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In vitro analysis of the binding of ADAR2 to the pre-mRNA encoding the GluR-B R/G site

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ABSTRACT

The ADAR family of RNA-editing enzymes deaminates adenosines within RNA that is completely or largely double stranded. In mammals, most of the characterized substrates encode receptors involved in neurotransmission, and these substrates are thought to be targeted by the mammalian enzymes ADAR1 and ADAR2. Although some ADAR substrates are deaminated very promiscuously, mammalian glutamate receptor B (gluR-B) pre-mRNA is deaminated at a few specific adenosines. Like most double-stranded RNA (dsRNA) binding proteins, ADARs bind to many different sequences, but few studies have directly measured and compared binding affinities. We have attempted to determine if ADAR deamination specificity occurs because the enzymes bind to targeted regions with higher affinities. To explore this question we studied binding of rat ADAR2 to a region of rat gluR-B pre-mRNA that contains the R/G editing site, and compared a wild-type molecule with one containing mutations that decreased R/G site editing. Although binding affinity to the two sequences was almost identical, footprinting studies indicate ADAR2 binds to the wild-type RNA at a discrete region surrounding the editing site, whereas binding to the mutant appeared nonspecific.

Keywords: ADAR; deamination; double-stranded RNA; dsRBM; RNA editing

INTRODUCTION

Adenosine deaminases that act on RNA (ADARs) convert adenosines to inosines within RNA. The enzymes have been found in every metazoan assayed (reviewed in Bass, 1997), and at least two distinct ADARs, ADAR1 and ADAR2, have been characterized (as cited in Bass et al., 1997). Although the enzymes are suspected to have many functions in a cell, at present only one has been proven: ADARs deaminate adenosines within codons so that multiple protein isoforms can be synthesized from a single encoded transcript. In this capacity, ADARs act as RNA-editing enzymes. ADARs are known to produce functionally important isoforms of the virally encoded protein, hepatitis delta antigen (Polson et al., 1996), as well as cellular proteins involved in neurotransmission, including serotonin receptors (Burns et al., 1997) and glutamate receptors (Egebjerg & Heinemann, 1993; Higuchi et al., 1993; Lomeli et al., 1994).

One of the most well-studied ADAR substrates is glutamate receptor B (gluR-B) pre-mRNA, which encodes a receptor subunit of the α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) type. Editing of gluR-B pre-mRNA results in the synthesis of receptor isoforms that form ion channels with altered properties (reviewed in Seeburg et al., 1998). For example, editing at the Q/R site, which alters a glutamine codon to an arginine codon, results in ion channels that are less permeable to Ca^{2+} (Hollman et al., 1991; Burnashev et al., 1992). Editing at a second site, the R/G site, results in an arginine-to-glycine change that produces ion channels that recover faster from desensitization (Lomeli et al., 1994).

Both ADAR1 and ADAR2 have highly conserved C-termini that contain the catalytic active site (Lai et al., 1995; Maas et al., 1996; Hough & Bass, 1997) and variable numbers of an amino acid sequence known as the double-stranded RNA (dsRNA) binding motif (dsRBM); ADAR1 has three dsRBMs and ADAR2 has two. The dsRBMs are ~65 amino acids long and exist in a variety of other dsRNA-binding proteins, including the bacterial protein, RNase III, the *Drosophila* protein, Staufen, and the mammalian dsRNA-dependent protein kinase, PKR (St. Johnston et al., 1992; Kharrat et al., 1995).

Many studies show that certain adenosines are deaminated more efficiently than others, but how this discrimination occurs is not well understood. Studies of dsRBMs in other proteins indicate that the dsRBM recognizes the A-form helix, but has little sequence spec-

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ificity (e.g., see Bass et al., 1994; Ryter & Schultz, 1998). Although few binding studies have been performed with ADARs, they are known to deaminate many different sequences, suggesting that they also bind with little sequence specificity. However, the binding specificity of ADARs has not been analyzed directly.

To determine if ADARs discriminate between different adenosines at the level of RNA binding, we studied binding of rat ADAR2 to a short RNA hairpin required for editing at the R/G site of rat gluR-B mRNA. We compared results of this hairpin to those obtained with a mutant hairpin that was edited with altered efficiency. We observed that ADAR2 binds the mutant and wild-type substrates with almost identical affinity. However, using a ribonuclease-footprinting analysis, we observed differences in where ADAR2 binds on the two substrates. Although both hairpins were protected along their entire lengths at high concentrations of protein, at lower concentrations of protein, a discrete region of protection was observed for the wild-type, but not the mutant, substrate.

RESULTS

Binding of ADAR2 to the R/G hairpin

Previously, binding of ADAR1 to an artificial substrate, a long, perfectly base-paired dsRNA, was characterized using a nitrocellulose filter-binding assay (Kim et al., 1994; Lai et al., 1995). These studies measured an equilibrium dissociation constant (K_d) between 0.1–0.2 nM and provided the first hint that, like other double-stranded RNA-binding proteins (dsRBPs), ADARs bind dsRNA with high affinity. To extend these observations we investigated binding of ADAR2 to a natural substrate. As ADARs will bind to many different sequences, we anticipated that a single molecule would have multiple binding sites. Multiple binding events cannot be visualized using a filter-binding assay, so we used a gel mobility shift assay that was previously used to study other dsRBPs (Bass et al., 1994).

We wanted to characterize binding of an ADAR protein to a cognate substrate and chose to study the binding of rat ADAR2 to the rat R/G RNA editing site. The rat protein was expressed in *Pichia pastoris*, a budding yeast (Fig. 1A), and the R/G hairpin was synthesized as a 75-nt RNA that folds into a stem-loop structure (Fig. 1B). This substrate is referred to as R/G 75 RNA. The stem-loop is phylogenetically conserved, and the structure of the human sequence has been verified using structure-specific ribonucleases (Aruscavage & Bass, 2000).

To determine the K_d for binding of rat ADAR2 to the R/G hairpin, a gel mobility shift assay was performed. RNA was incubated with varying amounts of purified rat ADAR2 and subsequently analyzed by electrophoresis on a native polyacrylamide gel (Fig. 2A). This electrophoresis system separates free RNA from RNA–protein complexes, and in this case, two different RNA–protein complexes were observed. For the data shown, the faster mobility shift (shift 1) was first observed at 0.29 nM rADAR2 (Fig. 2A, lane 5), and as protein concentration increased, this shift was chased into a second shift of slower mobility. At very high concentrations of protein (114 nM), the majority of the complex remained in the well without entering the gel.

Multiple experiments similar to those shown in Figure 2A were performed, and the data were used to determine a binding constant (Fig. 2B). For formation of the complex represented by the first shift, using the

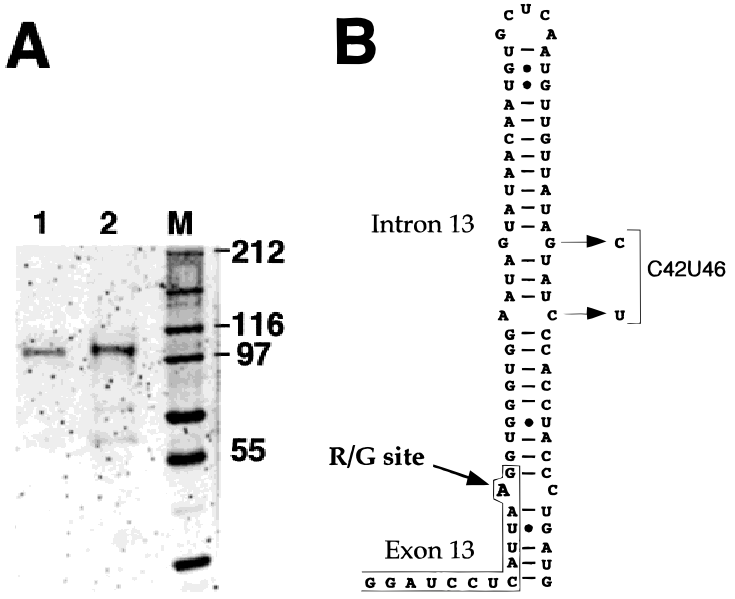

FIGURE 1. Protein and RNA used for the analysis. **A**: Rat ADAR2a-HIS 6 was expressed in *P. pastoris* and purified (see Materials and methods). Aliquots of 15 μL (lane 1) and 30 μL (lane 2) of the final protein preparation were analyzed by 7.5% SDS-PAGE and stained with Sypro-Red (Amersham). The predicted size of the protein, including a polyhistidine tag, is ~80 kDa, but the protein migrates at 100 kDa when compared to protein standards (M, kDa); other members of the ADAR family have also been observed to have aberrant mobilities by SDS-PAGE (Hough & Bass, 1994; O'Connell et al., 1997). **B**: Sequence and plausible secondary structure for the wild-type R/G-75 RNA used. The A at the R/G site is enlarged and shown in bold. The boxed sequence represents the 3′ part of exon 13, and the rest of the sequence corresponds to intron 13. The mutations C42U46 are indicated.

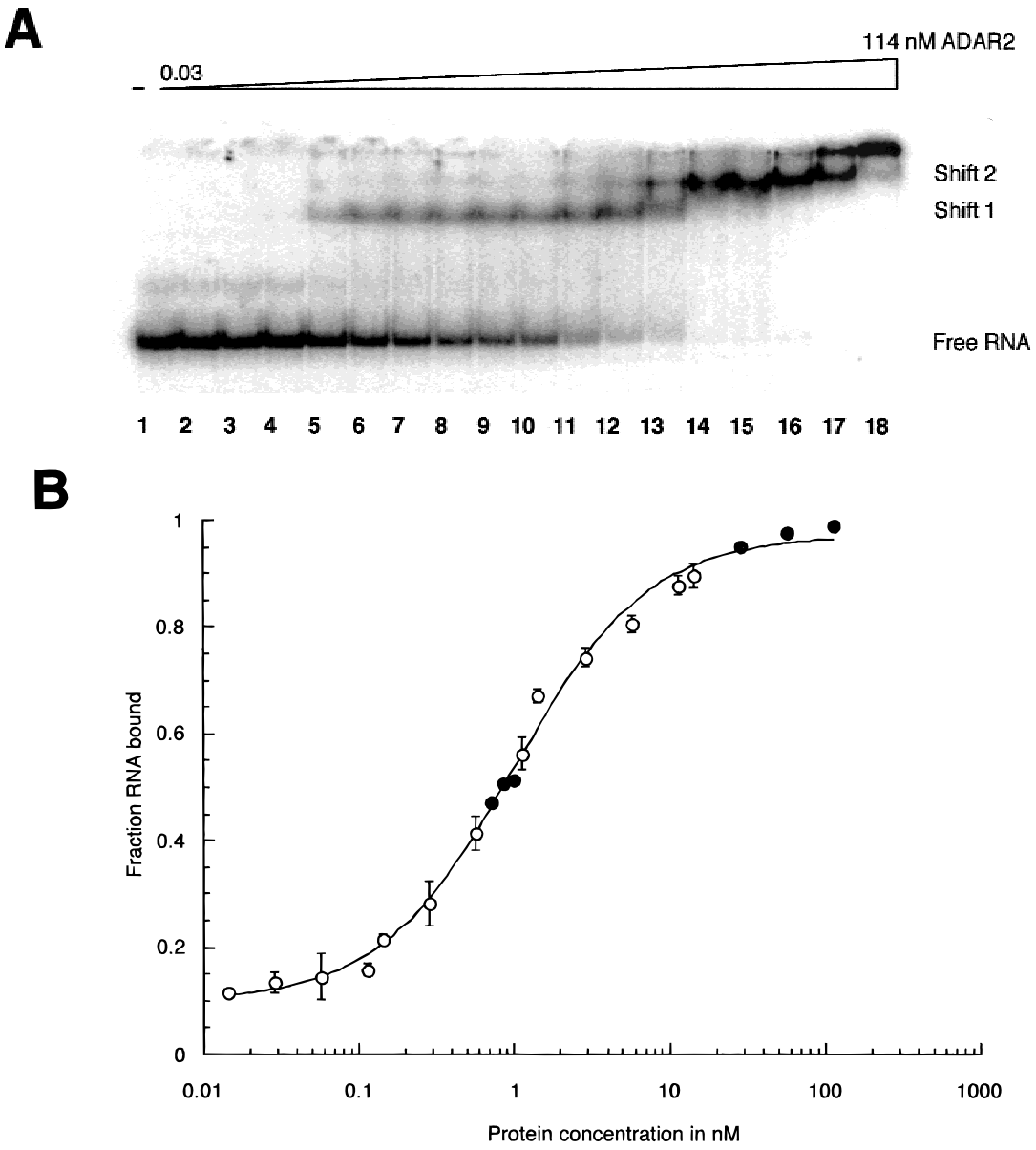

FIGURE 2. Determination of dissociation constants. **A**: Varying amounts of rADAR2 were mixed with wild-type R/G-75 RNA (10 pM), incubated for 15–20 min at 25 °C, and analyzed by electrophoresis on a native 6% polyacrylamide gel. The autoradiogram of the gel shows two RNA–protein complexes that are labeled shifts 1 and 2. ADAR2 concentrations (in nM) from lanes 1–18 were: 0, 0.03, 0.06, 0.1, 0.3, 0.6, 0.7, 0.9, 1.0, 1.1, 1.4, 2.7, 5.7, 11, 14, 29, 57, and 114. Two RNA–protein complex shifts are indicated as shifts 1 and 2. The band that appears between the free RNA and the first shift in samples containing no or low concentrations of protein is probably because of the ribonuclease cleavage of the loop in the RNA. The amount of protein sequestered by this intermolecular duplex does not change the protein concentration enough to affect the K_d. **B**: Multiple analyses similar to that shown in **A** were performed and used to determine a K_d. The data for ADAR2 binding to R/G 75 RNA were generated in 3–5 experiments for open circles and in a single time for closed circles. Values represented by circles correspond to the average, and error bars represent ± standard deviation from the mean.

described conditions, we measured a K_d of 1 nM for binding of rADAR2 to the R/G hairpin.

Is binding affinity influenced by the structure of the R/G hairpin?

Many studies show that ADARs deaminate different adenosines with different efficiencies (e.g., see Polson & Bass, 1994; Melcher et al., 1996). To determine whether altered editing efficiency could be correlated with altered binding, we studied a mutant R/G hairpin, C42U46, that has mutations that change all mismatches to base pairs, except at the R/G site (see Fig. 3A). Using a limited primer extension assay (Fig. 3B), we carefully quantified the amount of editing at the R/G site for the wild-type and mutant molecule (Table 1). At

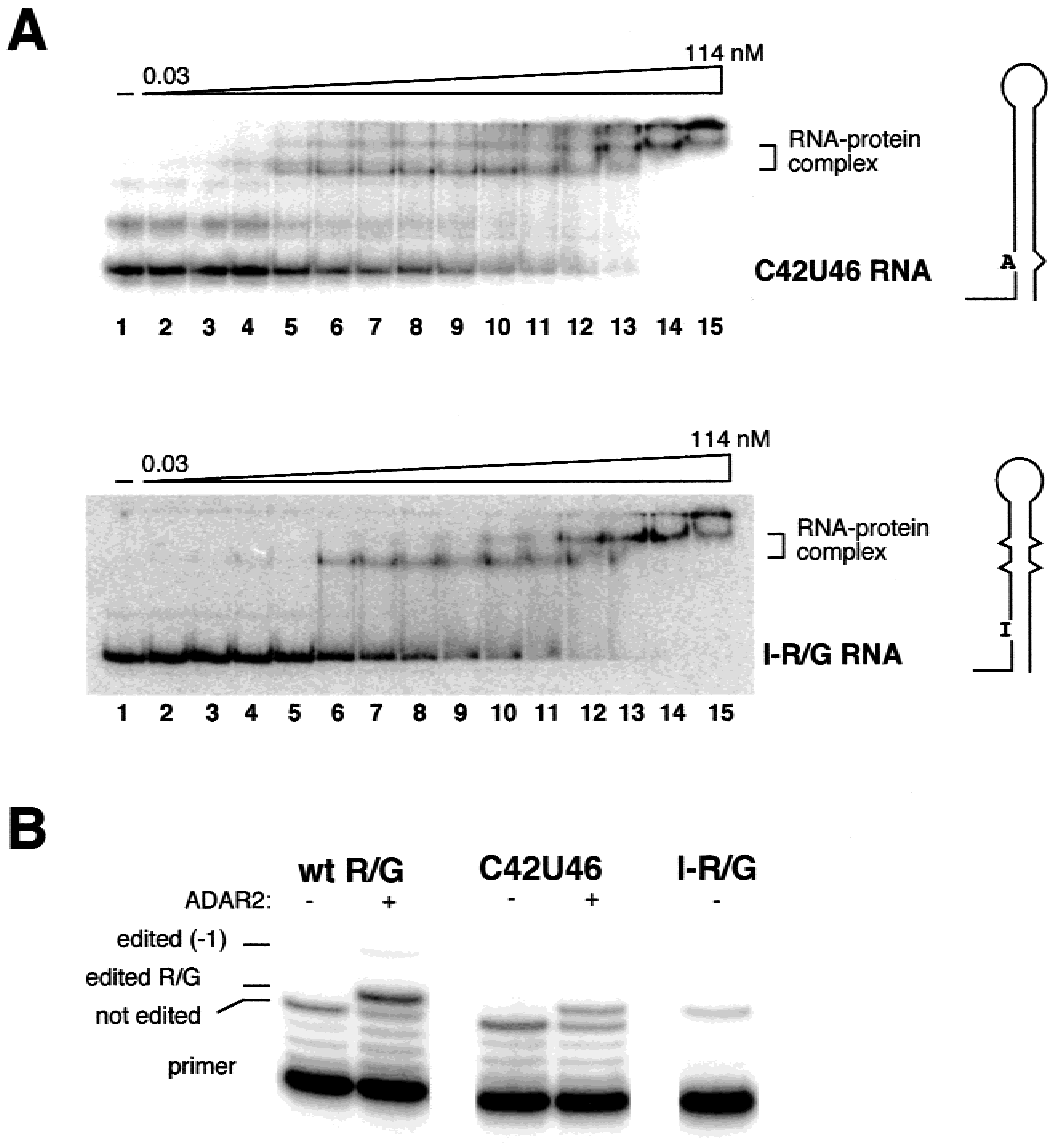

FIGURE 3. Comparison of wild-type and mutant R/G hairpins. **A**: The autoradiograms show representative gel mobility assays of ADAR2 binding to mutant RNAs, C42U46, or I-R/G RNA, and calculated K_ds are listed in Table 1. Conditions were as described in Figure 2, except fewer protein concentrations are shown for C24U46 RNA and I-R/G RNA. Protein concentrations (in nM) for lanes 1–15 were: 0, 0.03, 0.06, 0.1, 0.3, 0.6, 1.1, 1.4, 2.9, 5.7, 11, 14, 29, 57, and 114. The putative structure for the different mutants is illustrated to the right of each gel, with the nucleotide at the R/G site indicated. **B**: The efficiency of editing at the R/G site was determined for the wild-type R/G-75 RNA and the mutant hairpins (C42U46 and I-R/G RNA). A limited primer-extension assay was used to monitor the R/G site after incubation without rADAR2 (−) or with rADAR2 (+; see Materials and methods). Primer extension was performed in the presence of ddT so that reverse transcriptase terminated at the R/G site for unedited molecules (not edited) and continued on to the next adenosine if the R/G site was edited (edited R/G). Note that we observed a small amount of editing at the adenosine 5′ of the R/G site, as evidenced by the faint band that terminated at the more distant adenosine [edited (−1)]. Editing efficiency was calculated as described in Materials and methods.

complete reaction, we observed that the mutant hairpin was edited to ~40%, whereas the wild-type molecule showed about 75% editing at the R/G site. Similar editing efficiencies were observed under conditions for protein binding, using our gel mobility shift buffer (data not shown). We also performed gel mobility shift assays for the mutated hairpin (Fig. 3A) and calculated dissociation constants (Table 1). Although rADAR2 showed a reduced affinity for the mutant hairpin, the change in the measured K_d was within experimental error; C42U46 exhibited a K_d of 1.4 ± 0.3 nM, compared to 1 ± 0.1 nM for the wild-type substrate (Table 1).

We extended the editing analysis and determined R/G editing at early time points (Fig. 4). At the earliest measurement (30 s), the wild-type substrate was already edited to about 50%, whereas editing of the C42U46 mutant had only reached 14%. After 15 min, editing at the R/G site was complete and reached its

TABLE 1. K_d and editing of wild-type and mutant R/G RNA.

Substrate	wt R/G	C42U46 R/G	I R/G
K_d (in nM)	0.99 ± 0.07	1.44 ± 0.32	2.03 ± 0.37
Percent editing at the R/G site	74 ± 6	41 ± 6	N.A.[a]

Analyses were performed as in Figures 2 and 3. K_d values were averaged from at least three experiments; editing values represent an average of 2–3 experiments.

[a]N.A.: not applicable.

final level for both molecules. As listed in Table 1, the final level of R/G editing in the mutant molecule is about half of the wild-type substrate.

Can ADAR2 bind to the edited RNA product?

We wondered if ADAR2 turnover was facilitated by a reduced affinity for the reaction product. To test this idea, we synthesized a product mimic, an RNA with an inosine at the R/G site of the stem-loop structure (I-R/G). Using the gel mobility shift assay, we determined that rADAR2 bound the I-R/G hairpin with a K_d of 2 ± 0.4 nM, about twofold higher than the K_d measured for binding to the-wild type hairpin (Fig. 3A and Table 1). Although our studies indicate that ADAR2 does bind to its product with a lower affinity, the difference is slight, and future studies will be required to determine how significant this difference is to the overall reaction.

Where does ADAR2 bind on the R/G hairpin?

To determine whether a specific ADAR2-binding site could be detected on the R/G stem loop, we performed an RNA-footprinting experiment using ribonuclease V1. Ribonuclease V1 cleaves double-stranded or helical RNA without significant base specificity (Ehresmann et al., 1987). Interestingly, despite the fact that ADARs will deaminate many different RNA sequences, we observed a faint but discrete region of protection for ADAR2 binding to the wild-type R/G-75 RNA. In particular, we observed a protection of 16 nt, beginning about 8 nt 5′ of the R/G site and extending close to the mismatch, 7 nt 3′ of the R/G site (Fig. 5A). Note that the 5′ end of the protected region begins in a region that is not shown as base paired in the secondary structure (Fig. 1B). However, V1 cleaves the RNA in this region, in the absence of ADAR2 (see Fig. 5A, left lane), consistent with the idea that these bases are in a helical conformation that can be recognized by ADAR2. Although less well resolved, there is a reciprocal region of protection at the top of the gel, suggesting that the discrete protection encompasses both strands of the stem. Importantly, the discrete footprint is first observed when the first shift is observed by gel mobility shift assay, that is, at 0.1–0.2 nM of ADAR2 (compare Figs. 5A and 5B).

As higher concentrations of ADAR2 were used for footprinting, the entire stem-loop was completely protected (Fig. 6A), and under these conditions, all RNA migrated with the second shift by gel-shift analysis (Fig. 6B). These data suggest that the entire RNA stem is coated with ADAR2 at these higher protein concentrations, consistent with previous observations that suggest ADARs can deaminate many different sequences.

We also performed footprinting experiments with the mutant RNA (C42U46). As for the wild-type substrate, protection could first be observed at protein concentrations that correlated with the appearance of the first shift (Fig. 7). However, the protection pattern was quite different. For the wild-type substrate, the first shift correlated with an obvious decrease in V1 cleavage in a discrete region surrounding the R/G site. In contrast, the protection of the mutant substrate decreased cleavage only slightly, and this slight protection was not localized, but observed throughout the stem. As observed for the wild-type substrate (Fig. 6), complete protection of the entire stem was observed for the mutant substrate at high concentrations of protein (data not shown).

DISCUSSION

ADARs are capable of two very different types of deamination: a highly selective deamination in which one or a few specific adenosines are targeted, and a very promiscuous deamination in which 50–60% of the adenosines are deaminated in a single molecule (reviewed in Bass, 1997). The promiscuous deamination is found in RNAs that are predominantly double-stranded, whereas the more selective deamination occurs in molecules whose double-stranded regions are interrupted by mismatches, bulges, and loops. Thus, selectivity is thought to depend on nonhelical disruptions to the overall helical structure, and recent studies of internal loops in ADAR substrates provide support for this hypothesis (Lehmann & Bass, 1999).

How loops, bulges, and mismatches allow selectivity is unclear, but one possibility is that these structural features alter ADAR binding sites or the enzyme's affinity for its substrate. To explore these ideas, we studied binding of rat ADAR2 to the R/G hairpin of rat gluR-B pre-mRNA. This substrate contains three mismatches and is selectively edited at a single site in vivo, the R/G site (reviewed in Seeburg et al., 1998). Our in vitro studies showed that mutating two of the mismatches to base pairs altered editing at the R/G site, but did not alter ADAR2 affinity, as measured by gel shift analyses. However, footprinting analyses showed that these mutations affected where ADAR2 bound the R/G hairpin. At lower amounts of ADAR2, corresponding to concentrations that show only the first shift by gel shift analyses, protection of the wild-type sequence occurred at a

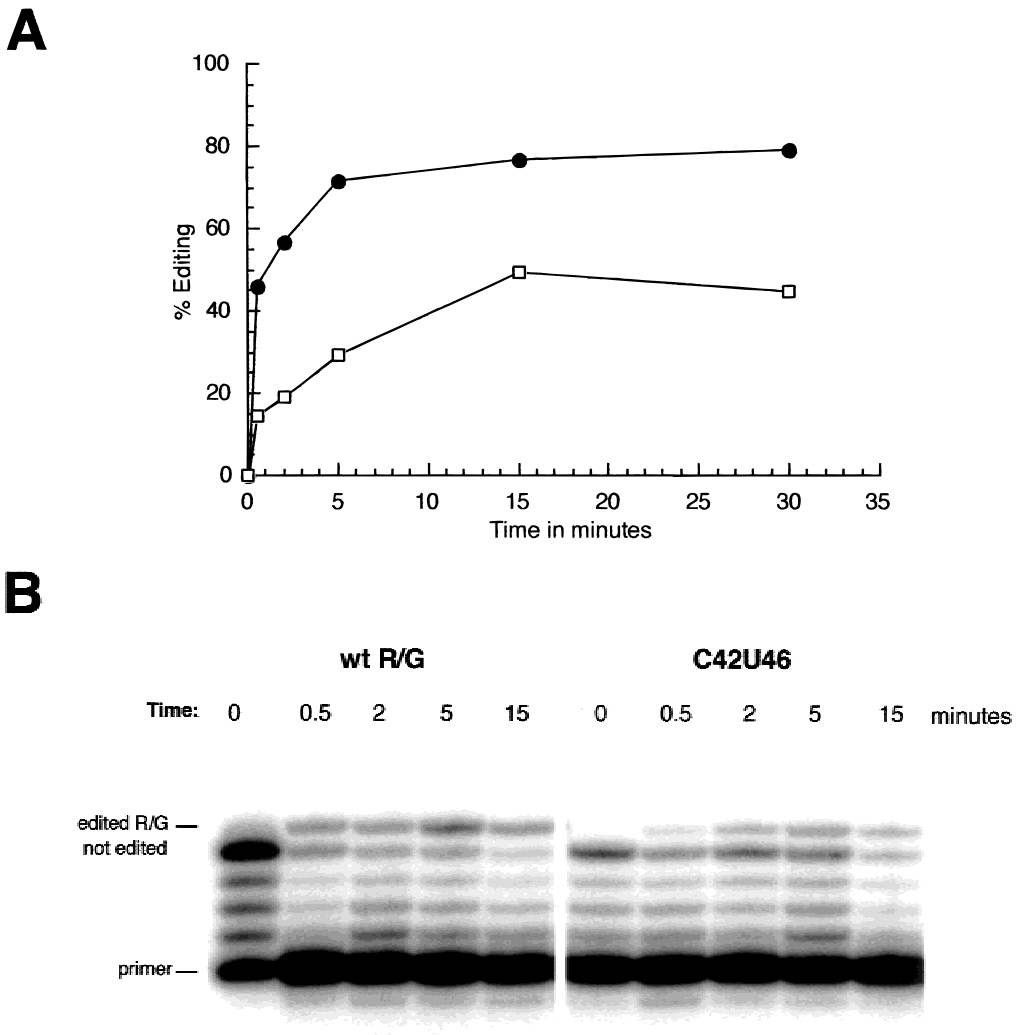

FIGURE 4. Efficiency of editing at different time points. Reactions of wild-type R/G RNA or C42U46 RNA (both at 5 nM) were sampled after 0, 0.5, 2, 5, 15, and 30 min of incubation with 50 nM of rADAR2 (see Materials and methods). A limited primer-extension assay was used to determine the percent editing at the R/G site as in Figure 3. **A**: Efficiency of editing graphed as % editing at the R/G site after different times of incubation. Closed circles show data using the wild-type substrate and open squares represent the C42U46 mutant RNA substrate. **B**: A representative autoradiogram from a limited primer-extension assay used to obtain the data shown in **A**.

discrete region that encompassed the R/G site. In contrast, protection of the mutant molecule occurred nonspecifically throughout the stem and to a lesser extent. Our studies provide the first demonstration of specificity for binding of an ADAR, and suggest mismatches are involved in directing ADARs to a specific site.

ADARs bind tightly to many different dsRNA molecules

Using a gel shift assay we measured K_d values for binding of ADAR2 to three versions of the R/G hairpin: the wild-type sequence (wt R/G, 0.99 $\pm$ 0.07 nM), a mutant where two mismatches were changed to pair (C42U46 R/G, 1.44 $\pm$ 0.32 nM), and a molecule that had an inosine at the R/G site and thus mimicked the reaction product (I R/G, 2.03 $\pm$ 0.37 nM). ADAR2 bound tightly to all three molecules and exhibited only slight differences in affinity.

Previous studies of ADAR1 binding to a dsRNA of 575 bp measured an affinity about fivefold greater (0.1–0.2 nM; Kim et al., 1994; Lai et al., 1995). Future studies will be required to determine if the values reflect differences between the binding properties of ADAR1 and ADAR2, or the different conditions used in the two studies. Importantly, because ADARs do not show strict sequence specificities, longer substrates have more binding sites and might be expected to exhibit higher affinities. Thus, the different K_d values may simply reflect the different substrates used in the studies.

ADAR2 binds to a discrete position on the wild-type but not the mutant hairpin

For all R/G hairpins, gel shift analyses showed two shifts, representing two different RNA–protein complexes. The second shifts were only observed at high concentrations of protein, and only after all of the RNA had moved into the first shift. This suggests that the second shift corresponds to binding of two protein monomers to one RNA, rather than one protein monomer binding to two RNA molecules. At present we do not know if the active form of ADAR2 is a single or multiple polypeptide, and we use the term monomer to mean one active protein species.

Despite the similar appearance of the gel shift analyses for the different hairpins, footprinting experiments suggest the initial shift represents binding to a discrete region for the wild-type sequence and a heterogeneous mixture of single-binding events for the mutant molecule. Although not extensively studied, the ADAR reaction is thought to occur distributively, with each binding event directing one or two deamination events (Polson & Bass, 1994). In this light, the footprinting results are consistent with the time course of the editing reaction for the wild-type and mutant substrates (Fig. 4). For the wild-type sequence, protein binding is localized to a region encompassing the editing site, and the R/G adenosine is rapidly deaminated (50% by 30 s). In contrast, binding to C42U46 occurs randomly, and only a small percentage of the RNA population has protein bound at the R/G site, and correspondingly, only a small percentage of the molecules are deaminated at the R/G site (14% by 30 s). For the wild-type RNA population, most of the enzyme is proposed to be bound near the R/G site, whereas ADAR2 would be bound randomly to the mutant hairpin, and thus other adenosines may be deaminated in this molecule. Future studies will be required to confirm this.

At higher concentrations of ADAR2, the entire stem is footprinted, even for the wild-type sequence. However, previous studies show that not all adenosines in a given molecule can be deaminated, and the number of deaminations observed at complete reaction is limited by the length and stability of the molecule. As AU base pairs are converted to the less stable IU mismatch, ADAR substrates become increasingly single stranded in character, and it is thought that this is why not all adenosines can be deaminated in a single mol-

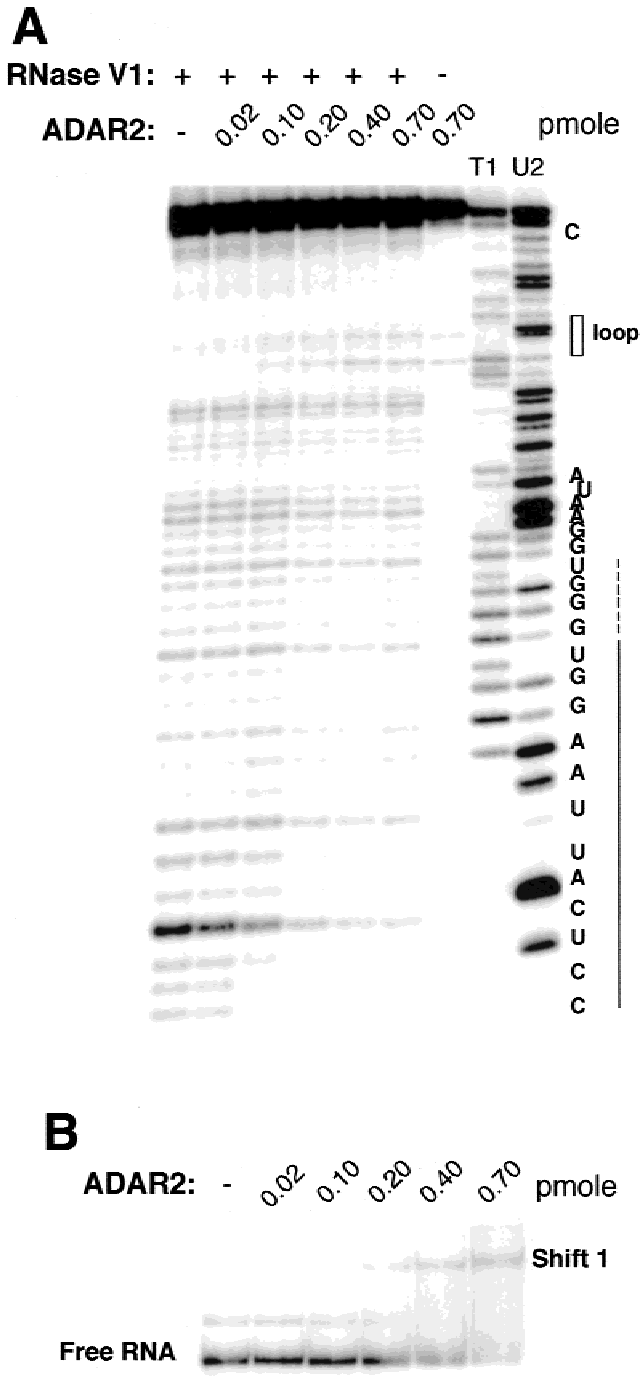

FIGURE 5. A: Ribonuclease V1 footprinting of rADAR2 bound to wild-type R/G-75 RNA (as described in Materials and methods). A constant amount of RNA was treated with (+) or without (−) 0.1 U of RNase V1 as indicated, in the absence of rADAR2 (−) or with increasing amounts of ADAR2 (lanes 2–6 contain 0.02, 0.10, 0.20, 0.40, or 0.70 pmol). A control reaction containing rADAR2 without V1 contained 0.70 pmol of rADAR2. To orient the bands with the RNA sequence, R/G-75 RNA was treated with RNases T1 and U2 under denaturing conditions (far right lanes). To the right of the gel, the five-base loop of the R/G stem-loop structure is labeled with a rectangle. The region of ADAR2 protection is indicated by a solid line, or a dotted line where a weaker protection was observed. The letter C close to the top at the right-hand side of the gel corresponds to the mispaired C opposite the R/G site on the opposite strand. **B**: Gel mobility shift assay of wild-type R/G-75 RNA binding to ADAR2 using the same concentrations and conditions as in the protein-binding step of the footprinting experiment in **A** (see Materials and methods). A single RNA–protein complex is indicated as shift 1 to the right of the gel. See Figure 2 for discussion of the faint band between free RNA and first shift.

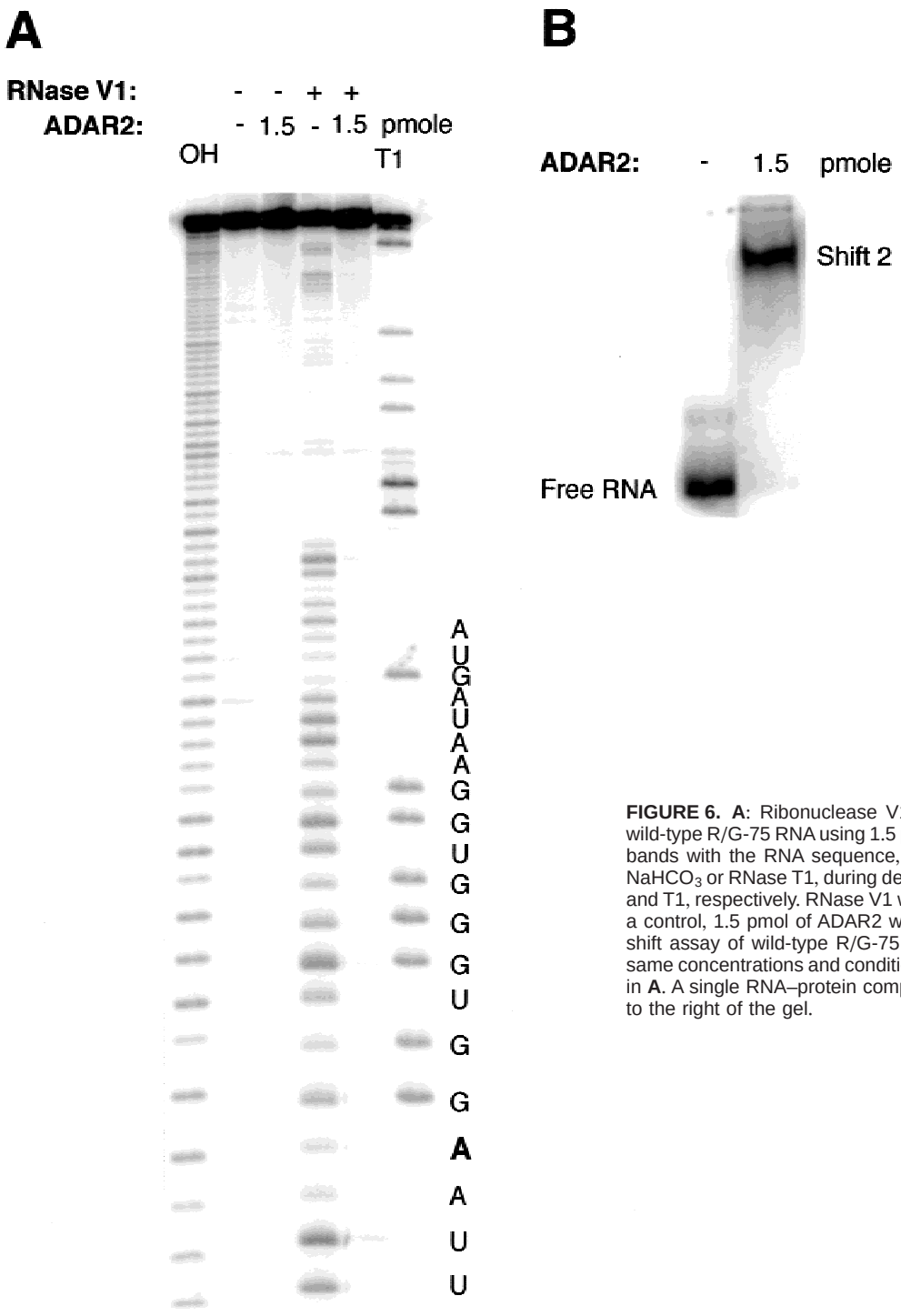

FIGURE 6. **A**: Ribonuclease V1 footprinting of rADAR2 bound to wild-type R/G-75 RNA using 1.5 pmol of ADAR2 protein. To orient the bands with the RNA sequence, the R/G-75 RNA was treated with $NaHCO_3$ or RNase T1, during denaturing conditions, and labeled OH and T1, respectively. RNase V1 was excluded from lanes 2 and 3. As a control, 1.5 pmol of ADAR2 was added in lane 3. **B**: Gel mobility shift assay of wild-type R/G-75 RNA, binding to ADAR2 using the same concentrations and conditions as in the footprinting experiment in **A**. A single RNA–protein complex indicated as shift 2 is indicated to the right of the gel.

ecule. This may explain why even after long incubation times, where the reaction is complete, editing at the R/G site of the mutant molecule is only half of the amount observed in the wild-type sequence (Fig. 3B and Table 1). Additional binding sites in this molecule would be predicted to lead to additional deamination events elsewhere in the molecule that would preclude deamination at the R/G site.

A model: Mismatches direct binding-site position

Our data suggest that mismatches in the wild-type sequence are important for establishing the register of ADAR2 binding, and help to position the enzyme over the R/G site. Interestingly, the crystal structure of the second dsRBM from *Xenopus laevis* protein A in com-

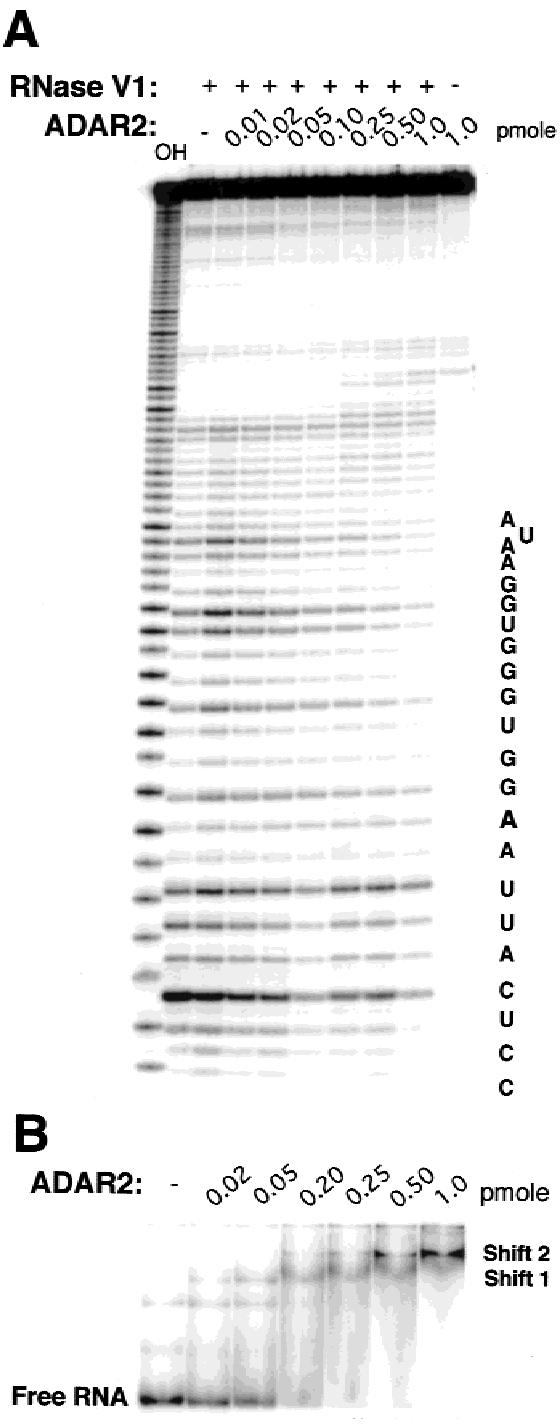

FIGURE 7. A: Ribonuclease V1 footprinting of rADAR2 bound to C42U46 RNA. RNA samples of constant concentrations (as described in Materials and methods) were treated with (+) or without (−) 0.1 U of RNase V1 as indicated, in the absence of rADAR2 (−) or with varying amounts of ADAR2 (lanes 3–9 contain 0.01, 0.02, 0.05, 0.10, 0.25, 0.50, or 1.0 pmol). As a control, 1.0 pmol of ADAR2 was added in the absence of RNase V1 (far right lane). The R/G 75 RNA was treated with $NaHCO_3$ during denaturing conditions and used as a marker, labeled OH, to orient bands. **B**: Gel mobility shift assay of C42U46 RNA, binding to ADAR2 using the same conditions as in the footprinting experiment in **A** showing a subset of concentrations.

plex with a dsRNA showed the protein interacting with two consecutive minor grooves as well as the major groove (Ryter & Schultz, 1998). Although the major groove is often too narrow for interactions with a protein, in this case the groove occurred at the interface of two independent stacked helixes, and thus was considerably widened. A mismatch could also help to widen the helix, and we speculate the mismatches in the wild-type R/G hairpin may serve to promote specific major-groove interactions that help to establish the register of protein binding.

MATERIALS AND METHODS

Rat ADAR2 cloning, expression, and purification

The rat ADAR2a clone was kindly provided by Dr. John Casey. PCR amplification was done using primers "rRED 5′ long" (5′-ACCATGGATATAGAAGACGAAGAGAATATGAGT TCCAGCAGCATTGATGTTA-3′) and "rRED end" (5′-TCT AGAGGAGTGAAGGAGAACTGGTC-3′) to delete a 47-nt intron sequence shown to be prohibitive to protein production. The PCR product was subcloned into pGEM-T (Promega), and the resulting plasmid cut with *Sac*II and *Xba*I to give a 2.1-kb fragment containing the ADAR2 gene. The ADAR2 fragment was inserted into the yeast vector pPICZ-B (InVitrogen) cut with the same enzymes. Insertion at this location of the vector gives an expressed protein with an N-terminal polyhistidine $(his)_6$ tag for further purification on Ni-NTA (see below). The ADAR2/pPICZ plasmid was recombined into the *P. pastoris* genome according to the manufacturer's protocol (InVitrogen).

Rat ADAR2a was expressed in *P. pastoris* by modifying a protocol for expression of human RED1-S (Gerber et al., 1997). Cells were grown as described, and harvested after 96 h. Cell pellet from 1 L of culture was resuspended in a total volume of 40 mL using buffer A (50 mM Tris-HCl, pH 8, 5 mM EDTA, 10% glycerol, 1 mM DTT, 0.5 mM PMSF, 0.7 μg/mL pepstatin, 0.4 μg/mL leupeptin). The suspension was lysed with 2–3 passes using a French press. Cell debris was separated by centrifugation (100,000 $\times$ *g*, 1 h). The supernatant was batch bound to a Macro Prep High Q matrix (Bio-Rad) for 15 min. Proteins were step eluted at low salt, buffer B (50 mM Tris-HCl, pH 8, 10% glycerol, 1 mM DTT, 0.5 mM PMSF) containing 150 mM KCl, and at high salt, buffer B containing 350 mM KCl. ADAR activity was traced to the high-salt eluate by assaying the deamination of CAT dsRNA (Hough & Bass, 1994). The eluate was precipitated with 40% ammonium sulfate, resuspended in 2.5 mL of buffer C (buffer B containing 0.7 μg/mL pepstatin, 0.4 μg/mL leupeptin, 200 mM KCl, and 0.05% NP-40), and desalted on a PD-10 G-25 gel-filtration column (Pharmacia). Protein was loaded onto a nickel-NTA column and eluted (see Gerber et al., 1997). Finally the buffer was exchanged on a PD-10 G-25 column leaving the pure ADAR2 protein in a storage buffer containing (50 mM Tris-HCl, pH 7.9, 200 mM KCl, 5 mM EDTA, 20% glycerol, 1 mM DTT, and 0.01% NP-40). Purified protein was assayed for enzymatic activity using a natural substrate containing the R/G site (see Fig. 3B and below). The protein

concentration was estimated to be 200 nM by comparing the intensity of ADAR2 bands to different concentrations of BSA on a 7.5% SDS-PAGE, stained with Sypro-Red, and scanned using a Storm Molecular Dynamics PhosphorImager.

Preparation of RNA substrates

A PCR product from a template containing rat gluR-B sequence, surrounding the R/G site, was made using the primers (5′-TAATACGACTCACTATAGGATCCTCATTAAGGTG GGT-3′) and (5′-TTAGATACACATCAGGGTAGGTGGGA TAC-3′). A *Bsa*B1-recognition sequence was added to the 3′ of the PCR product by the downstream primer. The R/G PCR product was cloned into the pGEM-T vector system (Promega) and named pGMRG75. A 75-nt wild-type gluR-B R/G RNA was synthesized by transcription using T7-MEGAshortscript (Ambion) with pGMRG75, linearized with *Bsa*B1, as template. The C42U46 mutant template was synthesized as above, but the downstream primer was (5′-TTAGATACACATCAGGGTAGGTGG**A**ATA**G**TATAAC-3′). For quantification, RNA substrates were labeled with uridine 5′-triphosphate [5, 6-^{3}H] during transcription. 5′-^{32}P-labeled RNAs were prepared by treatment of dephosphorylated transcripts with T4 polynucleotide kinase in the presence of [γ-^{32}P] ATP (6,000 Ci/mmol).

An RNA with a single I at the R/G site was synthesized by ligating two half molecules. The template for transcription of the 3′-half contained an SP6 promoter followed by 62 nt of the gluR-B sequence immediately following the R/G site. Transcription was initiated with GMP (5:1 molar ratio, GMP:GTP). The 5′-half RNA molecule (5′-GGAUCCUC AUUAI-3′) was synthesized on an Applied Biosystems 394 RNA/DNA synthesizer, gel purified, and ligated to a gel-purified 3′-half transcript as described (Moore & Sharp, 1992), except reactions (60 μL) contained 600 pmol 5′-half molecule; 200 pmol 3′-half molecule; 400 pmol bridging oligodeoxynucleotide (5′-ACATTGTTATACTATTCCACCCACC CTAATGAGGATCC-3′); and 150 U of T4 DNA ligase (Promega) in ligase buffer (Promega). The ligation product was gel purified and the concentration determined by OD at 260 nM. RNA was 5′-^{32}P labeled using [γ-^{32}P] ATP (6,000 Ci/mmol) and T4 polynucleotide kinase.

Gel mobility shift assay

Mobility shift assays were done essentially as described (Bass et al., 1994). Assays (15 μL) used purified rat ADAR2 protein (30 pM–114 nM) and 10 pM of the different RNA substrates. In a time-course experiment, no difference in fraction RNA bound was seen between 5–60 min, and an incubation time of 20 min was used in all binding reactions. Binding reactions were stopped by loading onto a 6% (19:1 acrylamide/bis) native polyacrylamide gel, and electrophoresis was at 160 V for 1.5 h in 1× TBE buffer.

Radioactivity in the band corresponding to free RNA and that of the entire lane was quantified using a Storm Molecular Dynamics PhosphorImager, and the fraction of RNA bound by protein calculated [fraction bound = 1 − (free RNA/total RNA)]. K_d values were extracted from binding isotherms as described (Bass et al., 1994), except the program KaleidaGraph (Abelbeck Software) was used.

ADAR-modification assay

Fifty femtomoles (0.5 nM) of RNA and 130 ng of ADAR2 were incubated at 30 °C for 30 min in standard assay buffer, unless otherwise indicated (Hough & Bass, 1994). The reaction was stopped by adding proteinase K, followed by phenol/chloroform extraction. Editing at the R/G site was assayed using a limited primer-extension assay. One picomole of 5′-^{32}P-labeled glu-R/G RT primer (5′-ATTGTTATACTATTCCACCC-3′) was hybridized to reacted RNA by heating at 70 °C (10 min), and then at 55 °C (2–4 h) in reverse transcriptase buffer (Boehringer-Mannheim). The RNA was extended using 4 U of AMV reverse transcriptase (Boehringer-Mannheim), 10 μM dATP, 10 μM dCTP, 10 μM dGTP, and 250 μM of dideoxy-TTP. The reaction was incubated at 42 °C for 45 min and stopped by adding 7 μL 80% formamide and dyes. Reaction products were separated on a 20% denaturing PAGE containing 7 M urea. Percent editing was calculated by dividing the radioactivity associated with edited bands by the sum of the radioactivity in the nonedited and edited bands. Radioactivity was quantified using a Storm Molecular Dynamics PhosphorImager. Percent editing values are given with reference to control reactions using the I-R/G RNA, which represented 100% editing at the R/G site.

Ribonuclease V1 footprinting assay

The footprinting assay was performed using cobra venom ribonuclease V1 (Pharmacia). Each reaction contained 50,000–75,000 cpm of RNA (40–50 fmol), and prior to digestion RNA was incubated for 10 min at 25 °C in 1× MSB (Bass et al., 1994) with or without ADAR2 protein, as indicated in the text, in a volume of 8 μL. RNase V1 (0.1 U) and 2 μg of tRNA were added and samples were incubated (10 min, 25 °C) in a total volume of 10 μL. Reactions were stopped by extracting with phenol and chloroform, followed by ethanol precipitation. Cleavage positions were identified using R/G 75 RNA treated with RNases T1 and U1, respectively, under denaturing conditions as described (Schlegl et al., 1997), or by using an alkaline ladder of the RNA (Donis-Keller et al., 1977).

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