mutant derivatives were labeled with [^{35}S-]methionine, bound to antibody–Sepharose beads, and reacted with ^{32}P-labeled dsRNA. For the single mutants, LS2-LS8, the results agreed closely with those derived from the dsRNA–Sepharose-binding assay, although the immobilized DAI assay appeared to be more sensitive to small differences in binding efficiency. Thus, LS3, LS5, and LS8 bound 85, 60, and 116% as much dsRNA as wild type and LS6 and LS7 bound only ~10–15% as much dsRNA as the wild-type polypeptide. Binding was insignificant in LS2 and LS4. Another mutant in the first basic region, LS13, which contains two Lys → Ala substitutions separated by two unaltered residues (Fig. 1B; Table 1), also failed to bind detectable quantities of dsRNA (Fig. 5, lane 10). These data show that some, but not all, of the basic residues in the amino-terminus are critical for binding and confirm that both basic regions are important, although mutants in region 1 are more severely impaired.

As expected from previous results (Fig. 4B), in general double mutants bound less dsRNA than the more severely impaired of the single mutants that constituted them (e.g., c.f. lane 12 with lanes 11 and 17). This was true even for those mutations (LS3, LS5, and LS8) that exerted minimal effects on their own, with one notable exception. Although the LS8 mutation caused a synergistic effect when combined with other region 2 mutations close to its own location (in LS6,8 and LS7,8), it exerted only a marginal effect when combined with more distant mutations in region 1 (LS3,8 and LS5,8). This observation raises the possibility that the two binding regions function semiautonomously.

Definition of an RNA-binding motif

Up to this point we have focused on the basic residues located in the amino-terminal third of the DAI molecule. Most mutations of such residues were deleterious to dsRNA binding, but it was not possible to decide on the basis of these experiments alone whether it was the charge change or the structural consequence of the mutation that was important. To address this question, we made further mutations altering nonbasic amino acids in the proximity of basic residues that are required for dsRNA binding. Mutants LS9–12 were constructed to alter residues near LS13, LS2, LS4, and LS6, respectively.

The introduction of the LS10 mutation reduced the ability of DAI to bind dsRNA only slightly, despite the fact that alterations in the preceding 3 amino acids in LS2 destroyed all ability to bind dsRNA (Fig. 5, lanes 8,33). On the other hand, the LS12 mutation reduced dsRNA-binding ability to 10% of the wild-type level, as seen for the adjacent mutation of basic residues, LS6 (Fig. 5, lanes 21,35). The LS9 and LS11 mutations impaired binding even more severely (Fig. 5, lane 32; see Fig. 7, lane 17, below). Therefore, changes in nonbasic residues within the amino terminus of DAI can also affect its dsRNA-binding ability, and the basic regions do not function simply as regions of positive charge.

Consistent with this conclusion, inspection of the sequence of this and other RNA-binding proteins sug-

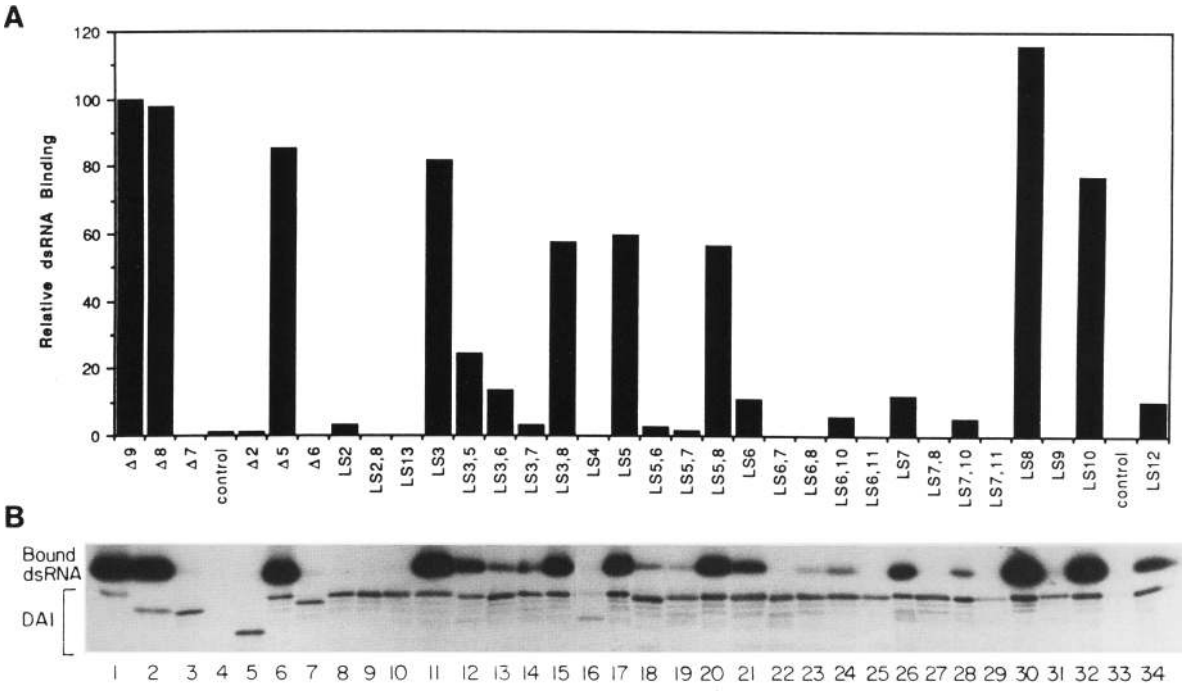

Figure 5. Quantitation of dsRNA binding to DAI mutants using the immobilized DAI assay. ^{32}P-Labeled dsRNA (85 bp) was adsorbed to ^{35}S-labeled proteins immobilized on antibody–Sepharose beads. (*A*) The amount of bound dsRNA was determined by direct counting of radioactivity and is expressed relative to that adsorbed by the wild-type Δ9 protein. (*B*) RNA–protein complexes were analyzed by electrophoresis in 20% polyacrylamide–SDS gels and autoradiography. All mutant proteins were truncated as for Δ9, except for Δ8 and Δ7 (lanes *3*,*4*). The controls in lanes *4* and *33* contained an unprogramed wheat germ extract and a mutant protein that is not recognized by the polyclonal antibody, respectively.

gested the existence of a possible RNA-binding motif. Many of the deleterious mutations fall in highly conserved regions of the consensus sequence (see Discussion). Because the motif is present once in each of the basic regions, it seemed possible that the duplication of one region would restore binding activity to a mutant from which the other region was deleted. To test this idea, we constructed mutants sub 1 : 1 and sub 2 : 2 containing tandem repeats of regions 1 and 2, respectively (Fig. 6B). The region 1 duplication bound dsRNA nearly as efficiently as wild-type DAI, whereas the region 2 duplication was severely defective for dsRNA binding (Fig. 6A), indicating that the two regions are not equivalent. Surprisingly, the mutant sub 2 : 1, in which the order of the two regions is reversed compared with the wild-type molecule, bound dsRNA with undiminished efficiency (Fig. 6). Similar data were obtained with VA RNA (not shown). These observations support the view that tandem copies of the motif are required for RNA binding but that motif 2 is less effective than motif 1.

Binding of short RNA duplexes and VA RNA

DAI interacts with dsRNA in a length-dependent fashion: Duplexes of ≥85 bp bind DAI and activate the enzyme as efficiently as very long natural dsRNAs; short duplexes of ≤30 bp bind and activate the enzyme very weakly, and their most prominent effect is to inhibit activation by long dsRNAs; duplexes of intermediate size exhibit intermediate properties and also form electrophoretically distinct complexes with DAI (Manche et al. 1992). Such qualitative and quantitative differences might be reflected in differential effects of DAI mutations on the binding of dsRNAs of varying sizes. For example, differential effects would be expected if short duplexes bound to a separate inhibitory site (Galabru et al. 1989). On the other hand, if there is a single site for binding both long and short dsRNA, residues at the extremities of the dsRNA-binding site might be dispensable for the binding of the smaller ligand. To determine whether DAI mutant proteins were capable of distinguishing between dsRNAs of different sizes, a panel of 17 mutants immobilized on antibody–Sepharose beads was challenged with ^{32}P-labeled dsRNA of 40 or 55 bp, as well as the 85-bp duplex used in previous experiments. The shorter dsRNAs bound less efficiently as expected (Manche et al. 1992), but the relative dsRNA binding was quantitatively unchanged among the DAI mutants (Fig. 7A). Thus, the relative binding efficiency of these mutants is independent of the length of the dsRNA, and DAI mutations that discriminate between longer and shorter RNA duplexes were not identified.

Adenovirus VA RNA$_I$ is a small highly structured single-stranded RNA that binds to DAI and inhibits the binding of dsRNA (Kostura and Mathews 1989), as well as the activation of DAI by dsRNA (Kitajewski et al.

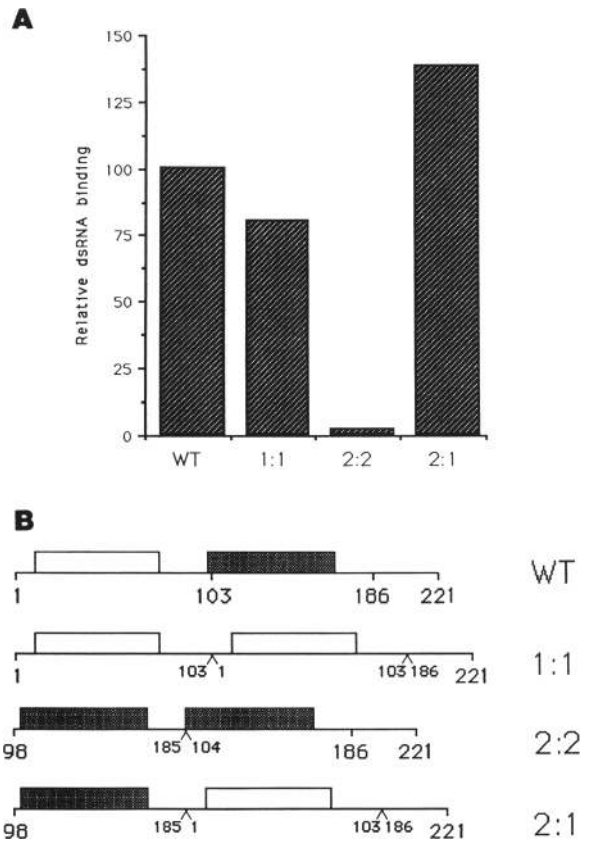

Figure 6. Interchange and duplication of RNA-binding motifs. (*A*) ^{32}P-Labeled dsRNA was adsorbed to immobilized DAI mutant proteins. The amount of bound dsRNA is shown relative to that adsorbed by the wild-type DAI, truncated at amino acid residue 221. (*B*) Schematic representation of the proteins used in *A*. Numbers correspond to amino acid residues in the original DAI sequence.

1986; O'Malley et al. 1986). It is not clear whether this is the result of direct competition between VA RNA_I and dsRNA for a single binding site on DAI (Kostura and Mathews 1989) or whether there are separate binding sites for the activator and inhibitor (Galabru et al. 1989). To address this question, we tested the same 17 DAI mutants for their ability to bind VA RNA_I. The immobilized DAI assay gave a nonspecific background of VA RNA_I binding presumably owing to interaction with a wheat germ protein capable of binding VA RNA_I; therefore, we used the VA RNA–Sepharose assay for this study. The ability of the DAI mutants to bind to VA RNA_I (Fig. 7B) matched the pattern obtained for dsRNA binding to immobilized DAI (Fig. 7A). As also seen with the truncation mutant Δ7 (Fig. 3A, lane 8), however, DAI mutants that are unable to bind dsRNA often interact to a small but detectable extent with VA RNA–Sepharose. Because Sepharose beads lacking RNA ligands do not bind any detectable DAI (Fig. 2B, lane 8), this observation implies that DAI mutations that abrogate dsRNA binding may still permit a weak interaction with VA RNA_I. Further investigation of this phenomenon is under way.

Discussion

DAI is a cellular protein kinase, subject to both positive and negative control by RNA effectors. Several functions have been attributed to DAI, the most studied of which is its role in the interferon-induced host antiviral response. Activation of the kinase by virus-derived dsRNA shuts down protein synthesis and limits virus multiplication. As a counter-measure, some viruses synthesize short, highly structured single-stranded RNAs, such as VA RNA, which block the activation of DAI. To explore the molecular basis for these interactions we undertook a mutational analysis to define the RNA-binding domain(s) in DAI.

Two regions of DAI interact with RNA

Noting that the amino terminus of the protein is rich in basic residues, we hypothesized that the positively charged side chains would form ionic bonds with the phosphodiester backbone of RNA. The basic amino acids are concentrated in three regions within the amino terminus of the protein. Deletion of the third basic region had no effect on RNA binding, but deletion of either the

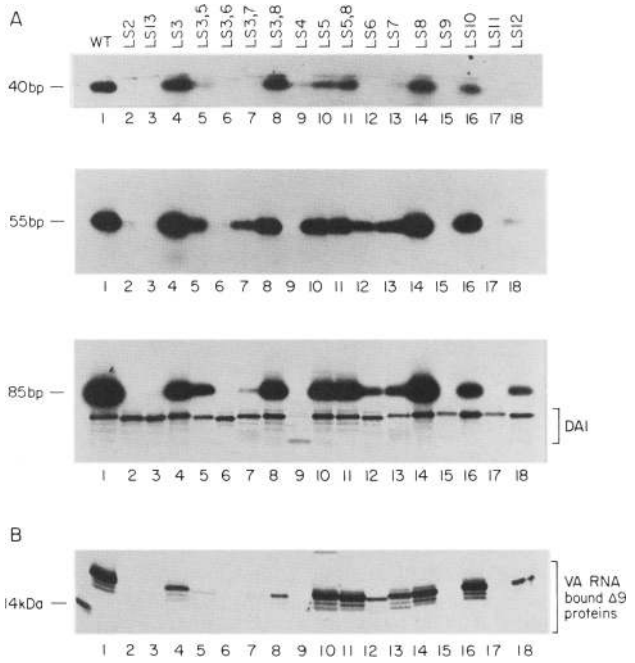

Figure 7. Binding of short dsRNAs and VA RNA. (*A*) Binding of dsRNA to immobilized DAI. RNAs of 40, 55, and 85 bp were exposed to mutant DAI proteins truncated as for Δ9. RNA–protein complexes were analyzed by electrophoresis in 20% polyacrylamide–SDS gels and by autoradiography. The gels were exposed for 12, 8, and 4 hr, respectively, to allow for the differing affinities of DAI for the three RNAs. (*B*) Binding of DAI to VA RNA–Sepharose. Equal radioactive counts of translated DAI mutant Δ9 proteins were incubated with VA RNA–Sepharose beads. The resultant protein–RNA complexes were analyzed by electrophoresis in 20% polyacrylamide–SDS gels and autoradiography.

first or second basic region rendered DAI unable to bind RNA. In agreement with recent results from other laboratories (Katze et al. 1991; Chong et al. 1992; Feng et al. 1992; McCormack et al. 1992; Patel and Sen 1992), a truncated protein, comprising the first 171 amino acids and containing regions 1 and 2 in their entirety, was capable of binding RNA. Removal of an additional 14 amino acids, encroaching on basic region 2, eliminated dsRNA binding, suggesting that some of these 14 residues also contribute to the function of the molecule. We have demonstrated further that a bacterially produced protein consisting of residues 1–184 of DAI is able to give a gel mobility shift with dsRNA (Manche et al. 1992), suggesting that post-translational modifications are not required for the RNA-binding activity of the protein.

Analysis of clustered point mutations in the first 184 residues, and of molecules with duplications of the two basic regions, confirmed that both regions are required for RNA binding and suggested that they function coordinately by binding to different parts of the RNA molecule. Two observations support this view. First, although double mutants were generally more severely affected than either of the parental single mutants, they were always more debilitating if the two mutations were in the same region than if one mutation was in region 1 and the other was in region 2. This implies that the two regions function as partially independent units. Second, the observation that the positions of the two basic regions can be interchanged without loss of activity implies that they cooperate to give stable binding, presumably by interacting with different parts of the RNA molecule. Yet the two regions are nonequivalent: Point mutations in region 1 were more debilitating than mutations in region 2, and a tandem repeat of region 1 was active while that of region 2 was not. Similarly, Feng et al. (1992) recently reported that deletions in region 1 are more deleterious than deletions in region 2. In the absence of region 2, a TrpE–DAI fusion protein containing only the amino-terminal 98 residues of DAI, was able to bind RNA (McCormack et al. 1992). We and others (Patel and Sen 1992) find that region 1 fails to bind in the absence of the TrpE sequences, suggesting that the bacterial protein can supply the region 2 function at least under some circumstances.

RNA-binding motif and helix

Their initial designation as basic regions notwithstanding, binding was abrogated by the substitution of either basic or non-basic residues. Although current data do not distinguish unequivocally between changes affecting residues that interact directly with RNA and changes that impact on the higher order structure of the enzyme, these results imply that the binding between DAI and RNA is more complex than a simple ionic interaction between the positively charged amino acids and the negatively charged RNA. The shift in electrophoretic mobility caused by LS4 suggested that this mutation lies in a region of stable secondary structure, and computer predictions suggested the existence of an α-helix with a distinct basic face and several other potential hydrogen bond-forming residues (Fig. 8B). The LS4 mutation removes two basic residues and introduces a glycine residue, which may affect the stability of the helix (Chou and Fasman 1974). To examine the importance of this helical domain, we made two additional mutations. First, the original alanine residues at the site of the LS9 mutation were not expected to play a role in direct interactions with RNA, so their substitution with two helix-breaking residues (Gly and Pro) would be expected to disrupt the helical structure without altering interactive residues. Second, in LS13 two lysine residues were changed to alanine, thereby preventing the original basic residues from making electrostatic contacts with RNA while having a minimal effect on the predicted protein structure (Chou and Fasman 1974). Both of these mutant proteins were defective for dsRNA binding, implying that the charge and the structure of this α-helix are important for the interaction.

Region 2 also contains a predicted α-helix, located between residues 148 and 160, that displays sequence homology with the helix in region 1 and with sequences in a number of viral and cellular RNA-binding proteins. A potential RNA-binding motif was derived from this consensus sequence (McCormack et al. 1992) and has since been found in other known RNA-binding proteins such as the TAR RNA-binding protein [TRBP (S. Venkatesan, pers. comm.)]. This core motif encompasses the α-helix in region 1 that we have identified as essential for dsRNA binding, thereby establishing that the motif is an essential component of the DAI dsRNA-binding domain. Further scrutiny reveals that the motif can be extended upstream of the original consensus sequence in that amino acids 11–77 and 101–167 of DAI display regions of homology throughout their length with other RNA-binding proteins [Fig. 8A (D. St Johnston and S. Venkatesan, pers. comm.). Several considerations support the view that this extended homology is of significance. First, most of regions 1 and 2 are included in these stretches of extended homology, and in each case, the original core motif is located in the area of greatest homology. Second, the extended motif incorporates residues between 157 and 171, which are important for binding, and there is no repeat of this motif in region 3, which is dispensable for RNA binding. Third, mutations in region 1 that are essential for function (LS2, LS4, LS9, LS11, and LS13) lie in conserved amino acids, whereas mutations that have little effect on dsRNA binding (LS3, LS5, and LS10) are in regions of weak homology or nonconserved regions. In keeping with the lesser dependence on region 2, mutations in this region are less deleterious even though some region 2 mutations lie in similar positions within the extended homology to those in basic region 1 (LS6, LS8, and LS12). The most striking difference is seen with LS8, which partially overlaps the LS9 site, but the significance of this discrepancy is attentuated by the fact that the changes within the two consensus sequences are not identical. Finally, in TRBP, deletions of a region corresponding to one of the highly con-

A

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Pvtiiney.cQitkrdwsfriesv.GPsn..sptFyacvdidgrvf.dkadGksKrdAknnAAKlAvdkllg  Vaccinia E3L
Plirlndc.ktkyGidiicrfyivldndgs.iihmcymr.tG..saeavakGrsKkeAkriAAKdildqigl  Rotavirus NS34

Pisllqey.gtriGktpvydllkaeGqaH..qpnFTfrvtvGd..tsctgqGpsKkaAkhkAAevAlkhlkg  TRBP 1
PvgalqelvvqkgwrlpeytvtqesGPaH..rkeFTmtcrv.er.fieigsGtsKklAkrnAAakmllrvht  TRBP 2

mdk.lnkyr.QmhGvaitykelstsGPpH..drrFTfqvlidekefg.eakGrsKteArnaAAKlAvdildn  mDAI 1
yig.lvnsfaQkkklsvnyeqcepnselp...qrFickckiGqtmyg.tgsGvtKqeAkqlAAKeAyqkllk  mDAI 2

          LS2LS10                  LS3          LS11    LS4LS13 LS9
mee.lnty.rQkqGvvlkyqelpnsGPpH..drrFTfqviidgrefp.egeGrsKkeAknaAAKlAvEilnk  hDAI 1
yig.linriaQkkrltvnyeqcas.Gv.Hgpeg.Fhykckmgqkeys.igtGstKqeAkqlAAKlAylqils  hDAI 2
       LS12LS6                        LS7                        LS8

Pi  LN    QK G    Y      GP H     FTy   IG   F   G G SKKEAKn AAK Ald L    Consensus
 l  iq     r      f              f   v    y   a   t rd rq      ve i
 v                                                             v
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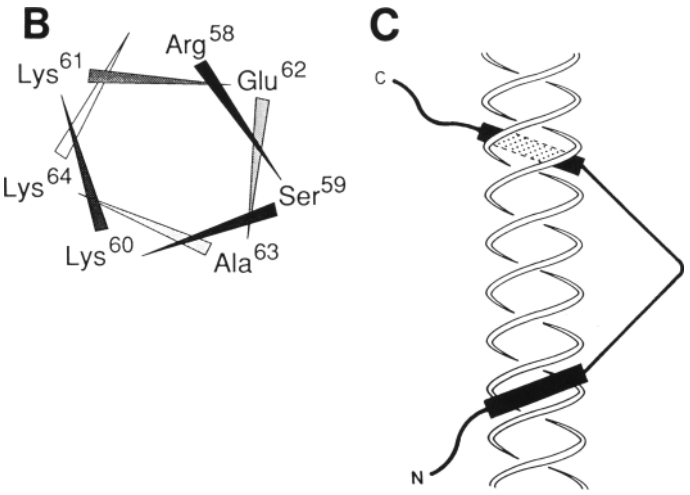

Figure 8. RNA-binding motifs. (*A*) Consensus sequence for an extended RNA-binding motif. Amino acids 11–77 and 101–167 of the human DAI sequence are compared with other RNA-binding proteins (vaccinia virus E3L protein, rotavirus NS34 protein, TAR RNA-binding protein, and the mouse DAI homolog TIK). Similar or identical amino acids present at the same position in four or more of the sequences are shown in boldface type as lowercase or uppercase letters, respectively. (*B*) The amino terminus of the predicted α-helix in region 1. The first 7 amino acids of the α-helix are diagramed to demonstrate the potential for interactions between the helix and RNA. (*C*) Bivalent binding to dsRNA. The model illustrates how the two RNA-binding motifs could interact with the dsRNA helix. Optimal binding occurs when the two motifs span ~80 bp (Manche et al. 1992). We speculate that binding to shorter duplexes entails increased strain at the "hinge" between the two motifs, thereby lowering binding efficiency, and that only monovalent binding is possible with duplexes of 30 bp or less.

served motifs abrogate RNA binding, and a 24-residue peptide analogous to this motif is able to interact with RNA (A. Gatignol, pers. comm.).

The 18-residue core motif, which we have identified as essential for dsRNA binding, is located at the amino terminus of a predicted α-helix in all of the RNA-binding proteins considered here. In DNA-binding proteins, three well-characterized motifs, the zinc finger, the helix–turn–helix, and the leucine zipper, all make protein–DNA interactions by inserting an α-helix into the major groove of the DNA helix. In the case of the helix–turn–helix and zinc finger motifs, it is the amino terminus of the α-helix that makes these interactions. The dsRNA-binding proteins may make similar contacts, although the A-form configuration of dsRNA would require the α-helices to be inserted into the minor groove because the major groove is too narrow in this conformation (Steitz 1990). An example of this kind of interaction occurs in the complex between glutaminyl tRNA synthetase and its uncharged cognate tRNA (Rould et al. 1989).

Regulation of DAI

Of the RNA-binding proteins discussed here, the viral proteins have one copy of the core motif, whereas the cellular proteins have two copies. When two motifs are present, they appear to be functionally nonequivalent: The first of the TRBP motifs is dispensable (A. Gatignol, pers. comm.) and the second motif in DAI plays an essential but subsidiary role. What, then, is the function of the second binding motif in DAI? One possibility is that region 2 is responsible for presenting the region 1 α-helix in the correct position or orientation for interaction with dsRNA. Alternatively, both regions may be involved in direct interactions with dsRNA, either acting in conjunction to create one binding site or binding to different areas of the dsRNA.

Both hypotheses would fit with the results of the Δ5 and Δ6 deletions, because a slight reduction in the spacing between the two essential regions might be tolerated, whereas a large deletion could prevent the two regions from functioning in unison. We strongly favor the second model because it ascribes similar functions to the homologous regions, consistent with the results obtained with tandem duplications and rearrangements, and provides a natural explanation for the differing affinities of DAI for dsRNAs of different sizes. Earlier work showed that as dsRNA chain length is increased, maximal enzyme activation and RNA-binding efficiency are reached at 85 bp (Minks et al. 1979; Manche et al. 1992).

We propose that this length affords optimal spacing for the two RNA-binding motifs to interact with separate sites on the duplex (Fig. 8C). The shorter the dsRNA, the lower the probability that DAI will form the correct conformation for this interaction and, therefore, the lower the efficiency of binding. This hypothesis could also explain the discontinuous behavior of DAI–dsRNA complexes in gel mobility analyses (Manche et al. 1992). In these experiments, complexes were formed with dsRNAs of 40–65 bp; but when the chain length increased past a critical value (~85 bp), the predominant complexes abruptly migrated considerably faster in native gels. We assume that longer dsRNAs permit the formation of a more compact protein–RNA conformation with a resulting increase in gel mobility and that this conformational change allows for DAI activation. The stretch of polypeptide sequence between the two binding regions might act as a hinge that is set under progressively greater strain as the length of the duplex is reduced from 85 to 30 bp, resulting in lower binding affinity (Fig. 8C). Below 30 bp, the range of the hinge's movement is exceeded and the protein can only interact with the duplex through a single motif, resulting in greatly reduced affinity and failure to detect complex formation.

The regions of DAI that interact with dsRNA are also involved in VA RNA_I binding. Within these regions, amino acid substitutions have very similar effects on the binding of both dsRNA and VA RNA_I implying that both copies of the RNA-binding motif and the same amino acids are required for VA RNA_I binding. VA RNA_I is comprised of two duplex regions, the apical and terminal stems, separated by a region of more open structure, termed the central domain (Mellits and Mathews 1988; Furtado et al. 1989), but it does not contain long stretches of continuous duplex. DAI binds to the apical stem (Mellits et al. 1990a) and the central domain (Ghadge et al. 1991; P. Clarke, unpubl.), perhaps because one motif binds to the apical stem and the other to the central domain. Why, then, is VA RNA an inhibitor? One strong possibility is that although VA RNA_I binds to both motifs, the structure of the resultant RNA–protein complex is incompatible with the conformational change that permits the autophosphorylation and activation of DAI.

In conclusion, we have shown that two copies of an extended RNA-binding motif are essential for RNA binding by DAI. The motif is rich in basic residues and contains a potential α-helical structure. We propose that both motifs interact with RNA by inserting the α-helical domain into the minor groove of the RNA double helix and causing a conformational change in the enzyme that is required for its activation. It remains to be seen whether similar RNA–protein interactions participate in the functioning of other proteins that share this motif.

Materials and methods

Vectors

Plasmid p84a (Meurs et al. 1990; provided by B. Williams, Cleveland Clinic Foundation, Cleveland, OH) contains the cDNA encoding DAI inserted between the *Xba*I and *Hin*dIII sites of the vector Bluescript KS. A derivative, p84b, was generated by joining the *Pst*I site in the 3′-untranslated region of the cDNA to the *Sal*I site in the vector with a *Bam*HI linker (CGGATCCG). Plasmid pPK1, (a gift of J. Field, Cold Spring Harbor Laboratory) contains the T7 RNA polymerase promoter driving the synthesis of an influenza virus HA1 epitope fused to the amino terminus of cyclic AMP-dependent protein kinase. To create a vector expressing an HA1–DAI fusion protein, pPK1 was digested with *Sal*I and p84b was digested with *Hin*dIII (at nucleotide −30 in the 5′-untranslated region of the DAI sequence). The resultant 3′-recessed ends were blunted and both DNAs were digested further with *Bam*HI. After gel purification, the 1.8-kb fragment containing the entire DAI-coding region was ligated into the pPK1 background (in place of the cAMP-dependent protein kinase sequences) to produce pSRG2. Subsequently, to remove the HA1 leader sequences, pSRG2 was cleaved with *Nde*I (at the start of the HA1 sequence) and *Hin*dIII, both recessed ends were blunted, and the DNA was religated. The resultant plasmid, pSRG2ΔL, contains the 5′-untranslated sequence of DAI immediately downstream from the T7 promoter. Proteins derived from this vector initiate at the natural DAI start codon.

Construction of DAI mutants

Most constructs were prepared in plasmid pUC118D, containing the *Hin*dIII–*Bam*HI DAI fragment from p84b inserted between equivalent sites in pUC118. After mutagenesis, the relevant fragments were excised from pUC118D by digestion with *Hin*dIII and *Msc*I and inserted into a comparably cut pSRG2ΔL background. Three types of mutant were produced: Deletions and rearrangements of large blocks of sequence were made using conveniently located restriction enzyme sites, whereas small deletions and substitutions were made by site-directed mutagenesis. The mutants are diagramed in Figure 1 and were all confirmed by DNA sequencing.

Deletion Δ1 was prepared by digesting SRG2 with *Nde*I and *Nco*I. Both 3′-recessed ends were filled, and the plasmid reclosed by self ligation. Δ2 was constructed by digesting pUC118D with *Fnu*4HI and filling in the 3′-recessed end. The DNA was digested further with *Bam*HI, and the resultant 1320-bp fragment was ligated into pUC118D digested with *Stu*I and *Bam*HI. Δ3 was constructed by digesting pUC118D with *Bsm*AI. The 5′ extension was removed by treatment with mung bean nuclease (New England Biolabs), before further digestion with *Hin*dIII to liberate a 723-bp fragment. A second fragment was prepared by digesting pUC118D with *Ase*I and filling in the 3′-recessed end. This linearized plasmid was digested further with *Eco*RI to produce a 283-bp fragment. These two fragments were then ligated into pUC118 digested with *Hin*dIII and *Eco*RI in a triple fragment ligation. To construct Δ4, pUC118C was made by digesting pUC118D with *Hin*dIII and *Acc*I. The ends were blunted and religated so that only the carboxyl terminus of DAI remained. pUC118C was then digested further with *Xmn*I and *Sau*I; again, the ends were blunted and the vector was religated. The deletion was transferred into an SRG2ΔL background by exchanging *Msc*I–*Bam*HI fragments.

Three mutants contain rearrangements of the amino terminus of DAI. Sub 2 : 1, in which two blocks of sequence are inverted, was produced by inserting DNA encoding amino acids 1–103 at the *Bsa*AI site of Δ1. The same fragment was inserted between the *Stu*I and *Bsa*AI sites of SRG2ΔL to create sub 1 : 1, which contains a duplication of region 1 and a deletion of region 2. Sub 2 : 2, which has a duplication of region 2 and a deletion

of region 1, was produced by inserting a *Stu*I–*Bsa*AI fragment from SRG2ΔL into the *Bsa*AI site of Δ1.

The two smaller deletions, Δ5 and Δ6, and the linker scanning (LS) mutants were constructed in pUC118D, using the oligonucleotide-directed mutagenesis technique (Kunkel 1985), and were subsequently transferred into the SRG2ΔL background. With two exceptions, the oligonuceotide primers used to construct the LS mutants contained the central sequence GGGGCCCT, which converted the target amino acid sequence to Gly–Ala–Leu. In LS9, the central sequence of the mutagenic oligonucleotide was GGGGCCCC, modifying the amino acid sequence to Gly–Ala–Pro. In LS13, Lys^{61} and Lys^{64} were both changed to Ala by mutating their codons to GCA. The amino acid changes are summarized in Table 1. Oligonucleotide-directed mutagenesis was also carried out to substitute Arg for Lys^{296}, the residue essential for the proton transfer activity involved with kinase function. After converting the AAA triplet to AGA, the mutation was transferred from pUC118C into an SRG2 background to produce K296R.

Production of polyclonal antibody against DAI

The *Escherichia coli* strain BL21(DE3) (Studier and Moffatt 1986) was transformed with plasmid pSRG2, and induction of DAI was carried out as described (Sambrook et al. 1989). Although trace amounts of DAI were detected by Western blot analysis, the cells died before significant amounts of protein had accumulated. However, large quantities of protein were produced by cells expressing the mutant protein K296R, which is nonfunctional for kinase activity (Katze et al. 1991). This protein, which was visible by Coomassie brilliant blue R staining of polyacrylamide gels containing crude cell extracts of such cells, was found in both the supernatant fraction and inclusion bodies. To purify the mutant DAI, a culture grown at 25°C was lysed and inclusion bodies were prepared as described previously (Marston 1987). The washed inclusion bodies were sonicated in modified buffer B (Kostura and Mathews 1989) containing 20 mM potassium phosphate (pH 6.8) (instead of HEPES) and 8 M urea to solubilize the DAI. This suspension was then passed over an S-Sepharose (Pharmacia) column, and the bound DAI was eluted with 150 mM KCl. Peak fractions were pooled and dialyzed overnight against 100 volumes of modified buffer B containing 20 mM Tris-HCl (pH 9.0) (instead of HEPES) and 8 M urea. The dialysate was loaded onto a Q-Sepharose (Pharmacia) column, and again the bound DAI was eluted with 150 mM KCl. DAI-containing samples were fractionated by electrophoresis through 12.5% polyacrylamide–SDS gels, and the band corresponding to DAI was excised. The gel fragment was prepared for immunization and injected into rabbits as described (Harlow and Lane 1988). A high level of antibody was detected after three injections.

Synthesis of DAI proteins

A wheat germ translation extract was prepared as described (Roberts and Paterson 1973). Translation reactions were carried out in a final volume of 25 μl, containing 7.5 μl of lysate, 160 μCi/ml of [^{35}S]methionine (ICN Biomedicals), 24 mM HEPES (pH 7.4), 25 mM potassium acetate, 2 mM DTT, 1 mM ATP, 25 μM GTP, 8 mM creatine phosphate, 250 μM spermidine, 25 μM amounts of each amino acid except methionine, and 12.5 μg/ml of calf liver tRNA. Reactions were programed with 0.5 μg of RNA and incubated at 20–25°C for 2 hr. From each translation reaction, 2 μl was spotted onto Whatman 3MM paper, and proteins were precipitated by boiling in 10% (vol/vol) TCA for 5 min. The relative amounts of radioactive protein synthesized were determined by liquid scintillation.

Uncapped RNA was generated as described (Mellits et al. 1990b) and used as a template for synthesis of truncated DAI. Capped RNA was used for the synthesis of full-length DAI: It was generated in transcription reactions containing 0.2 mM GTP and 0.5 mM m^7GpppG. All 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 at pH 7.4, 1 mM EDTA) and passed twice through Sephadex G-50 medium (Pharmacia) spin columns (Sambrook et al. 1989). The eluted RNA was precipitated with ethanol and dissolved in TE.

Binding of DAI to RNA–Sepharose matrices

Two kinds of RNA matrix were prepared, dsRNA–Sepharose and VA RNA–Sepharose. *Penicillium chrysogenum* dsRNA was a gift from H. Robertson (Cornell University Medical College, NY). VA RNA_I was generated by transcription with T7 RNA polymerase as described (Mellits et al. 1990b) and purified by electrophoresis through a native polyacrylamide gel. In both cases, the RNA ligand was linked to CNBr-activated Sepharose (Pharmacia) as described (Kadonaga and Tjian 1986). DAI-binding reactions contained equal amounts of ^{35}S-labeled wild-type and mutant proteins (5–10 μl of translation products) and were conducted with 50 μl of RNA matrix by the method of O'Malley et al. (1986), with the exception that binding to VA RNA–Sepharose was carried out on ice.

Binding of RNA to DAI–Sepharose matrix

Equal amounts of DAI protein (~50,000 cpm; 5–10 μl of translation products) were adsorbed to protein A–Sepharose beads saturated with 5 μl of polyclonal antibody. Radiolabeled dsRNAs (Manche et al. 1992) and VA RNA_I (Mellits et al. 1990b) were generated in vitro. RNA binding was carried out as described previously (Mellits et al. 1990a) using final concentrations of 50 ng of dsRNA/ml and 100 ng of VA RNA_I/ml. Under the same conditions, single-stranded RNA of 85 nucleotides (Manche et al. 1992) gave only weak and nonspecific binding.

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S R Green and M B Mathews

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