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

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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.

[*Key Words*: Kinase; translational control; VA RNA; RNA-binding motif; double-stranded RNA; interferon]

Received August 6, 1992; revised version accepted September 21, 1992.

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

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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-

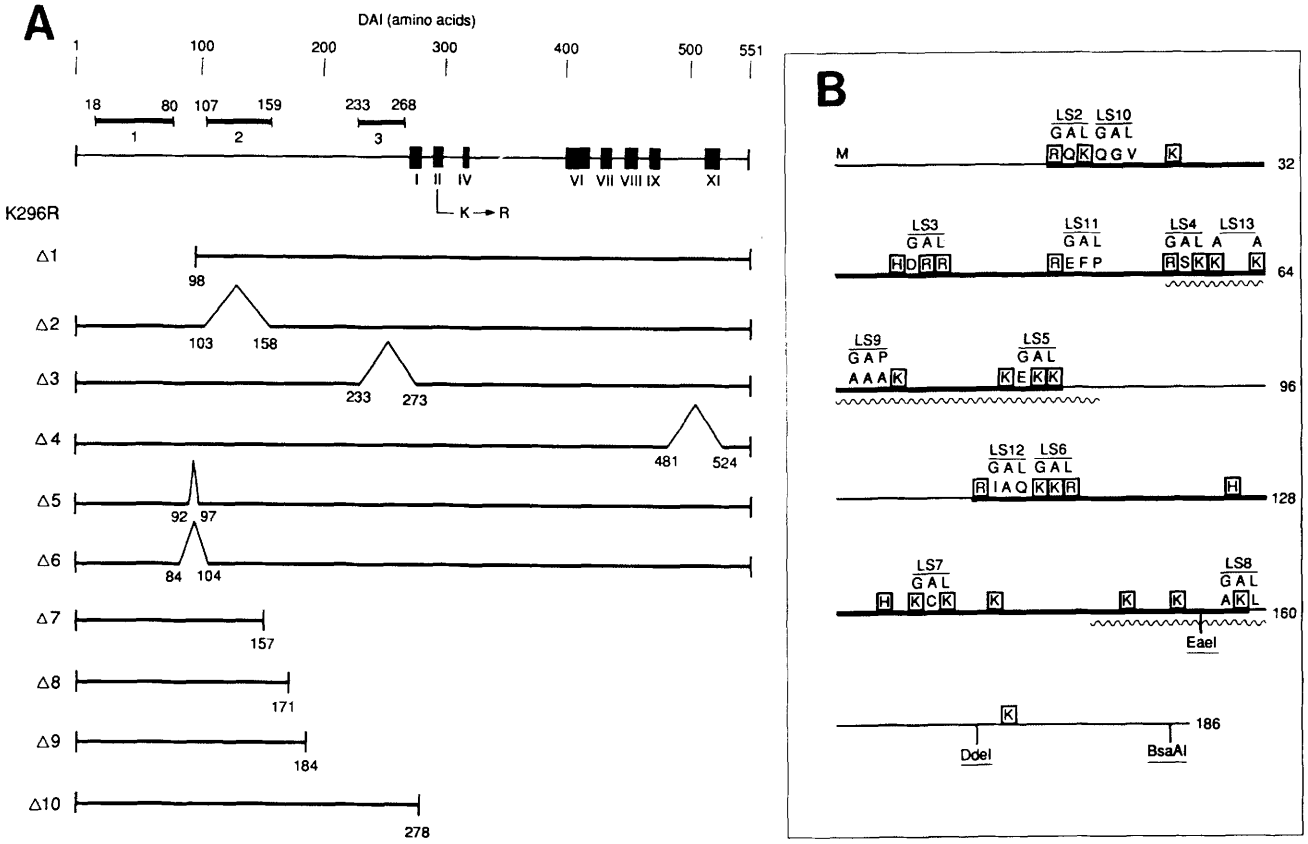

Figure 1. Structure of DAI and mutants. (*A*) Schematic representation of full-length DAI and mutants. The *top* line represents the linear DAI protein sequence indicating the highly conserved kinase domains in the carboxy-terminal half of the protein (boxes I–XI) and the three basic regions within its 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

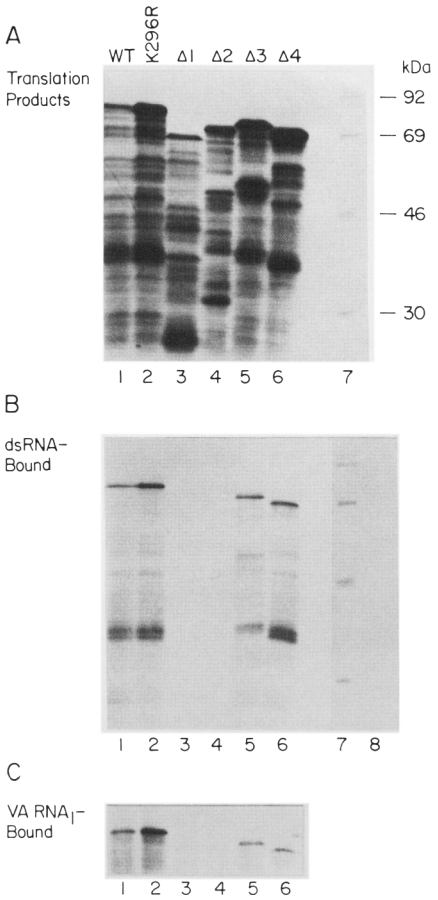

Figure 2. DAI translation and location of the RNA-binding domain. (*A*) Total translation products. Capped RNA transcripts synthesized by T7 polymerase in vitro, were used to program the wheat germ cell-free translation system. The resultant translation products were analyzed by electrophoresis in 12.5% 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.

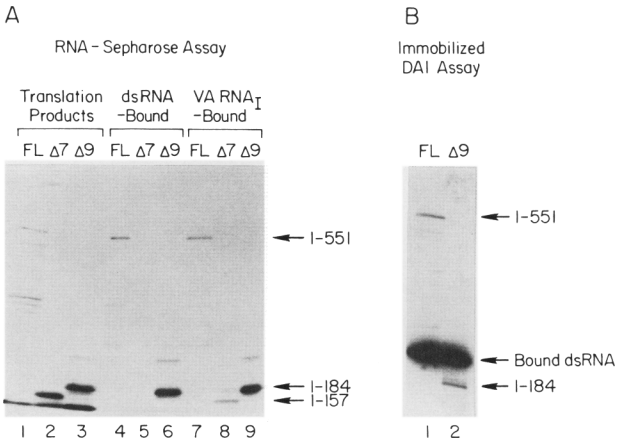

Figure 3. RNA-binding activity of truncated DAI. (*A*) RNA–Sepharose assay. RNA from full-length wild-type construct and the truncations Δ7 and Δ9 was translated. Equal volumes of the translation products (5 μl) were tested in both dsRNA–Sepharose and VA RNA–Sepharose-binding assays. Translation products 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

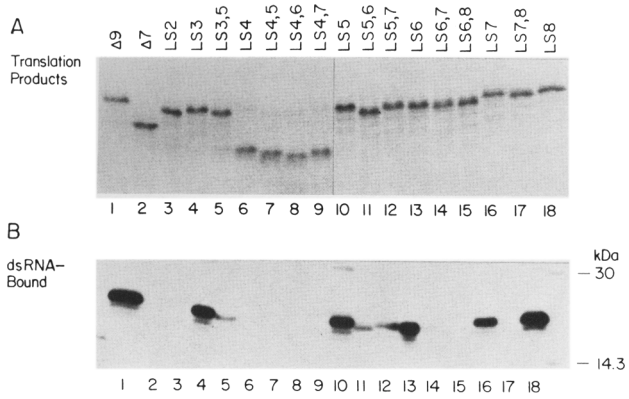

Figure 4. Binding of dsRNA to DAI mutants using the dsRNA–Sepharose assay. (*A*) Total translation products. Uncapped RNA was synthesized from a number of mutant templates truncated as for Δ9. Equal amounts of radioactive proteins were analyzed by electrophoresis in 20% polyacrylamide–SDS gels and autoradiography.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

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