THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 40, Issue of October 4, pp. 37624–37629, 2002

Printed in U.S.A.

Adenosine to Inosine Editing by ADAR2 Requires Formation of a Ternary Complex on the GluR-B R/G Site*

Received for publication, April 29, 2002, and in revised form, July 31, 2002
Published, JBC Papers in Press, August 5, 2002, DOI 10.1074/jbc.M204126200

Dominic C. J. Jaikaran‡, Cynthia H. Collins‡§, and Andrew M. MacMillan¶‖

From the ‡Department of Chemistry, University of Toronto, Toronto, Ontario M5S 3H6, Canada and the ¶Department of Biochemistry, University of Alberta, Edmonton, Alberta T6G 2H7, Canada

RNA editing by members of the ADAR (adenosine deaminase that acts on RNA) enzyme family involves hydrolytic deamination of adenosine to inosine within the context of a double-stranded pre-mRNA substrate. Editing of the human GluR-B transcript is catalyzed by the enzyme ADAR2 at the Q/R and R/G sites. We have established a minimal RNA substrate for editing based on the R/G site and have characterized the interaction of ADAR2 with this RNA by gel shift, kinetic, and cross-linking analyses. Gel shift analysis revealed that two complexes are formed on the RNA as protein concentration is increased; the ADAR monomers can be cross-linked to one another in an RNA-dependent fashion. We performed a detailed kinetic study of the editing reaction; the data from this study are consistent with a reaction scheme in which formation of an ADAR2·RNA ternary complex is required for efficient RNA editing and in which formation of this complex is rate determining. These observations suggest that RNA adenosine deaminases function as homodimers on their RNA substrates and may partially explain regulation of RNA editing in these systems.

Eukaryotic precursor-mRNAs (pre-mRNAs) are subject to a variety of post-transcriptional modifications that regulate gene expression. These modifications, collectively referred to as RNA processing reactions, include 5′- and 3′-end formation, RNA splicing, polyadenylation, and the recently characterized phenomenon of RNA editing (reviewed in Refs. 1–3). RNA editing involves changes in the information content of the primary transcript by the specific insertion, deletion, or chemical modification of nucleotides. This latter class of editing reactions includes the hydrolytic deamination of adenosine (A) to inosine (I) within the context of double-stranded RNA. Deamination events of this type have been demonstrated in both viral and cellular transcripts. For example, the life-cycle of the Hepatitis δ virus is regulated by an editing event in the antigenome in which a UAG stop codon is converted to a UIG tryptophan codon (4). As well, A to I editing is involved in the regulation of function of a growing number of cellular factors. Tissue-specific editing of the serotonin 5-HT2C receptor results in a 10–15-fold reduction in response to serotonin agonists and thus may be involved in the regulation of cellular response to neurotransmitters (5). Recently, A to I editing has been shown to be essential for erythropoeisis in mice (6). Transcripts for subunits of the neural-specific AMPA class of glutamate-gated (GluR) ion channels undergo A to I modification at two positions, the Q/R and R/G (see Fig. 1*A*) editing sites, which affect the properties of the resulting channels (7–11).

Relatively little is known about the determinants for specific A to I deamination in pre-mRNA substrates. In the case of GluR-B pre-mRNAs, it has been demonstrated that editing at both the Q/R and R/G sites requires a double-stranded structure formed between sequences proximal to the editing site and downstream intron sequences (8, 9, 12, 13). Mutation of the intron sequence to abolish the double-stranded sequence at the Q/R site prevents editing and is an embryonic lethal event in mice (14). The disposition of the edited nucleotide during the editing event is unclear; in the native Q/R substrate the target adenosine is contained within a perfect duplex, which is not the case with the R/G substrate (see Fig. 1*A*). It is also unclear whether there are specific sequences that direct the site of RNA editing or whether the targeting is dictated by a specific spatial arrangement of double-stranded RNA.

The first RNA deaminase to be cloned, ADAR1[1] (adenosine deaminase that acts on RNA) (15–18) was identified originally as an activity responsible for unwinding double-stranded RNA (19–21). Subsequently, this unwinding activity was correlated with deamination of A to I within double-stranded RNA substrates (22). ADAR1 exhibited low deaminase activity with a number of specific substrates, including the Q/R site of the GluR-B pre-mRNA, but has been shown to efficiently edit the R/G site of GluR-B as well as the anti-genome of Hepatitis δ virus (4, 23–25). Screening of a rat hippocampal cDNA library with probes complementary to the deaminase domain of ADAR1 resulted in the cloning of ADAR2 deaminase (26). ADAR2a, the shorter of two human isoforms, is an ~80-kDa protein containing two N-terminal double-stranded RNA binding domains as well as a C-terminal deaminase domain (27).

ADAR2 has been shown to efficiently edit both the Q/R and R/G sites of the GluR-B transcript, and in contrast to ADAR1 it does not exhibit activity at a position within intron 11 of GluR-B (see Fig. 1*A*) (23, 26). These facts, together with the biochemical purification of ADAR2 along with the Q/R deaminase activity (28) strongly support the identification of ADAR2 as the cellular factor responsible for editing of the GluR-B transcript. The mechanism of action of ADAR2 on its RNA substrates is unclear. Little is known about the mechanism of RNA binding or substrate activation for the deamination event. To study the molecular basis for RNA recognition and adeno-

* This work was supported by the Canadian Institutes for Health Research and by an establishment grant from the Alberta Heritage Fund for Medical Research. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Present address: Graduate Program in Biochemistry, California Inst. of Technology, Pasadena, CA 91125.

‖ To whom correspondence should be addressed. Tel.: 780-492-3803; E-mail: andrew.macmillan@ualberta.ca.

[1] The abbreviations used are: ADAR, adenosine deaminase that acts on RNA; DMS, dimethyl suberimidate hydrochloride; DTT, dithiothreitol; ADAT, adenosine deaminase that acts on tRNA.

sine deamination by members of the ADAR family, we have undertaken a series of studies involving hADAR2a using the R/G site of the human GluR-B pre-mRNA as a model system. We have characterized this minimal system using kinetic, gel shift, and cross-linking analyses. Interestingly, we have found that a ternary complex involving the association of two ADAR2 monomers with the RNA substrate is responsible for editing activity. Furthermore, the monomers can be cross-linked to one another in an RNA-dependent fashion, suggesting intimate association on the substrate. An analysis of RNA binding and R/G site editing using two deletion mutants indicates that association of ADAR2 with the editing site in a 1:1 complex is insufficient to yield efficient A to I editing. These results combined with previous observations suggest that editing of pre-mRNAs by members of the ADAR class of enzymes is contingent on homo- or hetero-dimerization on the RNA substrate and that this requirement is central to the regulation of editing by these enzymes.

EXPERIMENTAL PROCEDURES

Enzyme Overexpression and Purification—hADAR2a, with an N-terminal FLAG epitope and C-terminal His_6 tag, was expressed and purified from a recombinant *Pichia pastoris* clone (a generous gift of W. Keller, University of Basel) as described previously (27). Following chromatography on a Macro-Prep High Q column (Bio-Rad), fractions containing ADAR2, as identified by Western analysis with anti-FLAG antibody (Sigma), were pooled, mixed for 30 min with 2 ml of Ni^{2+} agarose (Qiagen) that had been pre-equilibrated with buffer C (200 mM KCl, 10% glycerol, 50 mM Tris (pH 7.9), 5 mM β-mercaptoethanol, 0.01% Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride, 0.7 μg/ml pepstatin, 0.4 μg/ml leupeptin), and poured into a 5-ml column. The column was washed with 10 ml of buffer C containing 20 mM imidazole, and the protein was eluted with buffer C containing 250 mM imidazole.

The fractions from the nickel nitrilotriacetic acid column containing ADAR2 were dialyzed against buffer E (10 mM Hepes pH 8, 100 mM KCl, 1 M NaCl, 0.5 M $(NH_4)_2SO_4$, 0.5 M DTT) and loaded onto a 1-ml Hi-Trap Phenyl column (Amersham Biosciences). The phenyl column was washed with 10-column volumes of buffer E and eluted with a 15-ml gradient from buffer E to buffer F (10 mM Hepes pH 8, 100 mM KCl, 0.5 mM DTT). The fractions containing ADAR2 were dialyzed into editing buffer (20 mM Hepes pH 8, 100 mM KCl, 0.5 mM DTT, 0.01% Nonidet P-40, 20% glycerol) and stored at −80 °C. Protein was quantified by comparing Sypro-Red (Molecular Dynamics) intensity to known amounts of bovine serum albumin using SDS-PAGE.

Preparation of RNA—R/G editing substrates of various lengths were synthesized by T7 RNA polymerase transcription using templates generated by PCR from a substrate cloned into pBS (Stratagene). Mobility shift and editing assays were performed using R/G substrates internally labeled with a single ^{32}P label 5′ to the edited adenosine. The internal label was introduced by the ligation of upstream and ^{32}P-end-labeled, ApG-primed, downstream T7 transcription products in the presence of a bridging oligonucleotide using T4 DNA ligase (300 pmol of 5′-RNA, 100 pmol of 3′-RNA, 100-pmol DNA bridge) (29). All RNAs were purified by denaturing PAGE (15%, 19:1) before use and stored in double distilled water at −80 °C.

Editing Reactions—Editing reactions containing 20 mM Hepes pH 8.0, 100 mM KCl, 0.5 mM DTT, 20% glycerol, 0.01% Nonidet P-40, RNasin (1 unit/μl, Roche Molecular Biochemicals) and varying amounts of ADAR2 were initiated by the addition of substrate RNA. A fixed RNA concentration of 0.5 nM was used for the protein titration experiments, and a fixed concentration of 50 nM ADAR2 was used in the RNA titration experiments. The substrate RNA was denatured at 80 °C for 2 min and allowed to renature at 30 °C for 10 min just prior to addition to the editing reactions. At each time point, an aliquot was removed and quenched by its addition to stop solution (6% SDS, 250 mM Tris, pH 8.0, 25 mM EDTA) at 80 °C. Each sample was diluted to 200 μl, made 0.3 M in NaOAc, and extracted once with phenol/chloroform/isoamyl alcohol and once with chloroform, and ethanol was precipitated. The pellet was resuspended in 10 μl of double distilled water and digested with Nuclease P1 (0.5 units/reaction; Amersham Biosciences), and the products were resolved by thin layer chromatography (saturated $(NH_4)_2SO_4$:0.1 M NaOAc:isopropanol; 79:19:2, v/v) (10) using cellulose-polyethyleneimine chromatography plates (J. T. Baker). The TLC plates were exposed to a Molecular Dynamics Phosphor screen which was scanned using a Molecular Dynamics Storm 860 PhosphorImager. The extent of editing was quantified using ImageQuant 5.0 software. Data from three separate experiments were averaged, and the extent of editing *versus* time was plotted; initial rates were determined by calculating the slope of the best fit through the first few time points. Two separate calculations of the rate constant for ESE formation (using data from experiments in which either substrate or enzyme concentration was varied) were performed using Dynafit (BioKin Ltd.).

Gel Mobility Shift Assays—Reactions were prepared as described above for editing assays and incubated for 10 min at 30 °C before being resolved by native 5% (89:1) PAGE. The gels were run at room temperature in TBE buffer (0.089 M Tris-base, 0.089 M boric acid, 0.2 mM EDTA) for 1.5 h at 150 volts. Gels were dried and exposed to a Molecular Dynamics Phosphor screen that was scanned on a Molecular Dynamics Storm 860 PhosphorImager. Data were analyzed using TotalLab V1.11 software (Phoretix).

Dissociation constants were determined from data obtained in at least three separate experiments by quantifying the fractions of free RNA (θ), RNA bound in the ES complex 1 (θ_1), and RNA bound in the ESE complex 2 (θ_2), and simultaneously fitting equations 2, 3, and 4 (Microsoft EXCEL). Because the gel shift analysis does not differentiate between substrate and product and the reaction proceeds quickly, it is assumed that ES is equivalent to EP and that ESE is equivalent to EPE in the equations below. Because ADAR2 is in large excess of the RNA substrate, E is approximately equal to the total amount of enzyme added to each reaction.

$$E + S \underset{}{\overset{K_{d1}}{\rightleftharpoons}} ES \quad ES + S \overset{K_{d2}}{\rightleftharpoons} ESE \qquad \text{(Eq. 1)}$$

$$\theta = \frac{K_{d1}K_{d2}[S]_T}{K_{d1}K_{d2} + K_{d2}[E] + [E]^2} \qquad \text{(Eq. 2)}$$

$$\theta_1 = \frac{K_{d2}[S]_T}{[E] + K_{d2} + K_{d1}K_{d2}/[E]} \qquad \text{(Eq. 3)}$$

$$\theta_2 = \frac{[S]_T}{1 + K_{d2}/[E] + K_{d1}K_{d2}/[E]^2} \qquad \text{(Eq. 4)}$$

Protein-Protein Cross-linking—Binding reactions (10 μl) containing 300 nM ADAR2, 20 mM Hepes pH 8.0, 100 mM KCl, and varying amounts of R/G RNA were set up on ice. The reactions were allowed to equilibrate for 15 min before cross-linking was initiated by the addition 2 μl of 45 mM dimethyl suberimidate hydrochloride (DMS; Pierce). After 4 h at room temperature the reactions were quenched by the addition of 2 μl of 1 M glycine. Samples were separated by SDS-PAGE (8%; 29:1). The gel was then transferred to polyvinylidene fluoride membrane (Bio-Rad). Western blotting was performed using anti-FLAG antibody, and products were visualized using the phototope-horseradish peroxidase detection kit (Cell Signaling Technology).

RESULTS

Adenosine to Inosine Editing in Model R/G RNAs—Recombinant ADAR2 was expressed and purified to homogeneity from a *P. pastoris* expression system yielding ~100 μg of protein per liter of culture. Using this protein we were able to demonstrate high levels of A to I conversion, using an inosine-specific cleavage assay (29), in a 500-nucleotide RNA containing the Q/R editing site of the human GluR-B pre-mRNA and a 78-nucleotide RNA containing the R/G editing site of the same pre-mRNA (data not shown). We focused further studies of ADAR2 mediated editing on RNAs based on the R/G site because it is smaller and simpler in structure. The strength of ADAR2-RNA interactions was measured by gel mobility shift and the efficiency of A to I conversion was measured using a nuclease/TLC assay (Fig. 1*B*) with RNA internally labeled adjacent to the target adenosine (29, 30).

Gel Mobility Analysis of ADAR2-RNA Interaction—It has been shown previously that ADAR2 can form two complexes on an RNA substrate (31), but it has been unclear which of these was associated with catalysis of editing. In our studies of ADAR2 using the 78-nucleotide R/G substrate, we also observed the formation of two complexes. We performed gel mobility shift experiments and editing reactions under identical

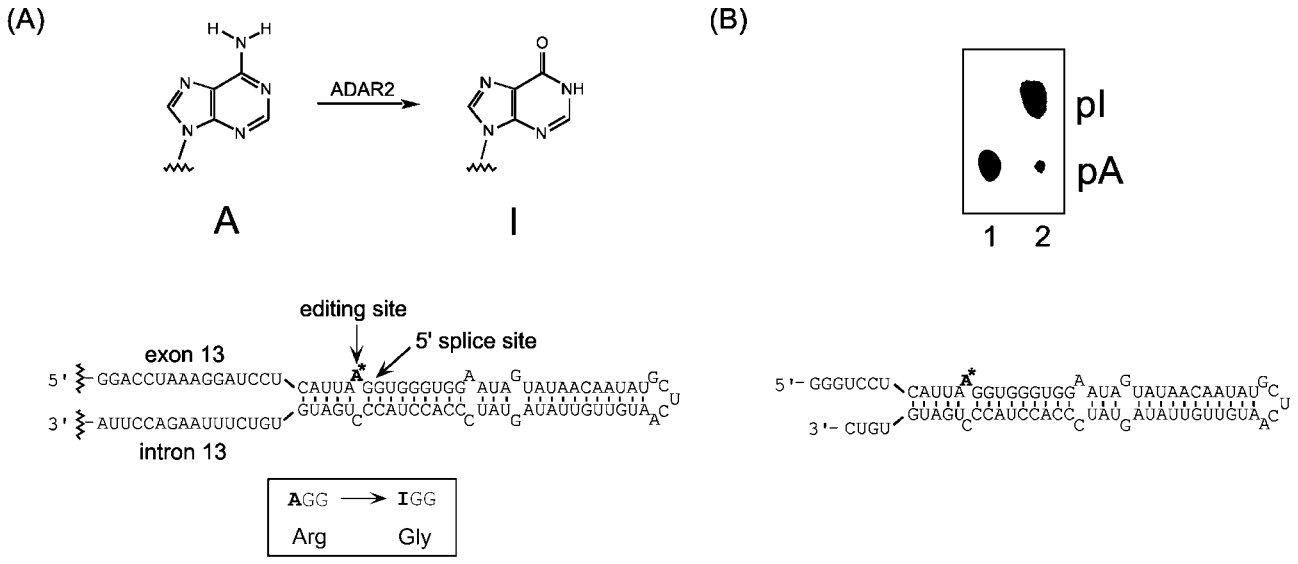

FIG. 1. **Editing at the R/G site of GluR-B pre-mRNA.** *Panel A*, ADAR2 catalyzes the conversion of adenosine (*A*) to inosine (*I*) within pre-mRNA substrates (*top*). RNA editing at the R/G site of the GluR-B pre-mRNA takes place within the context of a double-stranded RNA formed between nucleotides immediately proximal to the editing site and nucleotides downstream of intron 13 (*bottom*). The deamination of the indicated adenosine to inosine results in an arginine to glycine codon change in the mature mRNA (*inset*). *Panel B*, nuclease/TLC assay for editing at the R/G site catalyzed by recombinant ADAR2 using a 78-nucleotide RNA containing the R/G site internally labeled with ^{32}P 5′ to the editing site: *lane 1*, unedited RNA; *lane 2*, RNA in the presence of ADAR2.

conditions to investigate which complex was responsible for ADAR2 activity. Using a native gel analysis, all of the RNA probe could be shifted into an RNA-protein complex (ES; Fig. 2*A*) at low concentrations of protein and this in turn could be shifted into a higher order complex (ESE; Fig. 2*A*). The equilibrium dissociation constants for these two complexes were determined to be 4 and 21 nM, respectively. The total amount of editing observed with low and high concentrations of ADAR2 was similar over long time courses (24 h; ~85%). This result suggests that formation of the ESE complex does not inhibit R/G site editing. To investigate the mechanism of editing more closely, we carried out a detailed kinetic analysis of the reaction.

Kinetics of R/G Site Editing—We performed a kinetic analysis of the R/G editing event by determining initial rates of editing under conditions in which either substrate or ADAR2 concentration was varied. Intriguingly, we were unable to observe substrate saturation of the editing reaction. Under conditions in which the concentration of ADAR2 was held constant and substrate concentration was increased, the initial rate increased until the substrate concentration was roughly half of the protein concentration, and then it decreased (Fig. 3*A*). This observed inhibition is consistent with a mechanistic scheme in which formation of the ES complex is not sufficient for editing. Instead, efficient editing requires formation of the ESE complex; excess substrate sequesters ADAR2 in the inactive ES complex. If the ES complex was competent for editing, one would expect saturation at increased substrate concentrations (if the conversion of ES to EP was rate-limiting) or a linear dependence of rate on substrate concentration (if formation of ES was rate-limiting); neither was observed, consistent with the formation of ESE being required for editing.

In order to investigate the editing reaction further, we held the RNA concentration constant and varied the concentration of ADAR2. The initial rate increased over the entire range of enzyme concentrations tested, and a plot of initial rate *versus* enzyme concentration was linear (Fig. 3*B*). This suggests that the concentration of ESE in the reaction does not reach equilibrium before it is converted to product and that the rate-determining step of editing involves enzyme association with the ES complex.

RNA-dependent Cross-linking of ADAR2—We investigated the nature of the higher order RNA-ADAR2 complex by performing cross-linking experiments with the bifunctional amine-reactive reagent DMS. ADAR2 was incubated with various concentrations of R/G site RNA followed by cross-linking with DMS and analysis by SDS-PAGE and Western blotting. These experiments showed the formation of an RNA-dependent protein-protein cross-link with a molecular weight consistent with that of an ADAR2 dimer (Fig. 4). The cross-link was not sensitive to nuclease P1 treatment after cross-linking, indicating that it represents a protein-protein interaction (data not shown). The efficiency of cross-linking was dependent on RNA concentration (Fig. 4, *lanes 4–6*); the observed cross-link diminished at the highest RNA concentrations tested consistent with ADAR2 being sequestered in the 1:1 ES complex.

Substrate Deletion Analysis—RNA substrates with specific deletions were synthesized to determine which parts of the 78-nucleotide substrate were necessary for both binding and editing by ADAR2. A 58-nucleotide RNA substrate was synthesized that had a region distal to the hairpin loop removed and placed the target adenosine at the 5′-end of the substrate, and a 54-nucleotide RNA substrate was synthesized that had a region proximal to the hairpin loop removed (Fig. 5). Binding studies (Fig. 5*A*) revealed that the 58-nucleotide RNA could form two complexes with ADAR2, although the affinity of the protein for this RNA was ~7-fold less than that observed for the formation of complexes with the 78-nucleotide RNA. Only one complex was formed upon incubation of ADAR2 with the 54-nucleotide RNA; the dissociation constant for formation of this complex was ~18-fold greater than that observed for formation of the analogous complex on the 78-nucleotide substrate. Initial editing rates of both the 58- and 54-nucleotide RNAs, determined using ADAR2 concentrations that shifted 50% of the RNAs into the ES complex, were ~100-fold slower than those observed using the 78-nucleotide RNA (Fig. 5*B*). These results suggest that portions of both deleted regions are important for binding of the R/G site by ADAR2 and confirm that formation of the ES complex involves RNA binding in the immediate vicinity of the editing site; furthermore, a substrate severely compromised in its ability to form the ESE complex is edited inefficiently.

DISCUSSION

We have carried out a detailed kinetic study of RNA editing by a member of the ADAR enzyme class using an RNA that closely resembles the R/G editing site in the GluR-B pre-mRNA. Substrate inhibition of editing, as determined by measurement of initial rates, is consistent with a kinetic model (Scheme A) in which the formation of a ternary enzyme-substrate complex (ESE) is required for efficient editing. As substrate concentration increases, free enzyme is sequestered in the ES complex and is unavailable to form the active ESE complex. Under this scheme,

$$E + S \underset{}{\overset{K_S}{\rightleftharpoons}} ES \xrightarrow{k_1} ESE \xrightarrow{fast} EPE$$

SCHEME A

$$V_0 = \frac{k_1[E]^2[S]_T}{K_S + [E]} \qquad \text{(Eq. 5)}$$

the linear dependence of initial rate on enzyme concentration suggests that ES rapidly reaches equilibrium and that formation of the ESE complex is rate-limiting. When the concentration of enzyme is much higher than substrate, the initial rate (V_o) is given by Equation 5; the linear dependence of initial rate on total enzyme concentration is clear under conditions where [E] is much greater than K_s. Finally, we found a strong correlation between our kinetic analysis and Scheme A; we calculated the rate constant (k_1) for formation of ESE under conditions of excess substrate or excess enzyme and obtained very similar values of 0.20 ± 0.04 pM^{-1}s^{-1} and 0.21 ± 0.01 pM^{-1}s^{-1}, respectively.

It is possible that the association of a second ADAR2 protein with the RNA is independent of the first binding event and that the second event represents binding of the target adenosine to the enzyme active site with no participation of the other monomer. Several pieces of evidence argue against these suggestions. First, chemical and enzymatic footprinting of both ADAR2 complexes have shown that the first binding event corresponds to protection of the editing site with an extended footprint observed at higher protein concentrations (31). Second, we deleted distal regions of the R/G substrate, preserving the sequence proximal to the editing site, and observed a dramatic reduction in editing efficiency. Thus, initial binding of ADAR2 corresponds to protein-RNA association in the vicinity of the editing site, but the editing reaction itself requires association of a second ADAR2 monomer with the RNA substrate. It is unclear whether the chemistry of deamination is associated with the active site of the first or second monomer.

Gel filtration and glycerol gradient sedimentation experiments performed with another member of the ADAR family of enzymes, ADAR1, indicated that ADAR1 exists as a monomer in solution (32). Hough and Bass (33) reported that partially purified *Xenopus* ADAR1 formed complexes of ~215 kDa

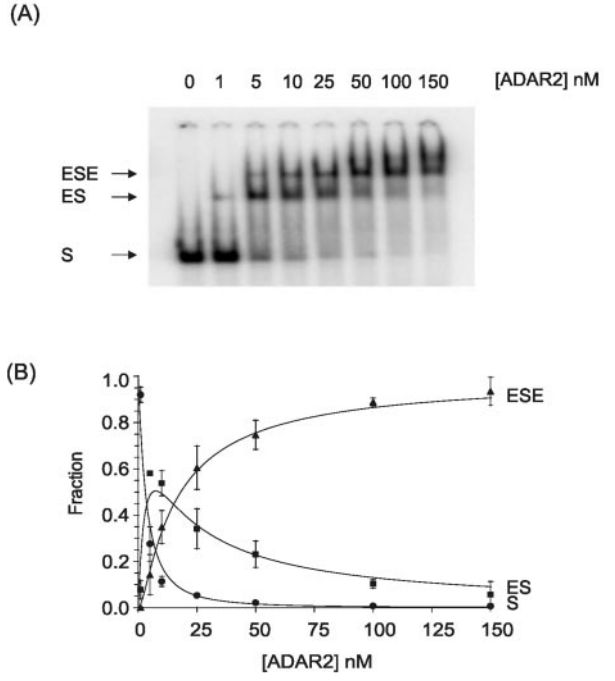

FIG. 2. **Native mobility shift analysis.** *A*, native mobility shift PAGE analysis of ADAR2 interaction with the 78-nucleotide R/G RNA showing the formation of two complexes under editing conditions. Reactions were incubated for 10 min at 30 °C and loaded directly onto 5% native gels (89:1). ADAR2 concentrations in *lanes 1–8* are 0, 1, 5, 10, 25, 50, 100, and 150 nM. The RNA concentration was kept constant at 0.5 nM. *B*, graphical representation of the native mobility shift PAGE analysis. Plot shows the fraction of species at differing ADAR2 concentrations (*filled circles*, S; *filled boxes*, ES; *filled triangles*, ESE). Dissociation constants were determined as described under "Experimental Procedures." Data points are the average of three independent experiments with the uncertainty represented by 1 S.D. The indicated fit is to a binding scheme in which ESE formation is contingent on initial binding of ADAR2 to the RNA to form ES (see "Experimental Procedures").

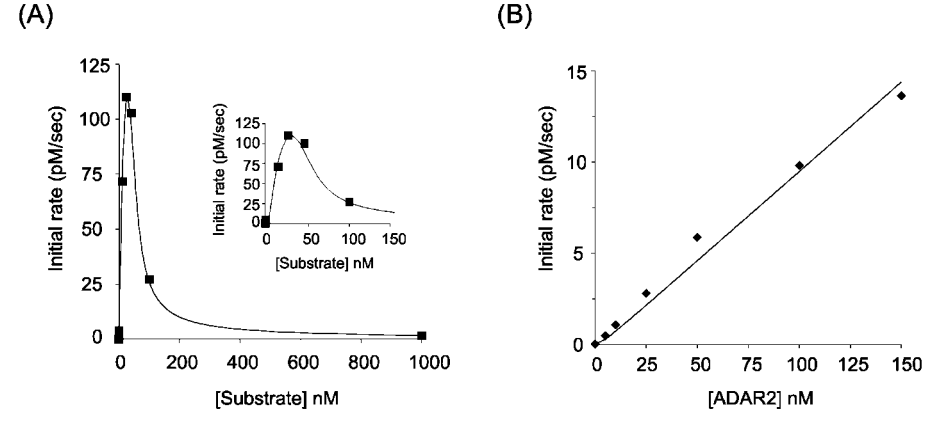

FIG. 3. **Kinetics of ADAR2 RNA editing.** *A*, initial rate of editing as a function of substrate concentration. Increasing amounts of an internally labeled 78-nucleotide R/G RNA were added to editing reactions containing 50 nM ADAR2, and the amount of product formed at various times was determined. Initial rates were calculated by measuring the slope through the first few points of the plot of product *versus* time (data not shown). The *inset* represents an expansion of the initial portion of the substrate titration. *B*, initial rate of editing as a function of substrate concentration. Initial rates were determined by calculating the slope through the first few points of the plot of product *versus* time.

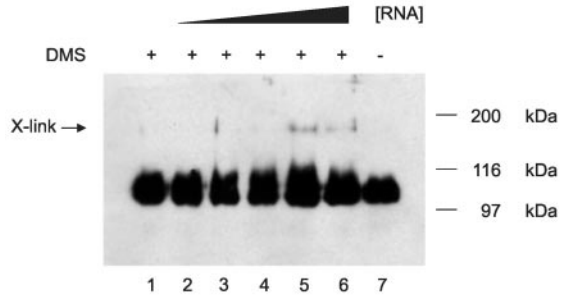

FIG. 4. **RNA-dependent cross-linking of ADAR2.** The enzyme (300 nM) was mixed with varying amounts of unlabeled R/G RNA and incubated with the protein-protein cross-linker DMS. Samples were run on SDS-PAGE and analyzed by Western blotting with anti-FLAG antibody. RNA concentrations (nM) for *lanes 1–7*: 0, 5, 50, 100, 500, 1000, and 0.

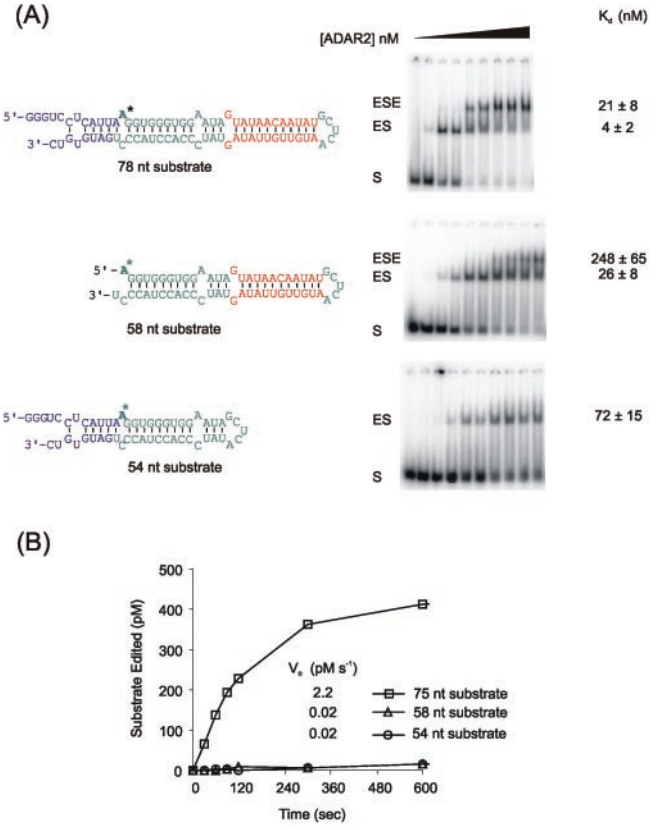

FIG. 5. **Substrate deletion analysis.** *A*, band mobility shift analysis of substrates based on the GluR-B R/G editing site. RNAs in which a region either proximal or distal to the loop was deleted were compared with the 78-nucleotide RNA using gel mobility shift analysis. Predicted secondary structures of the RNAs are shown to the *left* of the corresponding gel. Mobility shift assays were carried out with ADAR2 concentrations increasing from 0 to 180 nM. *B*, initial editing rates of R/G site-derived RNAs *versus* the amount of editing as a function of time. Editing reactions were carried out under conditions in which half of the substrate was bound in the ES complex at equilibrium (ADAR2 concentration is equal to K_{d1}).

(consistent with dimer formation) but behaved as monomers when fully purified. The presence of RNA in the partially purified protein preparation would explain these results. Cross-linking experiments with the bifunctional reagent DMS show that there is an intimate association between ADAR2 monomers on the R/G RNA and also that this association is RNA-dependent (Fig. 4).

Comparison of the ADAR class of enzymes with related enzymes suggests that dimerization may be conserved as a functional requirement. Relatively little is known regarding the catalysis of A to I deamination within the context of double-stranded RNA, but it has been assumed that the chemical mechanism is related to that of the adenosine and cytidine nucleotide deaminases (34). In both classes of enzymes an active site zinc, coordinated by cysteine or histidine residues, serves to activate water for attack on the purine or pyrimidine base. Sequence comparisons show relatively little homology between the ADAR family active sites and those of other deaminases with the exception of the presence, identity, and relative arrangement of the putative zinc coordinating signature residues; mutagenesis of these residues in ADAR1 supports their roles in catalysis. Interestingly, the identity and arrangement of the signature residues in the ADAR family is more reminiscent of the cytidine deaminases than of the adenosine deaminases. In the cytidine deaminases, zinc is coordinated by His, Cys, and Cys whereas coordination in the adenosine deaminases involves three Cys residues. Additionally, both the cytidine deaminases and ADAR enzymes contain the His-Ala-Glu triad; the acid residue likely acts as a proton donor and acceptor during the course of catalysis. The adenosine deaminases function as monomers, but many cytidine deaminases including *Escherichia coli* cytidine deaminase (35), the T4 bacteriophage cytidine deaminase (36), and the apoB mRNA editing deaminase (37) function as dimeric or polymeric species. The x-ray structure of the *E. coli* cytidine deaminase dimer shows that the active site is partially formed by contributions from each monomer (35).

Recently, a class of tRNA-specific adenosine deaminases has been characterized by Gerber and Keller (38, 39). Members of the ADAT (adenosine deaminases that act on tRNA) enzyme family share sequence homology in editing domains with both the cytidine deaminases and members of the ADAR family; it has been suggested that ADAT enzymes evolved into the ADAR class by the acquisition of double-stranded RNA binding domains. The fact that tad2p (ADAT2) and tad3p (ADAT3) form heterodimers supports the observation that ADAR2 functions as a dimeric species.

The enzymatic activity of the RNA-dependent protein kinase PKR has been shown to depend on dimer formation, although the mechanism of dimerization is the subject of controversy (40). The sequence of PKR includes two N-terminal double-stranded RNA binding domains and a C-terminal catalytic domain and is thus reminiscent of the domain structure of ADAR2. Future experiments with ADAR2 will involve the localization of the dimerization interface within the protein sequence.

Dimerization of ADAR2 on RNA may influence the editing activity of the enzyme in several ways. For example, the binding of a second ADAR2 monomer on its substrate may effect a conformational change in one or both monomers that is required for editing. Results from tryptophan quenching experiments are consistent with ADAR2 undergoing a conformational change upon the binding of RNA (41). An additional piece of evidence supporting the need for a conformational change in ADAR2 before editing is the observation that a potent inhibitor of adenosine deaminase, coformycin (K_d ~10^{-15} M), does not inhibit the activity of members of the ADAR family (15). Recently, Nishikura and coworkers (42) have demonstrated that the mixing of ADAR3 with either ADAR1 or ADAR2 attenuated the editing activity of the latter two enzymes, whereas mixing ADAR1 and ADAR2 lowered the site selectivity of the A to I editing activity; in light of the data reported here, we interpret these observations to indicate that members of the ADAR family can form hetero- as well as homodimers on RNA substrates. The requirement of ADAR dimerization for editing as well as

the modulation of activity by heterodimer formation are no doubt intimately involved in the regulation of the activity of these enzymes, including the site-specificity of editing.

Acknowledgments—We thank W. Keller for providing the *P. pastoris* hADAR2a expression construct, H. Shaikh, A. Franko, and K. Chilibeck for contributions to this work, and A. Gerber, D. Woolley, S. Chaulk, O. Kent, R. Zamel, R. Collins, and members of the Collins lab for helpful discussions.

REFERENCES

1. Bass, B. (1992) in *The RNA World* (Gesteland, R. F., and Atkins, J. F., eds), pp. 383–418, Cold Spring Harbor Press, Plainsview, NY
2. Scott, J. (1995) in *Pre-mRNA Processing* (Lamond, A. I., ed), pp. 157–161, R. G. Landis, Austin, TX
3. Rueter, S. M., and Emeson, R. B. (1998) in *Modification and Editing of RNA* (Grosjean, H., and Benne, R., eds), pp. 343–361, ASM Press, Washington, DC
4. Polson, A. G., Bass, B. L., and Casey J. L. (1996) *Nature* **380,** 454–456
5. Burns, C. M., Chu, H., Rueter, S. M., Hutchinson, L. K., Canton, H., Sanders-Bush, E., and Emeson, R. B. (1997) *Nature* **387,** 303–308
6. Wang, Q., Khillan, J., Gadue, P., and Nishikura, K. (2000) *Science* **290,** 1765–1768
7. Sommer, B., Kohler, M., Sprengel, R., and Seeburg, P. H. (1991) *Cell* **67,** 11–19
8. Lomeli, H., Mosbacher, J., Melcher, T., Hoger, T., Geiger, J. R. P., Kuner, T., Monyer, H., Higuchi, M., Bach, A., and Seeburg, P. H. (1994) *Science* **266,** 1709–1713
9. Rueter, S. M., Burns, C. M., Coode, S. A., Mookherjee, P., and Emeson, R. B. (1995) *Science* **267,** 1491–1494
10. Yang, J. H., Sklar, P., Axel, R., and Maniatis, T. (1995) *Nature* **374,** 77–81
11. Melcher, T., Maas, S., Higuchi, M., Keller, W., and Seeburg, P. H. (1995) *J. Biol. Chem.* **270,** 8566–8570
12. Higuchi, M., Single, F. N., Kohler, M., Sommer, B., Sprengel, R., and Seeburg, P. H. (1993) *Cell* **75,** 1361–1370
13. Egebjerg, J., Kukekov, V., and Heinemann, S. F. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 10270–10274
14. Brusa, R., Zimmermann, F., Koh, D. S., Feldmeyer, D., Gass, P., Seeburg, P. H., and Sprengel, R. (1995) *Science* **270,** 1677–1680
15. Kim, U., Wang, Y., Sanford, T., Zeng, Y., and Nishikura, K. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 11457–11461
16. O'Connell, M. A., Krause, S., Higuchi, M., Hsuan, J. J., Totty, N. F., Jenny, A., and Keller, W. (1995) *Mol. Cell. Biol.* **15,** 1389–1397
17. Hough, R. F., and Bass, B. L. (1997) *RNA* **3,** 356–370
18. Liu, Y., George, C. X., Patterson, J. B., and Samuel, C. E. (1997) *J. Biol. Chem.* **272,** 4419–4428
19. Bass, B. L., and Weintraub, H. (1987) *Cell* **48,** 607–613
20. Rebagliati, M. R., and Melton, D. A. (1987) *Cell* **48,** 599–605
21. Bass, B. L., and Weintraub, H. (1988) *Cell* **55,** 1089–1098
22. Polson, A. G., Crain, P. F., Pomerantz, S. C., McCloskey, J. A., and Bass, B. L. (1991) *Biochemistry* **30,** 11507–11514
23. Hurst, S. R, Hough, R. F., Aruscavage, P. J., and Bass, B. L. (1995) *RNA* **1,** 1051–1060
24. Maas, S., Melcher, T., Herb, A., Seeburg, P. H., Keller, W., Krause, S., Higuchi, M., and O'Connell, M. A. (1996) *J. Biol. Chem.* **271,** 12221–12226
25. Herb, A. M., Higuchi, M., Sprengel, R., and Seeburg, P. H. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 1875–1880
26. Melcher, T., Maas, S., Herb, A., Sprengel, R., Seeburg, P. H., and Higuchi, M. (1996) *Nature* **379,** 460–464
27. Gerber, A., O'Connell, M. A., and Keller, W. (1997) *RNA* **3,** 453–463
28. Yang, J. H., Sklar, P., Axel, R., and Maniatis, T. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 4354–4359
29. Morse, D. P., and Bass, B. L. (1997) *Biochemistry* **36,** 8429–8434
30. Moore, M. J., and Sharp, P. A. (1992) *Science* **256,** 992–997
31. Ohman, M., Kallman, A. M., and Bass, B. L. (2000) *RNA* **6,** 687–697
32. Kim, U., Garner, T. L., Sanford, T., Spiecher, D., Murray, J. M., and Nishakura, K. (1994) *J. Biol. Chem.* **269,** 13480–13489
33. Hough, R. F., and Bass, B. L. (1994) *J. Biol. Chem.* **269,** 9933–9939
34. Carter, C. W. (1995) *Biochemie* **77,** 92–98
35. Betts, L., Xiang, S., Short, S. A., Wolfenden, R., and Carter, C. W., Jr. (1994) *J. Mol. Biol.* **235,** 635–656
36. Maley, G. F., Duceman, B. W., Wang, A. M., Martinez, J., and Maley, F. (1990) *J. Biol. Chem.* **265,** 47–51
37. Lau, P. P., Zhu, H. J., Baldini, A., Charnsangavej, C., and Chan, L. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 8522–8526
38. Gerber, A. P., and Keller, W. (1999) *Science* **286,** 1146–1149
39. Gerber, A. P., and Keller, W. (2001) *Trends Biochem. Sci.* 376–384
40. Wu, S., and Kaufman, R. J. (1997) *J. Biol. Chem.* **272,** 1291–1296
41. Yi-Brunozzi, H. Y., Stephens, O. M., and Beal P. A. (2001) *J. Biol. Chem.* **276,** 37827–37833
42. Chen, C. X., Cho, D. S., Wang, Q., Lai, F., Carter, K. C., and Nishikura, K. (2000) *RNA* **6,** 755–767