The role of binding domains for dsRNA and Z-DNA in the *in vivo* editing of minimal substrates by ADAR1

Alan Herbert*† and Alexander Rich‡

†Department of Neurology, Boston University School of Medicine, 715 Albany Street, Boston, MA 02118; and ‡Department of Biology, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, MA 02139

Contributed by Alexander Rich, August 9, 2001

RNA editing changes the read-out of genetic information, increasing the number of different protein products that can be made from a single gene. One form involves the deamination of adenosine to form inosine, which is subsequently translated as guanosine. The reaction requires a double-stranded RNA (dsRNA) substrate and is catalyzed by the adenosine deaminase that act on dsRNA (ADAR) family of enzymes. These enzymes possess dsRNA-binding domains (DRBM) and a catalytic domain. ADAR1 so far has been found only in vertebrates and is characterized by two Z-DNA-binding motifs, the biological function of which remains unknown. Here the role of the various functional domains of ADAR1 in determining the editing efficiency and specificity of ADAR1 is examined in cell-based assays. A variety of dsRNA substrates was tested. It was found that a 15-bp dsRNA stem with a single base mismatch was sufficient for editing. The particular adenosine modified could be varied by changing the position of the mismatch. Editing efficiency could be increased by placing multiple pyrimidines 5′ to the edited adenosine. With longer substrates, editing efficiency also increased and was partly due to the use of DRBMs. Additional editing sites were also observed that clustered on the complementary strand 11–15 bp from the first. An unexpected finding was that the DRBMs are not necessary for the editing of the shorter 15-bp substrates. However, mutation of the Z-DNA-binding domains of ADAR1 decreased the efficiency with which such a substrate was edited.

DBRM | RNAi | transcription | Interferon | base pair mismatch

Editing of codons by the adenosine deaminase that act on double-stranded RNA (dsRNA) (ADAR) family of enzymes can result in the site-specific substitution of amino acids by deaminating adenosine to form inosine, which is read out as guanosine by the translational apparatus (1). These changes can have important physiological effects, as evidenced by editing of pre-mRNAs encoding subunits of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid-sensitive glutamate receptor and the serotonin-2C receptor (2, 3). Editing can also create or destroy splice sites, altering the protein product made from a gene (4). The ADAR family shares a catalytic domain that is evolutionarily related to the cytidine deaminase of *Escherichia coli*, from which the related set of adenosine deaminases that act on transfer RNA (tRNA) (ADAT) are also derived (5). Comparison between ADAR and ADAT catalytic domains suggests that variations in their structure are important in determining whether they are specific for either mRNA or tRNA substrates (5). Although ADATs lack a separate RNA-binding domain, ADAR family members contain a variable number of domains typical of the dsRNA-binding motif (DRBM). ADAR1 is unique in that it also contains two Z-DNA-binding motifs. In contrast with other members of the ADAR family that are found in invertebrates such as *Caenorhabditis elegans* and *Drosophila melanogaster*, ADAR1 has been found only in vertebrates (6). This distribution suggests an evolutionary process whereby an intrinsic editing function present in the catalytic domain of ADAR has been modulated first by the addition of DRBMs, then later by the incorporation of Z-DNA-binding motifs.

ADAR1 is induced by interferon and is thought to constitute one arm of the antiviral response, as well as being essential for embryonic erythropoiesis (7, 8). Long and short forms of the enzyme can be produced through alternative splicing (9). Translation of the short form (M246) starts at a methionine equivalent to residue 246 of the long form. M246 lacks the Z-DNA-binding motif Zα and the nuclear export signal encoded in residues I to 245 of ADAR1 but retains the second Z-DNA motif Zβ, three DRBMs, and a catalytic domain (Fig. 1) (10).

Although the role of DRBMs in editing by ADAR1 has been accepted as self-evident in editing, the function of the Z-DNA-binding motifs is still unresolved. Z-DNA formation is energy dependent and occurs in areas where there is appropriate negative torsional strain such as that generated by the movement of an RNA polymerase during transcription (11). A model has been proposed in which Z-DNA targets ADAR1 to sites of active transcription, enabling ADAR1 to interact with nascent RNA (12).

These studies were initiated to find the minimal-size ADAR1 construct capable of editing, along with the minimal-size dsRNA substrate that could be edited. Constructs were then expanded to examine the role of DRBMs and Z-DNA-binding domains on editing efficiency. The dsRNA constructs were also varied to examine the effect of sequence on the extent to which particular adenosines were edited. All editing experiments were performed in tissue culture cell lines to provide an appropriate biological context.

Methods

Editing Substrates. All substrates were expressed by using the mammalian pCI vector (Promega) in which the *Hin*dIII site had been removed by digesting the plasmid with *Hin*dIII, filling in the site with Klenow enzyme, and religating the vector. Two synthetic oligonucleotides that overlapped by 10 bp, one corresponding to the 5′ end of the substrate and the other complementary to the 3′ end were annealed and filled in by using Klenow polymerase. The resulting double-stranded DNAs contained either an *Eco*RI or *Nhe*I restriction site at their 5′ end and an *Xba*I site at their 3′ end. After digestion with the appropriate enzymes, fragments were directionally cloned into a pCI vector that had been digested with the same enzymes and treated with alkaline phosphatase. All sequences were confirmed by dideoxy sequencing.

ADAR1 Constructs. The pK5 vector expressing rat ADAR1 was a gift of M. Higuchi and P Seeburg (Max Planck Institute for Medical Research, Heidelberg). Mutagenesis was performed by using the QuikChange kit (Stratagene). Primers are published as supporting information on the PNAS web site (www.pnas.org). All constructs were confirmed by sequencing. Expression was

Abbreviations: dsRNA, double-stranded RNA; ADAR, adenosine deaminase that act on dsRNA; DRBM, dsRNA-binding motif; ADAT, adenosine deaminase that acts on tRNA.

*To whom correspondence should be addressed. E-mail: aherbert@bu.edu.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

www.pnas.org/cgi/doi/10.1073/pnas.211419898

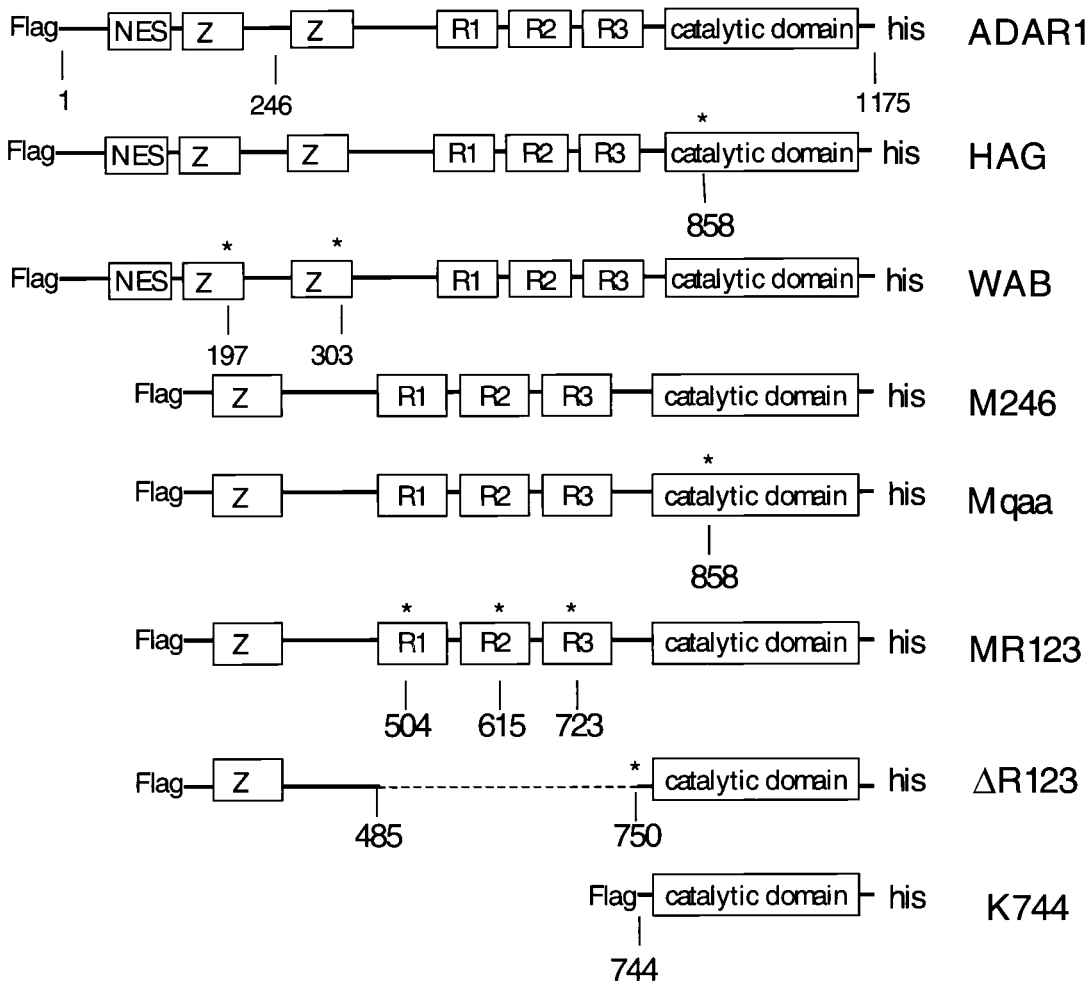

Fig. 1. ADAR1 constructs used in these studies. All constructs had a N-terminal FLAG tag and a C-terminal HIS tag. Both the long (ADAR1) and short (M246) forms of ADAR1 are shown. Sites at which mutations were made are indicated by an asterisk and by the position of the mutation. A *Swa*I site was created at position 750, allowing the in-frame deletion of the three DRBMs by using the naturally occurring *Swa*I site at position 485 to produce ΔR123. The nuclear export signal (NES) (residues 128–137; A.H., H. Knaut, A.R., and J. Nickerson, unpublished work), Z-DNA-binding domains (Zα and Zβ), and the DRBM (R1, R2, R3) are labeled.

checked by immunofluorescence and Western blotting by using the M2 anti-Flag antibody (Sigma).

Editing Assay. Transfection into HeLa cells of plasmids expressing ADAR1 constructs and editing substrates were performed by using Superfect reagent (Qiagen, Chatsworth, CA) or FuGENE 6 (Roche Biochemicals) into HeLa cells following the manufacturer's instructions. Typically, a 3-fold excess of the ADAR1-producing plasmid was used over that of the plasmid encoding the editing substrate, with 1.5 μg of total plasmid used per well of a six-well plate. RNA was extracted by using Trizol (Life Technologies, Gaithersburg, MD) 36 h after transfection and stored in H_2O at −80°C until use. After treatment with DNase I, RNA was annealed with either oligo-dT or random primers and reverse transcribed by using Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega) in the presence of Prime RNase Inhibitor (Eppendorf). Controls were prepared in parallel in which MMLV reverse transcriptase was omitted from the mixture. The cDNAs were amplified by using pCI sequencing primers for 35 cycles (94°C 30′, 60°C 15′, and 72°C 30′). The PCR product was then visualized on an agarose gel. The band observed depended on the construct used but was typically around 230 bp. Those reactions in which the reverse transcriptase minus control gave a band were discarded. Editing efficiency was determined by using the thermosequenance sequencing kit with a T7 primer and labeled dideoxynucleotides (Amersham Pharmacia). Products were resolved on a 7.5% polyacrylamide gel and quantitated by using a Molecular Dynamics PhosphorImager with a screen designed for ^{33}P detection. Background editing by HeLa cells was assessed by using an ADAR1 mutant in which the catalytic domain had been inactivated by mutation (13).

Results

The R/G-site of the GluR-B substrate was chosen for these studies as it forms a compact dsRNA stem, and there is evidence that it is an *in vivo* substrate for ADAR1 (14). We were interested in defining the minimum substrate necessary for *in vivo* editing of RNAs produced from transfected DNA templates. To ensure that sufficient enzyme was present for editing in cells, DNAs encoding ADAR1 were cotransfected with the substrates. This approach allowed us to investigate a variety of editing substrates as well as the effect of different ADAR1 mutations on the efficiency with which they were edited.

In GluR-B and other proposed ADAR1 substrates, the A that is edited has a mismatched cytosine residue on the complementary strand. The A-C mismatch has been shown to enhance editing (15, 16). We tested a variety of substrates in which this

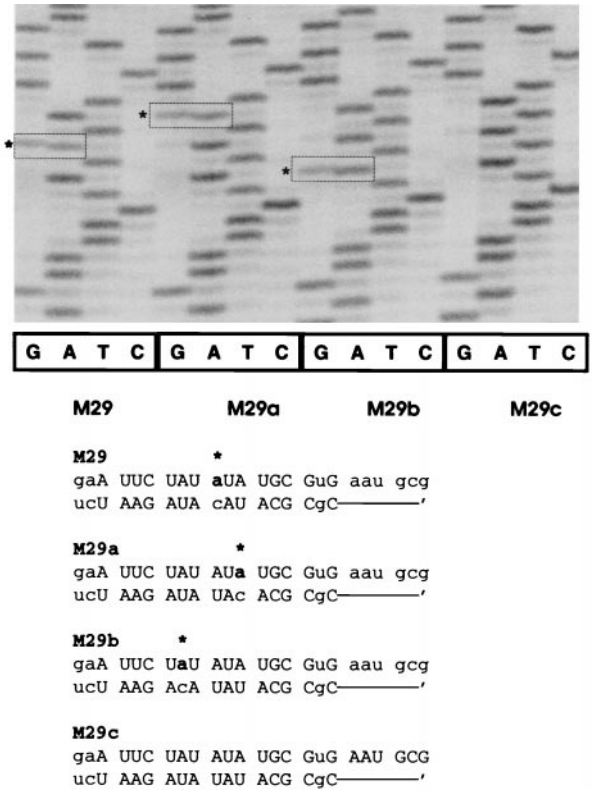

Fig. 2. The position of a mismatch determines which adenosine is edited. Four dsRNA editing substrates were examined that incorporated the sequence AUAUA in the top strand, as shown in the lower part of the figure. Each substrate differs in the position of a cytosine mismatch. M29a has a cytosine mismatch opposite the second A, M29b opposite the third A, and M29c opposite the first A. Editing was compared with the M29D substrate that had no mismatches. The upper part of the figure shows a sequencing gel where sites of A to G editing are boxed and marked with an asterisk. These sites are also marked with an asterisk in the sequences shown in the lower part of the figure. In these stem–loop structures, capital letters are used for Watson–Crick base pairs, lower case for mismatched or unpaired bases, the solid lines connect adjacent nucleotides, and the top line starts with the 5′ end of the sequence.

A-C mismatch was maintained but in which the dsRNA stem was shortened. Editing substrates with a dsRNA stem of 16 bp, including an additional GU mismatch (Fig. 2) and those with a 15-bp stem (Table 1), gave robust editing. Shortening the stem by 1 bp 5′ of the edited adenosine reduced editing efficiency to barely above background (data not shown).

We were able to confirm a previous report (15) that removing the A-C mismatch decreased editing efficiency (Fig. 2, M29c). We then investigated the effect of changing the position of the mismatch to test whether we could alter the specificity of editing by ADAR1 to cause modification of a particular adenosine. The dsRNA stem was designed to include the sequence AUAUA. The cytosine mismatch was placed opposite each of these As in turn. As shown in Fig. 2, the position of the cytosine specified the adenosine that was edited (compare M29 to M29a to M29b).

The sequence around the mismatch position was also varied by using a 15-bp dsRNA stem substrate (Table 1) maintaining the same base composition and the two nearest neighbors (i.e., UaG), as the 5′ residue has been reported previously to affect editing efficiency by ADAR (17). These experiments examined whether there was any phasing of purines or pyrimidines in the sequence that might affect editing efficiency by the ADAR1 catalytic domain. Stems with alternating purines and pyrimidines were examined as these are favored to form both Z-DNA and

Table 1. Editing of dsRNA substrates with a 15-bp stem

Clone		*	% editing (SD)
M14X	5′	gaa uuc GAU GCA **UaG** CGC AUA cgc aua	86 (3)
	3′	aga ucu CUA CGU AcC GCG UAU _____,	
M14X2	5′	gaa uuc GAU GCA **UaG** CCC AAA cgc aua	87 (1)
	3′	aga ucu CUA CGU AcC GGG UUU _____,	
M14X7a	5′	gaa uuc GAU GCA **UaG** CCC AAA ccc aaa	84 (5)
	3′	aga ucu CUA CGU AcC GGG UUU _____,	
M14X8a	5′	gaa uuc GUU CCU **UaG** CCC AAA cgc aua	98 (2)
	3′	aga ucu CAA GGA AcC GGG UUU _____,	
M14X9-2	5′	gaa uuc GUU CCU **UaG** CCC AAA ccc aaa	98 (3)
	3′	aga ucu CAA GGA AcC GGG UUU _____,	
M14X10	5′	gaa uuc GAU GCA **UaG** GCG UAU gcg uau	80 (7)
	3′	aga ucu CUA CGU AcC CGC AUA _____,	

Editing of 15-bp dsRNA stem–loop structures by the ADAR1 deletion mutant K744. The substrate name is shown in the lefthand column. The sequence of each substrate is shown in the middle column. All the dsRNA stems have the same base composition. Non-Watson–Crick base pairs are shown in lower case. The edited adenosine is asterisked and highlighted in bold. Quantitation of editing shown in the right-hand column is based on two experiments, with the standard deviation shown in parentheses.

Z-RNA, allowing the question to be examined of whether good Z-helix-forming substrates are also good editing substrates. Examination of Table 1 shows that the best substrates in these experiments are not the best Z-helix-forming substrates but rather M14X8a and M14X9–2, both of which contain the same pyrimidine-rich sequence 5′ of the editing site. Indeed, M14X9–2 does not contain any alternating purine/pyrimidine sequences. Loop sequences in these experiments were varied with no effect. dsRNA stems as short as 15 bp were thus edited efficiently in these experiments with a preference for pyrimidines 5′ of the editing site.

Multiple editing sites were observed in longer substrates. Results for the M7G construct, which has a 31-bp dsRNA stem containing two A-C and three G-U mismatches, are shown in Fig. 3. M7G represents a modification of the wild-type RG substrate (Fig. 3; modified residues are underlined). In addition to editing at the RG site (E2) and the adjacent adenosine (E1), additional editing sites (E3, E4, E5) were observed, as indicated by arrows in Fig. 3 (lane M246). E3, E4, and E5 are properly base-paired with no mismatch, indicating that a mismatch is not obligatory for editing by ADAR1. The two sites E2 and E5 are separated by 15 bp with the adenosines lying on opposite strands of the dsRNA stem. When the As in E3, E4, and E5 were moved to the same strand as E2, editing was not observed (data not shown).

The requirements for editing of longer substrates were examined further by using the M7L substrate, which has a dsRNA stem of only 23 bp (Fig. 4.). One change made in M7L was that E1 and E2 were moved two bases closer to the 5′ end of the duplex. Editing of E2 4 bp from the end was diminished, whereas editing of E1 just 3 bp from the end was lost, setting a limit on the distance an editing site can be from the end of the dsRNA stem. Another change in M7L was the reduction in spacing between E2 and E3 to 11 bp so that both sites are on the same face of the dsRNA stem, in contrast to M7G, where they were on different sides. Both sites were still edited. This result argues against the need for a particular spatial relationship between the two editing sites, as might be expected if ADAR1 functioned as a dimer. In contrast to M7G, editing of E2 in M7L is less efficient than editing of E3, indicating that the efficiency of editing at the two sites can be varied independently of each other. Comparison of M7G and M7L thus demonstrates that the structural requirements for editing in longer substrates are quite flexible.

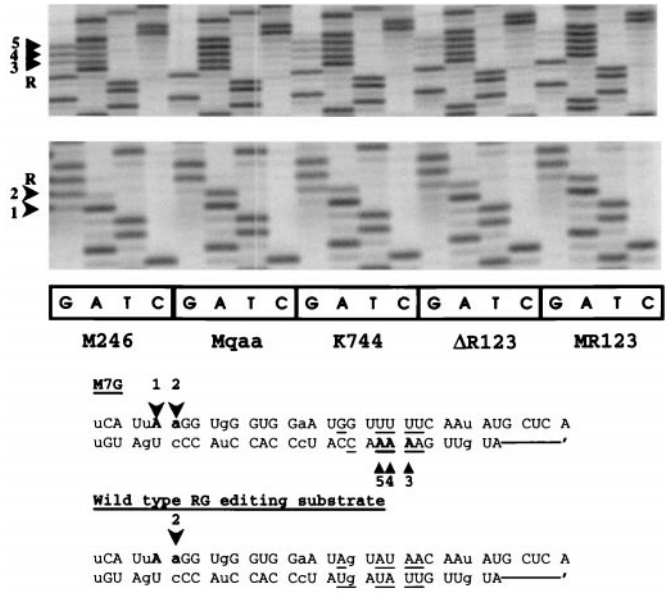

Fig. 3. The M7G editing substrate is edited on both strands of the dsRNA stem. Editing is observed even when ADAR1 DRBMs are absent or mutated. M7G was created from the wild-type GluR-B pre-mRNA RG site by replacing the residues that are underlined. Editing was observed at five sites in M7G (numbered 1–5, 5′ to 3′). The same numbering is used for the wild-type substrate. The ability of different ADAR1 constructs to edit the sites in M7G was compared (the band marked as R was used as a reference for quantitation). M246 is the short form of ADAR1 and serves as a positive control. Mqaa has the catalytic domain of M246 inactivated by mutation and serves as a negative control. K744 contains only the catalytic domain of ADAR1. ΔR123 has all three DRBMs in M246 deleted. MR123 has the following mutations that inactivate RNA binding: K504E, K615E and K723E.

The question then arose of the role played by the ADAR1 DRBMs in editing of these different substrates. The crystal structure of the second DRBM of *Xenopus laevis* RNA-binding protein A complexed with dsRNA shows that this domain interacts with 16 bp of dsRNA (18), whereas there are previous

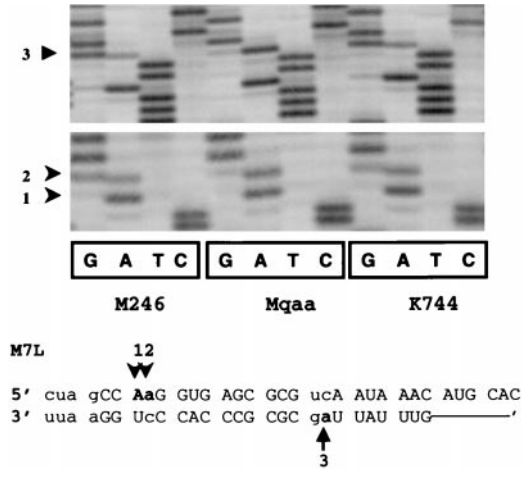

Fig. 4. The relative position of editing sites in a dsRNA is not fixed. M7L has editing sites E2 and E3 separated by 11 bp compared with 15 bp between E2 and E5 in M7G (Fig. 3.). This change in spacing places E3 in M7L and E5 in M7G on different sides of the dsRNA helix with respect to E2. Comparison of Fig. 4 with Fig. 3 also shows that editing of E2, which is placed 4 bp from the end of the duplex in M7L, is diminished when compared with M7G, which has E2 6 bp from the end. Editing of E1, which is 3 bp from the end of the dsRNA stem, is also greatly reduced in M7L.

Table 2. Effect of ADAR1 mutants on editing

Clone	% E1 (SD)	% E2 (SD)	% E4 (SD)
M246	41 (7)	78 (3)	70 (3)
Mqaa	10 (7)	17 (3)	3 (5)
K744	16 (4)	49 (7)	37 (7)
ΔR123	13 (9)	48 (4)	25 (4)
MR123	12 (3)	35 (4)	17 (6)

Quantitation of editing shown in Fig. 3 of the M7G substrate by M246, the catalytically inactive Mqaa mutant, the deletion mutants K744 and ΔR123 lacking the DRBMs of ADAR and MR123 in which the DRBMs have been inactivated by mutation. The results are based on quantitation of three experiments, with the standard deviation shown in parentheses.

reports that longer dsRNA stems are edited more efficiently by ADAR1 (1).

To investigate the role of dsRNA-binding domains in editing longer substrates, the following ADAR1 constructs were made (see Fig. 1): (*i*) K744, which locks all N-terminal residues including both Z-DNA-binding domains and the three DRBMs and has only the catalytic domain left (residues 744-1175); (*ii*) ΔR123, which has only the DRBMs deleted; (*iii*) MR123, which has a lysine to glutamic acid mutation in each of the three DRBMs, mutations previously shown to diminish dsRNA binding (19); (*iv*) Mqaa, which had the active site HAE motif in the catalytic domain mutated to QAA (20). These ADAR1 mutants were tested by using the M7G substrate described above. With this substrate, editing at all sites was equivalent and robust with the M246 construct, almost absent with the Mqaa construct, and diminished but still present with the constructs that removed the DRBMs (Fig. 3). The quantitation of editing by M246 shown in Table 2 indicates that for sites E2 and E4, at least 48% of transcripts are modified at both sites. The two ADAR1 constructs in which all three DRBMs were deleted and the third where key residues were mutated all show editing at E2 and E4 that are significantly above that of the catalytically inactive Mqaa (Table 2, Fig. 3), suggesting that the catalytic domain alone is capable of binding dsRNA and that the DRBMs are not obligatory. This result is reminiscent of ADAT, which do not have an RNA-binding domain that is distinct from the catalytic domain (5). These data suggest that the DRBMs increase the efficiency of editing of longer substrates but are not necessary for it to occur.

Next we investigated whether deletion of DRBMs affects editing of the shorter 15-bp dsRNA substrates. Editing of the M14X8a and related substrates was tested by using the K744 construct. Results are presented in Table 1. With editing as high as 98%, there is no apparent need for DRBMs for modification of this class of substrate. ADAR constructs where the deletion extents further into the catalytic domain than K744 were not able to edit this short substrate (results not shown). The system, consisting of the catalytic domain of ADAR1 and a 15-bp substrate, thus defines a minimal editing system.

The combined contribution of the Z-DNA-binding domains and the DBRMs on the efficiency of editing was examined by comparing editing by the ADAR1 and K744 constructs of a variety of longer editing substrates (Table 3). These substrates contained differing amounts of alternating purines and pyrimidines with a different potential to form a Z-helix. All substrates were edited by K744. Although editing by ADAR1 was more efficient, there again was no particular effect of potential Z-RNA-forming sequences. A substrate with only adding short runs of alternating purine and pyrimidines (RG1E) was edited as well as others containing sequences with moderate (RG2B) or good potential (RG3a, RG10a, RG15D) to form Z-DNA or Z-RNA (21). These results suggest that the Z-DNA-binding domains are not required to edit this class of substrate efficiently.

GENETICS

Table 3. Editing by ADAR1 and K744 of dsRNA substrates with a 31-bp stem

	E1 E2 * *		ADAR1	K744
RG1E	5′ uCA UU**A** **a**GG UgG GUG GaA UGC UAU AAC AAu AUg cuca	E1	48 (8)	22 (16)
	3′ uGU AGU cCC AuC CAC CcU ACG AUA UUG UUg UA_____,	E2	73 (3)	47 (11)
RG2B	5′ uCA UU**A** **a**GG UgG GUG GaA UGC AUA UAC AAu AUg cuca	E1	47 (3)	ND
	3′ uGU AGU cCC AuC CAC CcU ACG UAU AUG UUg UA_____,	E2	74 (3)	ND
RG3A	5′ uCA UU**A** **a**GG UgG GUG GaA UGC GCG CGC AAu AUg cuca	E1	49 (6)	25 (20)
	3′ uGU AGU cCC AuC CAC CcU ACG CGC GCG UUg UA_____,	E2	69 (7)	42 (13)
RG10A	5′ uCA UU**A** **a**GG UgG GUG GaG CGC GCG CAU AAu AUg cuca	E1	59 (12)	26 (9)
	3′ uGU AGU cCC AuC CAC CcC GCG CGC GUA UUg UA_____,	E2	74 (3)	52 (11)
RG15D	5′ uCA UU**A** **a**GG UgG GUG GCG CGC GCU AAC AAu AUg cuca	E1	43 (1)	28 (6)
	3′ uGU AGU cCC AuC CAC CGC GCG CGA UUG UUg UA_____,	E2	66 (7)	53 (12)

Z-forming substrate sequences do not enhance editing either by ADAR1 or the deletion mutant K744. A variety of editing substrates were tested, some of which contain alternating purine and pyrimidine sequences that are favored to form Z-DNA/Z-RNA. The two adenosines that are edited are asterisked and highlighted in bold in the middle column. The one on the left is referred to as E1, and the one on the right as E2, with E2 corresponding to the adenosine deaminated in the RG substrate *in vivo.* Quantitation for each substrate is given in the right-hand column, with the upper value corresponding to E1 and the lower value to E2. Quantitation is based on three experiments, with the standard deviation shown in parentheses.

We investigated the role of the Z-DNA-binding domains further by using an ADAR1 construct in which the Z-DNA-binding domains were inactivated by mutation (22): the conserved tryptophans in Zα and Zβ (W197 and W303) were changed to alanine (WAB) and tested against the longer RG10a substrate and against the 15-bp minimal editing construct M14X8a. A catalytically inactive version of ADAR1 in which the active site HAE was mutated to HAG was used as a control (HAG). Both ADAR1 and WAB edited the E2 site of RG10a with equal efficiency (71 $\pm$ 4% vs. 68 $\pm$ 4%) (Table 4). This result supports the conclusion that the Z-DNA-binding domain is not directly interacting with the editing substrate, which in this case contains a strong Z-forming sequence. Further, it demonstrates that both the DRBM and catalytic domains of WAB remained fully functional after mutagenesis of the Z-DNA-binding domains. As well, this result indicates that the expression levels of ADAR and WAB are equivalent in these experiments. The M14X8a substrate was then investigated because of its lack of dependence of editing on the DRBM, allowing a direct examination of the role of the Z-DNA-binding domains in editing. Compared with the RG10a substrate, there was a significant difference in the editing efficiency of M14X8A between wild-type ADAR1 (66 $\pm$ 6%) and WAB (51 $\pm$ 5%) that was also seen when two other independently produced clones of WAB were tested against the same substrate. This result shows that the Z-DNA-binding domains of ADAR1 are able to increase the efficiency of editing of this class of substrate. The M14X8a substrate does not have strong Z-RNA-forming sequences in the dsRNA stem, making it likely that the Z-DNA-binding domains of ADAR1 are binding to Z-DNA rather than Z-RNA (21).

Table 4. Effect of mutating the Zα and Zβ domains of ADAR1

	ADAR1	WAB	HAG
RG10A-E1	43 (2)	40 (4)	10 (8)
RG10A-E2	71 (4)	68 (4)	27 (3)
M14X8A	66 (6)	51 (5)	12 (7)

Mutation to the Zα and Zβ of ADAR1 lower editing of a 15-bp substrate but not that of a longer substrate containing a strong Z-forming segment. Editing by ADAR1 and WAB were compared to HAG, a catalytically inactive form of ADAR1. Quantitation for M14X8a (see Table 1 for the sequence) is based on five experiments. Quantitation of the two editing sites in RG10A (see Table 3 for the sequence) is based on three experiments. The standard deviation is shown in parentheses.

Discussion

The assays we used here are cell based, where rat ADAR1 constructs are transfected in high enough amounts to produce editing significantly above that caused by endogenous ADAR1. Mutation of the transfected constructs allows us to assess how various domains affect editing efficiency of a variety of editing substrates. Inactivating the deaminase domain also allows us to estimate the maximum possible contribution of endogenously produced ADAR1. Here we define a minimal editing system consisting of a 15-bp substrate with a single A-C base pair mismatch and the catalytic domain of ADAR1. We demonstrate that the deamination of a particular adenosine in these substrates can be altered by changing the position of the mismatch. In the case of M14X8a and M14X9–2 constructs, editing is enhanced when there is a polypyrimidine tract 5′ to the editing site, perhaps reflecting a lower energy of stacking that destabilizes the dsRNA stem. The demonstration that ADAR1 can edit dsRNA substrates with a 15-bp stem is of interest, considering the recent finding that RNA interference (RNAi) also uses relatively short dsRNA substrates (23). The potential thus exists for interplay between these two systems. It is also noteworthy that ADAR1 is induced by interferon, raising the possibility that this cytokine can modulate RNAi responses.

We show that substrates as short as 23 bp can selectively edit two sites at the 5′ end of the dsRNA and that the separation between these sites can vary between 11 and 15 bp. We also show that editing can occur from 4 to 8 bp from the 5′ ends of the helix. When both sites are placed 6–7 bp from the end, they appear to be edited with equal frequency. The results are consistent with a mechanism in which ADAR1 either rebinds rapidly to the substrates after completion of one editing event or can swivel on the substrate to edit both sites. The variable spacing between sites argues against any particular spatial constraint that would arise through dimers or multimers of ADAR1.

By using the M7G and M7L substrates, we show that editing by ADAR1 can also occur in the absence of a mismatch involving the adenosine, although in these substrates a GU mismatch occurs nearby, raising the possibility that ADAR1 scans from a mismatch until it finds a suitable adenosine to deaminate. We also confirm a role for the DRBMs in increasing the efficiency of editing without affecting the selection of the site that is edited (13, 15, 20). However, we demonstrate that the DRBMs are not necessary for editing some substrates *in vivo*, which is different from the results obtained from other studies *in vitro* (13, 15, 20). Either folding of the catalytic domain is more efficient in the

systems used here or other proteins associate with ADAR1 to improve the efficiency of editing *in vivo*.

Further, we show that the Z-DNA-binding domains of ADAR1 are unlikely to bind directly to the dsRNA editing substrates tested here. Placing Z-forming sequences in these substrates does not enhance editing, nor is editing of substrates containing Z-forming sequences reduced significantly by inactivating the Z-DNA-binding domains although mutation. However, we show that mutation of Z-DNA-binding domains decreases editing of the minimal substrate RG14X8a by 28% even though it lacks Z-forming sequences. Since the DRBMs are intact in these mutants, as shown by the efficient editing of the RG10A substrate, it is makes it unlikely that this outcome is due to diminished binding by WAB to the mRNA containing the editing substrate. Rather, this result suggests a role for the Z-DNA-binding domains in targeting ADAR1 to the DNA from which the editing substrate is produced.

It is likely that the potential effect of mutation to the Z-DNA-binding domains of ADAR1 is much larger than demonstrated here. A number of factors mitigate the effect of the mutations to $Z\alpha$ and $Z\beta$ in these experiments. First, ADAR1 is overexpressed in these systems and may be present at high enough levels to find a substrate without targeting by the Z-DNA-binding domains, reducing the apparent effectiveness of a mutation. Second, the substrates used are nonsplicing, increasing the time period during which editing can occur, thereby increasing background editing. Third, editing is not necessarily solely a nuclear event and may continue even after the mRNA is exported to the cytoplasm, again increasing background editing. In support of this possibility, K744 shows a punctate cytoplasmic distribution when examined by immunofluorescence and appears to lack nuclear targeting signals (24), making it likely that editing occurs in the cytoplasm.

The efficiency of editing by K744 even though it lacks dsRNA- and Z-DNA-binding domains may stem from an association with ribosomes or cytoplasmic RNA transport proteins that target it to cytoplasmic mRNAs. Such an eventuality is not unexpected, as we originally purified ADAR1 from a ribosomal pellet (25), and raises the possibility that some RNAs are mostly edited in the cytoplasm.

Recent work has shown that ADAR1 undergoes nucleocytoplasmic shuttling (A.H., H. Knaut, A.R., and J. Nickerson, unpublished work; ref. 24). Once ADAR1 shuttles back to the nucleus, binding to Z-DNA would allow its localization to actively transcribed genes. It is possible that Z-DNA forms in the wake of an actively transcribing polymerase, allowing for the dynamic localization of ADAR1 to a nascent transcript. One prediction of such a scenario would be that good Z-DNA-forming sequences near the editing site would influence editing. We find no evidence for this. A more likely model for the involvement of Z-DNA in editing involves nuclear architecture, where a Z-DNA-forming sequence is brought adjacent to the DNA from which the editing substrate is produced, perhaps by DNA looping. Such a sequence could be, for example, an open area of a chromosome such as near a promoter (25) and could involve DNA looping that brings the site of Z-DNA formation close to the region where transcription of the editing substrate occurs. Such a mechanism would allow both spatial and temporal control of editing to be regulated during development by altering the association of a particular transcript site with a region of Z-DNA formation. The plasmids used here for *in vivo* transcription of substrates are of small size and have a high rate of transcription. The open region around the cytomegalovirus promoter may facilitate the formation of Z-DNA and the localization of ADAR1 near the nascent editing substrates. This is a good model to analyze in further experiments.

From an evolutionary perspective, the data presented would support a scenario where the efficiency of editing of long substrates was increased by addition of the DRBMs to the ancient catalytic domain and that of short substrates by the addition of Z-DNA-binding domains.

GENETICS

We thank M. Higuchi and P. Seeburg for the gift of the rat ADAR1 expression clone. This work was supported by grants to A.R. from the National Institutes of Health (NIH) and the National Science Foundation by NIH/National Heart, Lung, and Blood Institute contract N01-HC-38038 (to Dr. Phillip Wolf).

1. Bass, B. L. (1997) *Trends Biochem. Sci.* **22,** 157–162.
2. Sommer, B., Kohler, M., Sprengel, R. & Seeburg, P. H. (1991) *Cell* **67,** 11–19.
3. Burns, C. N., Chu, H., Rueter, S. M., Hutchinson, L. K., Canton, H., Sanders-Bush, E. & Emeson, R. (1997) *Nature (London)* **387,** 303–308.
4. Herbert, A. (1996) *Trends Genet.* **12,** 6–9.
5. Gerber, A. P. & Keller, W. (2001) *Trends Biochem. Sci.* **26,** 376–384.
6. Palladino, M. J., Keegan, L. P., O'Connell, M. A. & Reenan, R. A. (2000) *RNA* **6,** 1004–1018.
7. Wang, Q., Khillan, J., Gadue, P. & Nishikura, K. (2000) *Science* **290,** 1765–1768.
8. Patterson, J. B. & Samuel, C. E. (1995) *Mol. Cell. Biol.* **15,** 5376–5388.
9. George, C. X. & Samuel, C. E. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 4261–4266.
10. Herbert, A., Kim, Y.-G., Alfken, J., Nishikura, K. & Rich, A. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 12875–12879.
11. Herbert, A. & A., R. (1996) *J. Biol. Chem.* **271,** 11595–11598.
12. Herbert, A., Lowenhaupt, K., Spitzner, J. & Rich, A. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 7550–7554.
13. Lai, F., Drakas, R. & Nishikura, K. (1995) *J. Biol. Chem.* **270,** 17098–17105.
14. Kohler, M., Burnashev, N., Sakmann, B. & Seeburg, P. H. (1993) *Neuron* **10,** 491–500.
15. Maas, S., Melcher, T., Herb, A., Seeburg, P., Keller, W., Krause, S., Higuchi, M. & O'Connell, M. A. (1996) *J. Biol. Chem.* **271,** 12221–12226.
16. Wong, K., Sato, S. & Lazinski, D. (2001) *RNA* **7,** 846–858.
17. Polson, A. G. & Bass, B. L. (1994) *EMBO J.* **13,** 5701–5711.
18. Ryter, J. M. & Schultz, S. C. (1998) *EMBO J.* **17,** 7505–7513.
19. Liu, Y., Herbert, A., Rich, A. & Samuel, C. E. (1998) *Methods* **15,** 199–205.
20. Liu, Y., George, C. X., Patterson, J. R. & Samuel, C. E. (1997) *J. Biol. Chem.* **14,** 4419–4428.
21. Brown, B. A., 2nd, Lowenhaupt, K., Wilbert, C. M., Hanlon, E. B. & Rich, A. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 13532–13536. (First Published November 21, 2000; 10.1073/pnas.240464097)
22. Schade, M., Turner, C., Lowenhaupt, K., Rich, A. & Herbert, A. (1998) *EMBO J.* **18,** 470–479.
23. Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K. & Tuschl, T. (2001) *Nature (London)* **411,** 494–498.
24. Eckmann, C. R., Neunteufl, A., Pfaffstetter, L. & Jantsch, M. F. (2001) *Mol. Biol. Cell* **12,** 1911–1924.
25. Schroth, G. P., Chou, P. J. & Ho, P. S. (1992) *J. Biol. Chem.* **267,** 11846–11855.