Cleavage of dsRNAs hyper-edited by ADARs occurs at preferred editing sites

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ABSTRACT

Long double-stranded RNAs (dsRNAs) may undergo covalent modification (hyper-editing) by adenosine deaminases that act on RNA (ADARs), whereby up to 50–60% of adenosine residues are converted to inosine. Previously, we have described a ribonuclease activity in various cell extracts that specifically targets dsRNAs hyper-edited by ADARs. Such a ribonuclease may play an important role in viral defense, or may alternatively be involved in down-regulation of other RNA duplexes. Cleavage of hyper-edited dsRNA occurs within sequences containing multiple IU pairs but not in duplexes that contain either isosteric GU pairs or Watson–Crick base pairs. Here, we describe experiments aimed at further characterizing cleavage of hyper-edited dsRNA. Using various inosine-containing dsRNAs we show that cleavage occurs preferentially at a site containing both IU and UI pairs, and that inclusion of even a single GU pair inhibits cleavage. We also show that cleavage occurs on both strands within a single dsRNA molecule and requires a 2'-OH group. Strikingly, we show that ADAR1, ADAR2 or dADAR all preferentially generate the preferred cleavage site when hyper-editing a long dsRNA.

INTRODUCTION

Eukaryotic cells have a number of sensitive defense mechanisms that recognize and respond to the presence of long double-stranded RNA (dsRNA) molecules (1). Such molecules may arise through antisense transcription, or more commonly may indicate the presence of viruses or other invading nucleic acid molecules. One general antiviral mechanism employed by the cell in response to dsRNA involves the induction of PKR (2) and oligoadenylate synthetase/RNase L (3,4). Alternatively, dsRNA may be utilized in the RNA interference (RNAi) pathway to silence the cognate gene (5,6). On the other hand, adenosine deaminases that act on RNA (ADARs) may catalyze the covalent modification of long dsRNA molecules (7). Modification by ADARs and RNAi appear to be mutually antagonistic processes (8–12).

ADARs constitute a family of enzymes that exist throughout the metazoa, including mammals [ADAR1 and ADAR2 (7)], frogs, worms and flies [dADAR (13)]. Conversion of adenosine (A) residues to inosine (I) residues within dsRNA by ADARs constitutes one type of RNA editing. Inosine differs from guanosine only by the presence of an exocyclic amine group, and preferentially pairs with cytosine residues. As inosine is decoded by the translation machinery as guanosine, selective editing by ADARs has the potential to change the coding capacity of an mRNA (7). Alternatively, ADARs may catalyze hyper-editing within long dsRNA molecules, which results in up to 50% of the adenosine residues being converted to inosine (14,15). Hyper-editing has the potential to alter not only the sequence of a dsRNA molecule, but also its structure as IU and UI pairs are less stable than the corresponding Watson–Crick AU and UA pairs (16). The presence of both IU and UI pairs may result in localized distortions of the A-form RNA duplex.

Several pathways have been identified in cells that may determine the fate of hyper-edited dsRNA. Hyper-edited dsRNA may be retained in the nucleus by a protein complex that comprises p54[nrb], PSF and matrin 3 (17). Moreover, it has recently been proposed that hyper-edited dsRNA binds to vigilins in the nucleus and may be involved in the formation of heterochromatin (18). Hyper-editing of dsRNA by ADARs has alternatively been proposed to form part of an antiviral mechanism whereby covalent modification may tag the dsRNA for subsequent disposal. Previously, we have identified a ribonuclease activity in various cell extracts (HeLa S100, *Xenopus laevis* oocyte extract) that specifically cleaves hyper-edited dsRNA (19). In contrast, dsRNAs containing only Watson–Crick base pairs or that contain GU pairs rather than IU pairs were not cleaved in either *Xenopus* oocyte or HeLa cell extracts. Cleavage of inosine-containing dsRNA occurs 5' of U residues within the sequence 5'-IIUI-3'/3'-UUIU-5' and

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leaves a 3′ phosphate (19). We have recently shown (12) that cleavage of hyper-edited dsRNAs involves Tudor Staphylo-coccal Nuclease (TSN), which was previously described as a component of the RNA-induced silencing complex necessary for RNAi (20). We showed that TSN binds specifically to dsRNAs containing multiple IU pairs, and that the addition of recombinant TSN to a limited amount of *Xenopus* extract caused an increase in cleavage of inosine-containing dsRNA. Moreover, specific inhibitors of TSN also inhibited cleavage. Nevertheless, it is likely that TSN constitutes only part of a protein complex necessary for cleavage of hyper-edited dsRNA (12). In addition to a potential role in viral defense, the TSN complex might target non-coding dsRNAs sequences that undergo hyper-editing by ADARs (10,21). While editing of such sequences is thought to prevent their entry into the RNAi pathway *per se*, removal from the cell via the TSN-mediated pathway would ensure their exclusion. Recent data showed that ADARs could edit pre-miRNA precursors (22); this observation raises the additional possibility that inappropriately edited miRNAs could be fed into a destruction pathway that specifically recognizes hyper-edited dsRNAs.

Here, we set out to further characterize the properties of hyper-edited dsRNAs that undergo cleavage in *Xenopus* oocyte extract. Cleavage of dsRNA substrates that contain a mixture of IU and GU pairs supports the previous observations that GU pairs are unable to substitute IU pairs and that multiple IU pairs are required for cleavage. We show that cleavage requires a 2′-OH residue, and that cleavage can occur on both strands within a single molecule. We use various substrates to show that cleavage occurs preferentially within a site

that contains both IU and UI pairs. Strikingly, editing of a long dsRNA (ΔKP) by ADAR2, ADAR1 or dADAR efficiently generates this cleavage site.

MATERIALS AND METHODS

RNA substrates

2′-protected RNA oligonucleotides were purchased from Dharmacon and deprotected before use according to the manufacturer's instructions. Sequences of synthetic dsRNAs are shown in Table 1. Double-stranded RNAs were prepared as described previously (19). RNA oligonucleotides were 5′ end-labeled with [γ-^{32}P]ATP (3000 Ci/mmol; Amersham) using T4 PNK (23). In each dsRNA only one strand of the duplex was labeled. ΔKP is a perfect dsRNA substrate (295 bp) that comprises spliced exons 2 and 3 of α-tropomyosin, generated by deletion between the KpnI and PvuII sites at the ends of the intron in construct pGC+DX (24). The sense RNA strand (296 nt) was synthesized using SP6 RNA polymerase (Strata-gene) following linearization of ΔKP with BamHI. The antisense RNA (329 nt) was synthesized using T7 RNA poly-merase from a PCR template generated from ΔKP (19). ΔKP dsRNA was prepared as described previously (19). The hairpin with a single internal label was prepared by RNA ligation (25). The sequences of the two RNA oligonucleotides to be ligated were 5′-ACUGGACAIIUICUCCGAGG (5′ RNA) and 5′-UACGCCUCGGAGUIUUUGUCCAGUAUC (3′ RNA), and the bridging DNA oligonucleotide sequence was 5′-AGGC-TCCGCATGGAGCCTCGTGGAC. The 3′ RNA was 5′ end-labeled with [γ-^{32}P]ATP to give a single label adjacent to the

Table 1. dsRNA substrates: sequences of the synthetic dsRNA oligonucleotides used in this study

Substrate	5′ Sequence 3′	dsRNA used to show that:
IIUI (wild type)	ACUGGACA**IIUI**CUCCGAGG UGACCUGU**UUIU**GAGGCUCC	GU pairs have a deleterious effect on cleavage (Figure 1)
GU (control)	ACUGGACA**GGUG**CUCCGAGG UGACCUGU**UUGU**GAGGCUCC	
IIUI/UUGU	ACUGGACA**IIUI**CUCCGAGG UGACCUGU**UUIU**GAGGCUCC	
GGUG/UUIU	ACUGGACA**GGUG**CUCCGAGG UGACCUGU**UUIU**GAGGCUCC	
IIUI-30	GAUGAACUGGACA**IIUI**CUCCGAGGCUCUC CUACUUGACCUGU**UUIU**GAGGCUCCGAGAG	Additional IU pairs increase cleavage efficiency (Figure 1D)
IIIUI-30e	GAUGAACUGGAC**IIIUI**CUCCGAGGCUCUC CUACUUGACCUG**UUIU**GAGGCUCCGAGAG	
dUT	ACUGGACA**IIdUI**CUCCGAGG UGACCUGU**UU IU**GAGGCUCC	2′-OH groups are important for cleavage (Figure 2)
dUB	ACUGGACA**I IUI**CUCCGAGG UGACCUGU**UdUIU**GAGGCUCC	
IIUI hairpin	ACUGGACA**IIUI**CUCCGAGG$\overset{*}{G}\overset{U}{}$A CUAUGACCUGU**UUIU**GAGGCUCC$_G$C	Cleavage of IIUI dsRNA occurs on both strands within a single molecule (Figure 3)
GU hairpin	ACUGGACA**GGUG**CUCCGAGG^{U}A CUAUGACCUGU**UUGU**GAGGCUCC$_G$C	
IIUI-pal	CUGGACA**IIUIUU**CUCCGAG GACCUGU**UUIUII**GAGGCUC	Cleavage occurs preferentially between the IU and UI pair in the sequence IIUI (Figures 4 and 5)
COMP	ACUGACA**UUUU**CCUGACA**IIUI**CCUGAGA UGACUGU**IIII**GGACUGU**UUIU**GGACUCU	

tetraloop sequence (underlined). Ligated RNAs were purified on a 16% (v/v) polyacrylamide gel before use (25).

Preparation of extracts and recombinant proteins

Xenopus laevis oocyte extracts were prepared according to a method described previously (26). The ADARs used in this study were the short form (p110) of human ADAR1 (27) (ADAR1), human ADAR2b (27) (ADAR2) and dADAR 3/4 (13) (dADAR). Recombinant ADARs were expressed in *Pichia pastoris*, as described previously (28).

RNA editing

ΔKP was edited using ADAR1, ADAR2 or dADAR according to a method described previously (28). Editing reactions resulted in ~30–45% of adenosine residues being converted to inosine, as determined by TLC. Edited RNAs were amplified using RT–PCR and cloned into pGEM-Teasy (Promega) as described previously (19). A total of 20–45 clones corresponding to editing by each enzyme were sequenced to analyze editing frequency at each position.

Ribonuclease assays

5′ end-labeled dsRNA substrates (50 fmol) were assayed in a 10 µl reaction that contained 10 mM HEPES-KOH, pH 7.9, 0.8 mM ATP, 1 µg tRNA and 10% *Xenopus* oocyte extract. Reactions were incubated at 25°C for 0–2 h and then treated with 6 µl Proteinase K (20 mg/ml) in a total volume of 100 µl 1× Proteinase K buffer for 30 min at 37°C. The RNA was extracted once with phenol/chloroform and recovered by precipitation with ethanol and carrier tRNA. The RNA pellets were dried, resuspended in formamide dyes and analyzed by denaturing PAGE (18% polyacrylamide gels). The gels were dried under vacuum and visualized by either autoradiography or using a Storm PhosphorImager. Quantification was additionally performed using a Storm PhosphorImager and Molecular Dynamics software. RNA melting experiments were carried out according to a previous method (29) where the assays were in 10 mM HEPES, pH 7.5, 100 mM NaCl.

RESULTS

Previously, we used short model dsRNA substrates to demonstrate that the presence of multiple IU pairs resulted in efficient cleavage of the dsRNA in *Xenopus laevis* oocyte extracts (12). Cleavage occurred efficiently within the sequence 5′-IIUI-3′/3′-UUIU-5′, which is referred to as the 'wild-type' sequence (IIUI, Table 1), and decreased as the number of IU pairs were reduced. Here, we describe a study aimed at further characterizing the properties of the cleavage site.
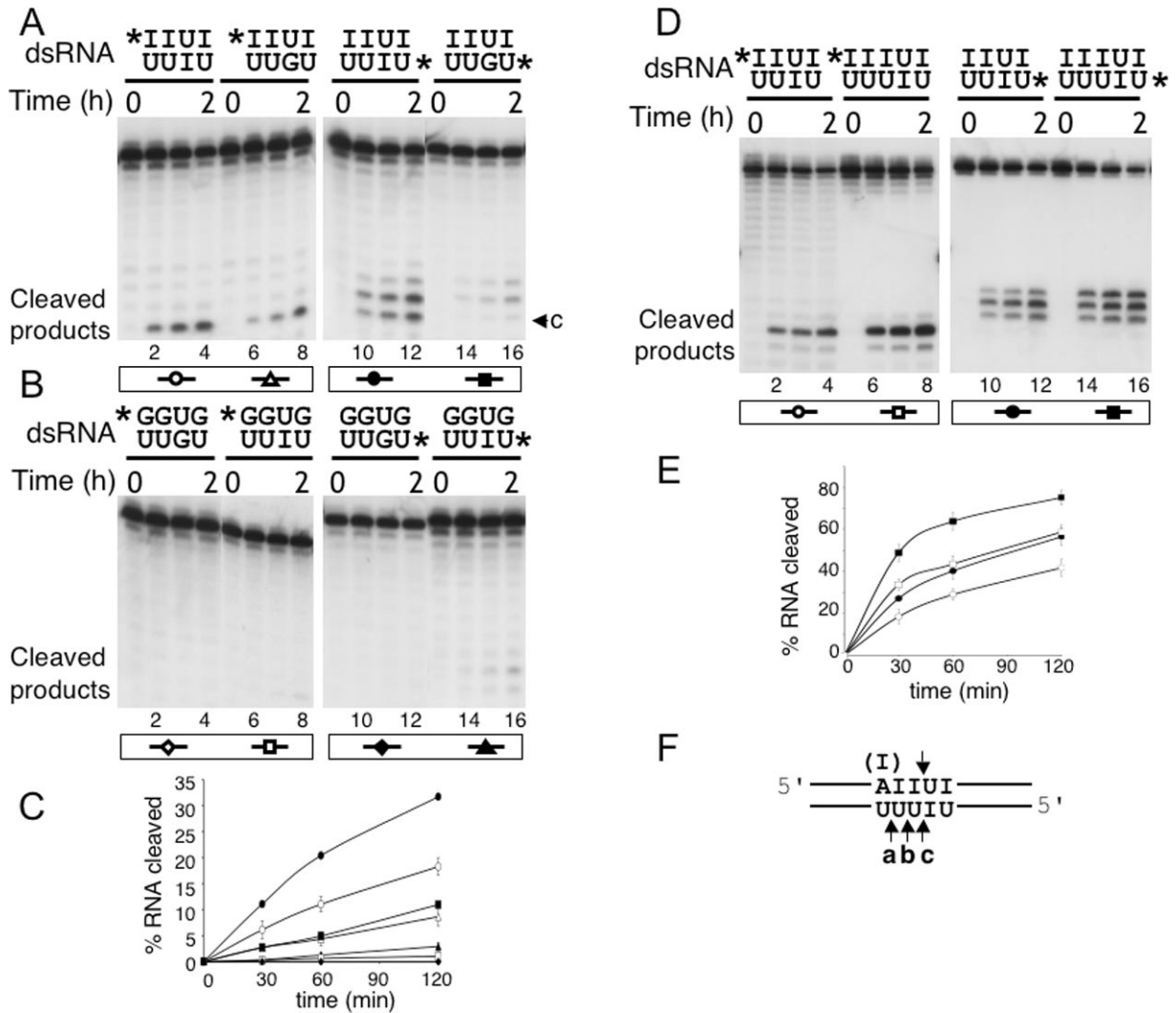
GU pairs have a deleterious effect on cleavage

While the IIUI dsRNA was cleaved efficiently in *Xenopus* oocyte extract, an equivalent control dsRNA that contained isosteric GU pairs (GU, Table 1) was stable (12,19). Synthetic RNA duplexes (20 bp) that contain a mixture of IU and GU pairs were now used to analyze cleavage in *Xenopus* oocyte extract (Table 1). The RNA duplexes were 5′ end-labeled on either the top or the bottom strand.

When the IIUI dsRNA labeled on the top strand was incubated in *Xenopus* extract, efficient cleavage occurred to give a single product (Figure 1A, lanes 1–4 and Figure 1C). When one UG pair was introduced into the IIUI dsRNA to give the substrate IIUI/UUGU (Table 1), cleavage on the top strand was reduced by at least 50% (Figure 1A, lanes 5–8 and Figure 1C). Efficient cleavage was observed when the wild-type IIUI dsRNA was labeled on the bottom strand and yielded several products (Figure 1A, lanes 9–12 and Figure 1C). However, the inclusion of a GU pair (IIUI/UUGU) reduced cleavage on the bottom strand by at least 70% (Figure 1A, lanes 13–16 and Figure 1C). Quantification of the individual cleaved products revealed that there was a disproportionately large reduction in cleavage at position 'c' (Figure 1F), which is adjacent to the UG pair (data not shown). These data show that the presence of even one GU pair in the context of multiple IU pairs was incompatible with efficient cleavage. As GU and IU pairs are isosteric, a single IU to GU substitution was unlikely to have a significant effect on the RNA structure. This idea is supported by RNA melting experiments (29) which show a single A_{260} transition with melting temperatures of 61.3 and 62.1°C for the IIUI and IIUI/UUGU dsRNAs, respectively (data not shown). The small difference in melting temperature is consistent with previous reports that IU pairs are less stable than GU pairs (30), but is unlikely to be a significant determinant for differential cleavage at 25°C. When the control GU substrate was labeled on either the top or the bottom strand, no cleavage was observed, as expected (Figure 1B, lanes 1–4 and 9–12, respectively, and Figure 1C). Similarly, when three GU pairs replaced IU pairs (substrate GGUG/UUIU, Table 1), no cleavage was detectable on the top strand (Figure 1B, lanes 5–8 and Figure 1C). A very small amount of cleavage was seen on the bottom strand (Figure 1B, lanes 13–16 and Figure 1C). These data are again consistent with the observation that GU pairs are unable to substitute for IU pairs, and that multiple IU pairs are required for efficient cleavage (12).

Additional IU pairs increase cleavage efficiency

As mentioned above, we have previously shown that cleavage efficiency decreased with decreasing number of IU pairs, where the hierarchy is 4 IU pairs > 3 IU pairs >>> 2 IU pairs (or GU pairs). Cleavage of a dsRNA substrate that contains 5 IU pairs (IIIUI-30e, Table 1) was next compared with cleavage of the wild-type IIUI sequence (IIUI-30, Table 1). In these experiments, the dsRNA substrates were 30 bp in length. Cleavage efficiency of the IIUI-30 was comparable with cleavage of the standard 20 bp IIUI duplex when compared in parallel assays (data not shown). When the IIUI-30 duplex labeled on the top strand was incubated in *Xenopus* oocyte extract one major cleaved product was again seen (Figure 1D, lanes 1–4). Cleavage of the IIIUI-30e dsRNA yielded the same product with greater efficiency (Figure 1D, lanes 5–8 and Figure 1E). When both IIUI-30 and IIIUI-30e dsRNAs were labeled on the bottom strand, multiple cleavage products were seen, as expected. Again, cleavage of IIIUI-30e was substantially more efficient than cleavage of the IIUI-30 duplex (compare lanes 13–16 and 9–12, respectively, and Figure 1E). The increase in cleavage efficiency of IIIUI-30e can largely be accounted for by enhanced cleavage at position 'a' (Figure 1F). These data therefore show that the cleavage

Figure 1. GU pairs have a deleterious effect on cleavage. (**A**) Cleavage assays were carried out using IIUI dsRNA molecules [5′ end-labeled on one strand (*)] and substrates where IU pairs were replaced by GU pairs (Table 1). Time points used in these assays were 0, 0.5, 1 and 2 h. (**B**) Cleavage assays were carried out using control GU dsRNA molecules [5′ end-labeled on one strand (*)], and substrates where GU pairs were replaced by IU pairs. Time points used in these assays were 0, 0.5, 1 and 2 h. (**C**) Data from cleavage assays as shown in (A) and (B) were quantitated following phosphorimaging ($n \geqslant 4$). The amount of cleaved product is given as the percentage of the total amount of dsRNA. (**D**) Cleavage assays using dsRNA substrates [5′ end-labeled on one strand (*)] that contain four or five IU pairs were carried out. Time points used in these assays were 0, 0.5, 1 and 2 h. (**E**) Data from cleavage assays as shown in (D) were quantitated as described above ($n \geqslant 4$). The amount of cleaved product is again given as the percentage of the total dsRNA. (**F**) A schematic diagram showing the position(s) of cleavage within the cleavage site sequence.

efficiency can be increased further by extension of the cleavage site by a single IU pair.
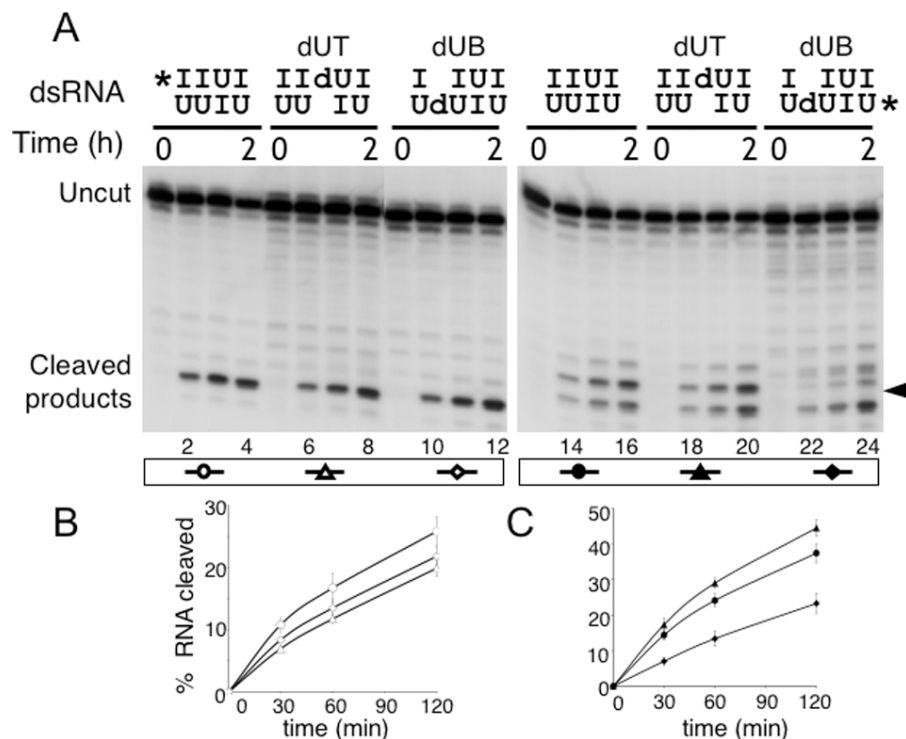
2′-OH groups are important for cleavage

Cleavage of inosine-containing RNA duplexes occurs 5′ of U residues to give a 3′ phosphate (19). dsRNA substrates that contained a single deoxy-uridine (dU) residue on either the top (dUT) or the bottom (dUB) strand (Table 1) were assayed for cleavage in *Xenopus* oocyte extract. When dsRNAs were labeled on the top strand, cleavage of either dUT or dUB yielded the same cleavage product as the wild-type IIUI dsRNA (Figure 2A, compare lanes 5–12 with 1–4), and with comparable efficiency (Figure 2B). When the dsRNAs were labeled on the bottom strand, cleavage of IIUI and dUT dsRNAs were indistinguishable (Figure 2A, lanes 13–20 and

Figure 2C). In contrast, cleavage of dUB was substantially reduced (Figure 2A, lanes 21–24 and Figure 2C). The reduction in cleavage can be explained by the reduction of cleavage at position 'b' (Figure 1F), which is immediately 3′ of the dU residue. This indicates that the 2′-OH group of the adjacent 5′ nucleotide is critical for cleavage. The presence of 2′-OMe U residues within the cleavage site of a substrate with four consecutive IU pairs [4I substrate (19)] also abolished cleavage, supporting the idea that the 2′-OH is necessary (data not shown).

Cleavage of IIUI dsRNA occurs on both strands within a single molecule

Cleavage is seen on either strand of the IIUI duplex depending on which strand is 5′ end-labeled, e.g. Figure 1A, lanes 1–4 and 9–12. However, it is impossible from these data to

Figure 2. 2′-OH groups are important for cleavage. (**A**) Cleavage assays were carried out using IIUI, dUT and dUB dsRNA molecules [5′ end-labeled on one strand (*)], to investigate the effect of dU residues on cleavage. Time points used in these assays were 0, 0.5, 1 and 2 h. (**B**) Data from cleavage assays as shown in (A) were quantitated following phosphor imaging ($n \geqslant 4$). The amount of cleaved product is given as the percentage of the total dsRNA.

determine whether cleavage is restricted to one strand of a duplex or whether both strands of a single duplex may be cleaved. dsRNA hairpins that contain either the wild-type IIUI sequence (IIUI hairpin, Table 1) or the control GU sequence (GU hairpin, Table 1) were used to analyze whether both strands of a single molecule undergo cleavage. The sequence of the RNA hairpins were identical to the equivalent RNA duplexes, but with the addition of a UNCG tetraloop sequence linking the strands. When the 5′ end-labeled IIUI hairpin was incubated in *Xenopus* oocyte extract, several cleavage products were observed that corresponded to cleavage within the IIUI cleavage site on either strand, as indicated (Figure 3A and B). Cleavage of the RNA hairpin on the top strand appeared to be most efficient (Figure 3A, lanes 1–4; open square). However, the product corresponding to cleavage on the bottom strand (closed square) may be underrepresented if the cleaved molecule was able to undergo further cleavage on the top strand. Conversely, no cleavage of the control GU hairpin was detectable (Figure 3A, lanes 9–12). In order to look directly at whether both strands could undergo cleavage, an IIUI hairpin substrate was used that was labeled internally at a single position immediately 5′ of the tetraloop sequence (indicated by an asterisk, Table 1 and Figure 3C). Cleavage of this substrate again yielded several products following incubation in *Xenopus* oocyte extract (Figure 3A, lanes 5–8 and Figure 3C). One set of cleavage products (∼34 nt) was the result of cleavage on either the top or bottom strand, which yielded products of a similar molecular weight (open circle; Figure 3A and C). In addition, a pair of cleavage products (∼24 nt) was detected that resulted from cleavage of both RNA strands within the same molecule (closed circle;

Figure 3A and C). These data therefore show that an IIUI dsRNA may be cleaved on both strands within a single molecule.

Cleavage occurs preferentially between the IU and UI pair in the IIUI sequence

Experiments using the IIUI hairpin suggested that cleavage 5′ of the U residue on the top strand was more efficient than cleavage of the bottom strand (Figure 3A, lanes 1–4). To test whether cleavage at such a position was preferred, an additional dsRNA substrate was used where the IIUI sequence was extended to give the palindromic sequence 5′-IIUIUU-3′/ 3′-UUIUII-5′ (IIUI-pal, Table 1). Cleavage of both the IIUI and IIUI-pal dsRNAs, labeled on the top strand, yielded a single product (Figure 4A, lanes 1–4 and 5–8, respectively, and Figure 4B). Although cleavage occurred at the same relative position within the IIUI sequence for both substrates (Figure 4C), the cleaved product of the IIUI-pal dsRNA was 1 nt shorter as the result of the position of the IIUI sequence within the dsRNA (Table 1). Cleavage of the IIUI-pal dsRNA appeared to be more efficient than the IIUI substrate, consistent with the observation that cleavage efficiency increases with increasing numbers of IU pairs (12). Cleavage of the IIUI dsRNA labeled on the bottom strand yielded several cleavage products, as expected (Figure 4A, lanes 9–12 and Figure 4B). In contrast, cleavage of the IIUI-pal substrate labeled on the bottom strand gave rise to one major cleaved product (Figure 4A, lanes 13–16 and Figure 4B). This product corresponded to cleavage at the same relative position as occurs on the top strand (Figure 4C). Furthermore, although cleavage occurred at a single position, cleavage of IIUI-pal
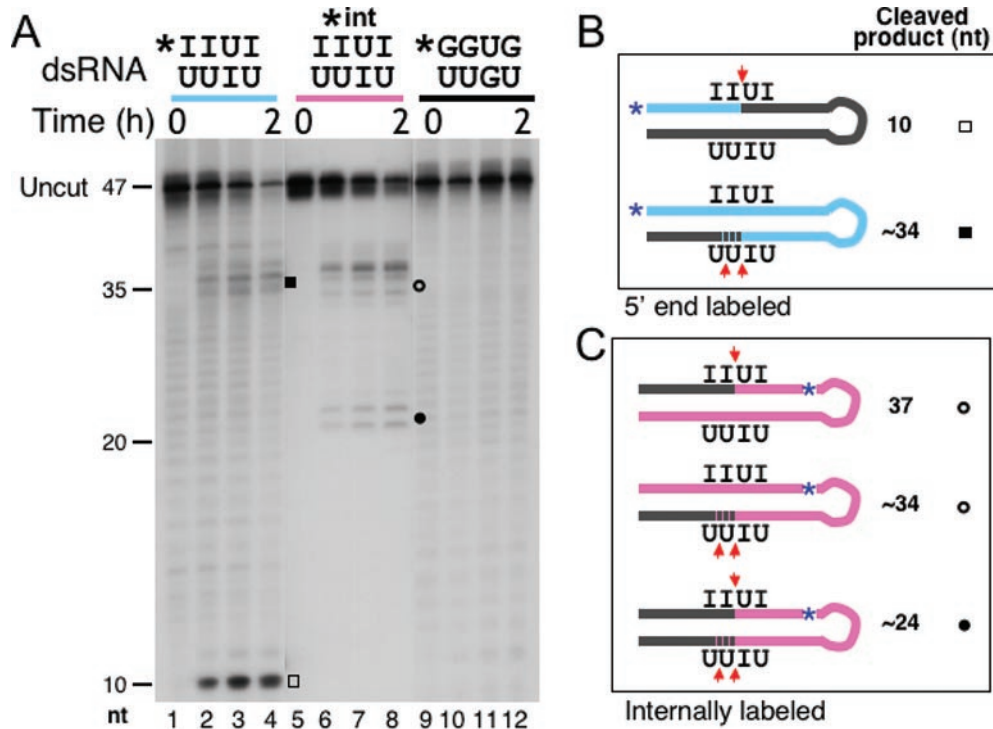
Figure 3. Cleavage of IIUI dsRNA occurs on both strands within a single molecule. (**A**) Cleavage assays were carried out using IIUI or GU hairpins (Table 1). The GU hairpin was 5′ end-labeled (*) while the IIUI hairpin was labeled either on the 5′ end (*) or internally, adjacent to the tetraloop sequence (*). Time points used in this assay were 0, 0.5, 1 and 2 h. Positions of cleavage within the 5′ end-labeled or internally labeled IIUI hairpins are shown schematically in (B) and (C), respectively. The product corresponding to cleavage on the top strand of the 5′ end-labeled IIUI hairpin is indicated by an open square while cleavage on the bottom strand is indicated by a closed square. Cleavage products corresponding to cleavage on either the top or the bottom strand of the internally labeled IIUI hairpin are indicated by an open circle (∼34–37 nt), while cleavage products corresponding to cleavage on both strands are indicated by a closed circle (∼24 nt). Molecular weight markers (nt) are shown at the left of the figure. (**B**) Cleavage of the 5′ end-labeled IIUI hairpin is shown schematically. The blue line represents the ³²P-labeled cleaved product(s) following cleavage at the site(s) indicated by the arrow(s). The striped line indicates cleavage at multiple positions on the bottom strand. (**C**) Cleavage of the internally labeled IIUI hairpin is shown schematically. The pink line represents the ³²P-labeled cleaved product(s) following cleavage at the site(s) indicated by the arrow(s). The striped line indicates cleavage at multiple positions on the bottom strand.

dsRNA was more efficient than the IIUI substrate (Figure 4B). These data therefore suggested that the preferred site of cleavage is between an IU and a UI pair, in the context of the IIUI sequence. Nevertheless, in the absence of the preferred cleavage site cleavage occurs efficiently 5′ of U residues when there are multiple IU pairs (e.g. Figure 1A, lanes 9–12).

IIUI is better than the 4I site for cleavage

Based on the experiments using IIUI-pal dsRNA, we predicted that cleavage within the sequence IIUI would be preferable to cleavage within a run of four UI pairs if they were in direct competition. To test this idea, a dsRNA was used for cleavage assays that contained both the 4I and IIUI cleavage sites (COMP, Figure 5A and Table 1). The cleavage sites were arranged such that the U residues of the 4I site (that are susceptible to cleavage) were on the same strand as the IIUI sequence of the wild-type cleavage site. The context of both cleavage sites was similar to the wild-type dsRNA, with regard to both flanking nucleotides and distance from the ends of the dsRNA (Figure 5A). When the dsRNAs were labeled on the top strand, cleavage of the wild-type (20 bp) IIUI dsRNA in *Xenopus* oocyte extract gave a single product (Figure 5B, lanes 1–4). In contrast, cleavage of the COMP dsRNA labeled on the top strand yielded products corresponding to cleavage at both the 4I (closed circle) and

IIUI (open circle) sequences (Figure 5B, lanes 5–8). As the dsRNA was labeled only at the 5′ end, it was likely that cleavage at the IIUI sequence was underrepresented if additional cleavage occurred at the 4I site. Nevertheless, cleavage at the IIUI site appeared to be at least 2.5-fold more efficient than at the 4I site in the COMP dsRNA (Figure 5C). The sum of cleavage at both sites was similar to the efficiency of cleavage of the 20 bp IIUI dsRNA. When the IIUI and COMP dsRNAs labeled on the bottom strand were assayed in *Xenopus* oocyte extract, both yielded several cleavage products with equal efficiency corresponding to cleavage at the IIUI sequence (Figure 5B, lanes 11–18 and Figure 5C). No cleavage occurred within the 4I site on the bottom strand of the COMP dsRNA due to the absence of U residues (Figure 5B, lanes 15–18). These data corroborated those described above that suggested that the preferred site of cleavage is between an IU and a UI pair in the sequence 5′-IIUI-3′/3′-UUIU-5′.

Hyper-editing of a long dsRNA substrate

Previously, we used a long (295 bp) dsRNA (ΔKP, Figure 6A) to analyze cleavage of dsRNAs hyper-edited by ADAR2 (19). This initial analysis revealed that cleavage occurred efficiently at a site containing IU and UI pairs, such as that described above. We have now used this long dsRNA substrate to carry out a more extensive analysis of hyper-editing by ADAR2.
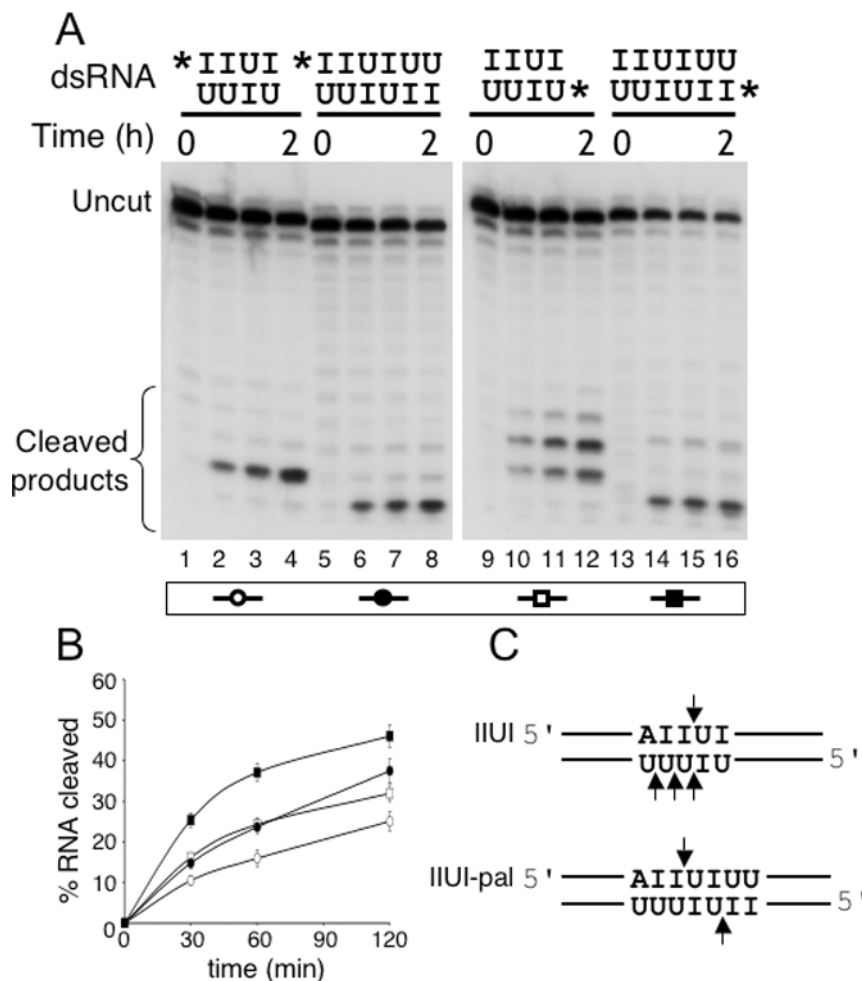
Figure 4. Cleavage occurs preferentially between the IU and UI pair in the IIUI sequence. (**A**) Cleavage assays were carried out using IIUI and IIUI-pal dsRNA molecules [5′ end-labeled on one strand (*)]. Time points used in these assays were 0, 0.5, 1 and 2 h. (**B**) Data from cleavage assays as shown in (A) were quantitated following phosphor imaging ($n \geqslant 4$). The amount of cleaved product is given as the percentage of the total amount of dsRNA. (**C**) A schematic diagram showing the position(s) of cleavage within the IIUI and IIUI-pal dsRNAs.

Hyper-editing of ΔKP by ADAR1 and dADAR was subsequently analyzed.

Editing preferences of ADAR2

ΔKP dsRNA was hyper-edited by ADAR2 such that 30–45% of adenosine residues were converted to inosine. Edited sequences were then amplified by RT–PCR and cloned for subsequent sequencing. Owing to the choice of oligonucleotide primers used for RT–PCR, editing within the sequences shown in gray at the 5′ and 3′ ends of ΔKP (Figure 6A) was not determinable. These sequences were therefore excluded from all data analyses described. Approximately 40 unique clones corresponding to the sense strand and 30 clones corresponding to the antisense strand were used to analyze editing of ΔKP by ADAR2.

The efficiency of editing of each adenosine residue on either strand was determined, where the efficiency of editing was expressed as the percentage of the total number of clones edited. These data are summarized in Figure 6B. Although there were approximately twice the number of adenosines on the sense strand as the antisense (29 and 14%, respectively), ~75% of all adenosines on each strand were edited. When

hyper-editing occurs within perfect dsRNA, the efficiency of editing of particular adenosines is influenced by the neighboring nucleotides (15,31). Neighbor preferences were therefore calculated for editing sites on both strands of ΔKP (Figure 6C). This analysis showed a 5′ neighbor preference of U = A > C > G which is similar to those determined previously for ADAR2 [U ≈ A > C = G (31)] and ADAR1 [U = A > C > G (15)]. Despite quantitative differences in the various studies, the hierarchy of neighbor preferences was preserved when analyzing editing of ΔKP. A similar analysis revealed that ADAR2 also appears to have a 3′ neighbor preference when editing ΔKP (Figure 6C, G > A = C = U). While this appears to differ from that determined previously for ADAR2 [U = G > C = A (31)], the only real difference appears to be the lack of preference for a U residue 3′ of the edited adenosine. This discrepancy may reflect the different methods used to analyze hyper-editing by ADAR2. Whereas this study involved the analysis of individual clones corresponding to edited sequences, previous studies employed methods where the whole population of edited dsRNAs was assessed simultaneously [e.g. nuclease protection, primer extension (31)]. Nevertheless, these data are largely consistent with those
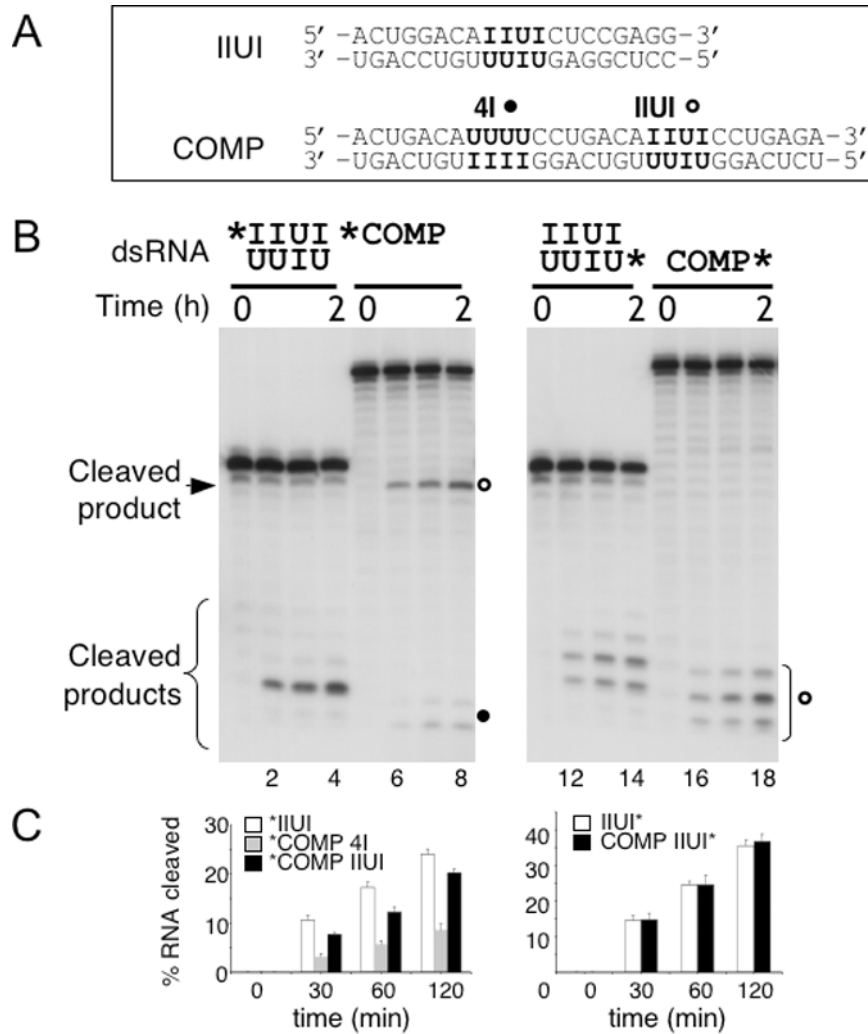
Figure 5. IIUI is better than the 4I site for cleavage. (**A**) Sequence of IIUI and COMP dsRNAs. The two cleavage sites in the COMP dsRNA are labeled as IIUI (open circle) and 4I (closed circle). (**B**) Cleavage assays were carried out using IIUI and COMP dsRNA molecules [5′ end-labeled on one strand (*)]. Time points used in these assays were 0, 0.5, 1 and 2 h. Cleavage on the top strand of the COMP dsRNA at the 4I site is indicated by a closed circle, while cleavage at the IIUI site on either strand of the COMP dsRNA is indicated by an open circle. (**C**) Data from cleavage assays as shown in (A) were quantified following phosphorimaging ($n \geqslant 4$). The amount of cleaved product is given as the percentage of the total amount of dsRNA. Cleavage of COMP dsRNA occurred at the IIUI sequence (COMP IIUI) or at the 4I sequence (COMP 4I).

previously reported with regard to neighbor preferences for editing by ADAR2. While editing by ADARs is not thought to be processive *per se* (15), it is interesting to note that when there are two or three adjacent adenosine residues editing efficiency appears highest at the 3′ adenosine and then decreases at each successive adenosine in a 3′ to 5′ direction [e.g. $A_{217-219}$ (red bars, Figure 6B)]. This observation is upheld for all adenosine dinucleotides and triplets in ΔKP, regardless of the neighboring nucleotides (data not shown). This preference may reflect an intrinsic property of the enzyme important for either binding or catalysis. Consistent with previous data (19), one sequence that was edited efficiently by ADAR2 on both strands was 5′-A_{200}AUA_{203}-3′ (Figure 6A and B), which gave rise to the cleavage site sequence 5′-IIUI-3′. Efficient editing of this sequence is in accordance with both the 5′ and 3′ neighbor preferences determined previously for ADAR2 (31).

These analyses confirmed that hyper-editing of ΔKP by ADAR2 was consistent with earlier studies where relatively short dsRNA substrates were used (15,31). ΔKP was therefore additionally used as a substrate for ADAR1 and dADAR to enable a comparison of hyper-editing.

ADAR1, ADAR2 and dADAR generate the preferred cleavage site

As described above for ADAR2, ΔKP was hyper-edited by ADAR1 or dADAR such that 30–45% of adenosine residues were converted to inosine. RT–PCR was used to amplify the edited sequences and the products were cloned for subsequent sequencing. A total of 20–40 individual clones corresponding to the antisense strand of ΔKP were analyzed for both ADAR1 and dADAR. Again, the editing efficiency at each position was determined and expressed as the percentage of the total number of clones edited. These data are summarized together with the data corresponding to editing of the antisense strand by ADAR2 (Figure 6D). These data show that while editing occurs at equivalent positions by all three ADAR enzymes,
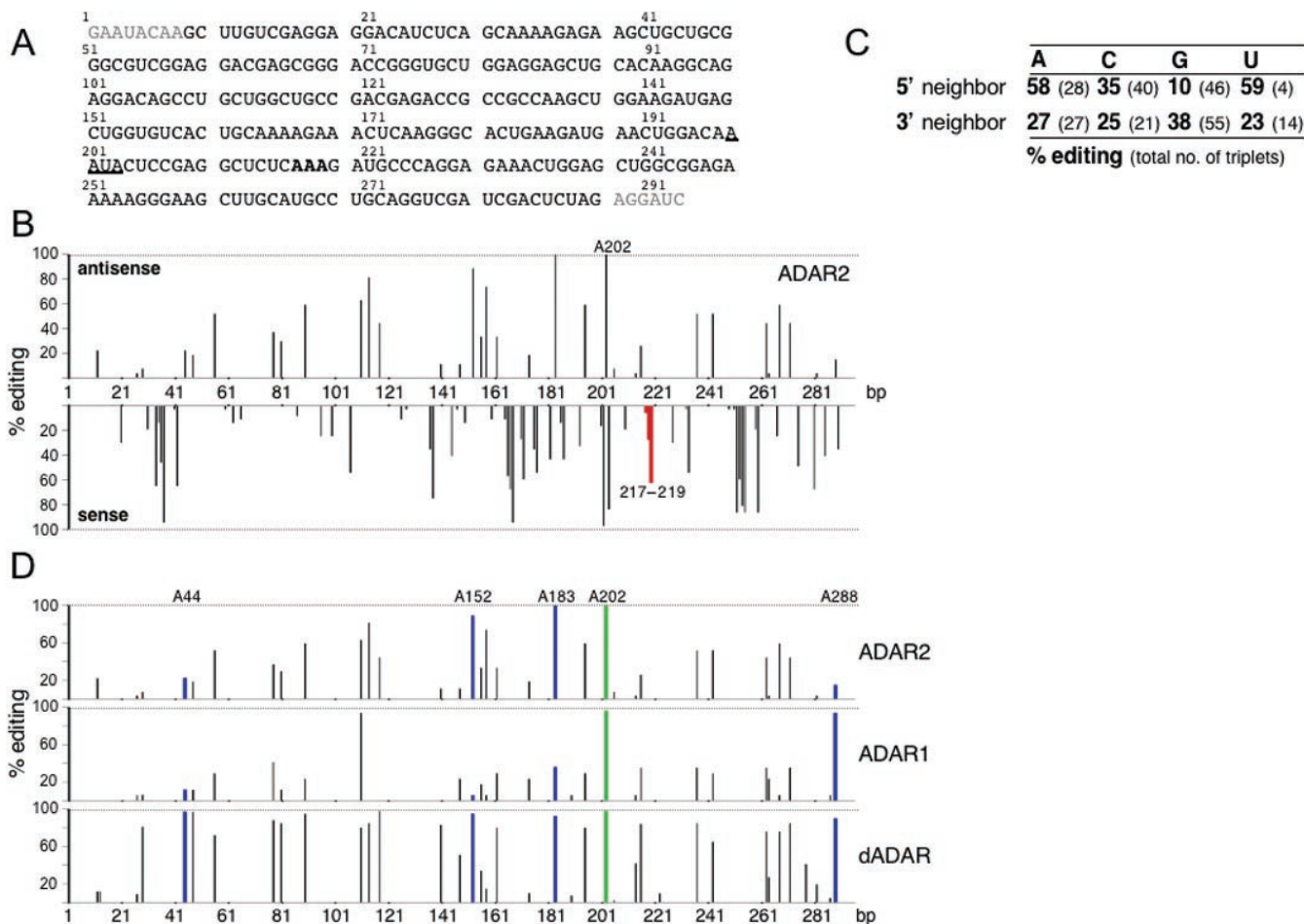
Figure 6. Editing by ADAR1, ADAR2 or dADAR generates the IIUI sequence. (**A**) Sequence of ΔKP RNA (sense strand). An example of an adenosine triplet ($A_{217–219}$) is shown in bold. The cleavage site sequence ($A_{200–203}$) is underlined. (**B**) Efficiency of editing of ΔKP by ADAR2. Efficiency of editing is expressed as the percentage of clones that contain an inosine residue at each edited position. Hundred percent editing is indicated by a dotted line. Editing of the antisense and sense strands are shown at the top and bottom of the graph, respectively. Red bars show an example of editing of an adenosine triplet ($A_{200–203}$). (**C**) The average editing (%) of each adenosine preceded by a particular nucleotide (AAX, CAX, GAX, UAX) was calculated to give 5′ neighbor preferences. Similarly, the average editing (%) of each adenosine followed by a particular nucleotide (XAA, XAC, XAG, XAU) was calculated to give 3′ neighbor preferences. The total number of adenosines in each context is shown in brackets. (**D**) Efficiency of editing of ΔKP (antisense strand) by ADAR2, ADAR1 or dADAR. Efficiency of editing is expressed as the percentage of clones that contain an inosine residue at each edited position. Hundred percent editing is indicated by a dotted line. Blue bars indicate particular adenosine residues differentially edited by the three ADARs. The green bar indicates the adenosine residue within the cleavage site sequence.

the efficiency of editing at each site varies. For example, editing of adenosine 183 occurred in 100% of the clones edited by ADAR2, 92% of clones edited by dADAR but only 36% of clones edited by ADAR1 (blue bars, Figure 6D). Other examples of variation are readily seen at adenosines 44, 152 and 288 where the editing efficiency is considerably different for each enzyme (blue bars, Figure 6D). These data therefore show that ADAR1, ADAR2 and dADAR have distinct but overlapping specificities. This is consistent with previous findings using ADAR1 and ADAR2 (31). Of the three enzymes, dADAR appears to edit numerous positions more efficiently than either ADAR1 or ADAR2. Nevertheless, both ADAR1 and dADAR show similar 5′ neighbor preferences, where U was preferred and G was least favorable (data not shown). This is consistent with the 5′ neighbor preferences of both ADAR1 and ADAR2 described above. As the triplet XAA was underrepresented on this strand it was difficult to draw conclusions about the 3′ neighbor preferences of ADAR1

or dADAR. Although differential editing was seen at most positions, it was striking that adenosine 202 was edited efficiently by all three ADARs—94, 98 and 100% for ADAR1, dADAR and ADAR2, respectively (green bars, Figure 6D). This adenosine is contained within the sequence that constitutes the cleavage site sequence when edited. When just the deaminase domain of dADAR was used for editing assays, adenosine 202 was still edited despite the overall efficiency of editing being <5% (data not shown). Adenosine 202 was also preferentially edited by both ADAR1 and ADAR2 when the overall level of editing was relatively low (<10% adenosines converted to inosine; data not shown). Similar observations were made when analyzing editing on the sense strand at adenosines 200–203 (data not shown). Strikingly, these data together indicated that each of the ADARs tested would efficiently generate the cleavage site sequence. The preferred cleavage site therefore corresponds to a preferred editing site.

DISCUSSION

Previously, we have described a ribonuclease present in various cell extracts that specifically cleaves dsRNA hyper-edited by ADARs (19). Here, we have described experiments aimed at further investigating the cleavage of hyper-edited dsRNAs. Furthermore, we have analyzed hyper-editing of a long dsRNA substrate by various ADARs and show that they all give rise to the preferred cleavage site.

Cleavage of inosine-containing dsRNA occurs within the sequence 5′-IIUI-3′/3′-UUIU-5′. In contrast, when GU and UG pairs replace the IU and UI pairs, respectively, cleavage is abolished. Two explanations have previously been proposed to explain the difference in susceptibility to cleavage of dsRNAs containing either IU or GU pairs. While the geometry of IU and GU pairs is essentially identical, the additional minor groove amine groups present on guanosine may interfere with cleavage. Alternatively, the IU- or GU-containing dsRNAs may adopt different structures that give rise to differential cleavage. However, as the melting profile of IU- and GU-containing dsRNAs were comparable, our favored explanation for the specific cleavage was recognition of specific distorted dsRNA structures (19). We have now used substrates that contain a mixture of IU and GU pairs for cleavage assays. When a single UI was replaced by a UG pair in the substrate IIUI/UUGU, cleavage on the top strand was reduced by at least 50%, and on the bottom strand by at least 70% (Figure 1A). As the conformation of the UI and UG pairs were expected to be comparable, these data show that the single exocyclic amine group within the cleavage site was sufficient to interfere with cleavage. It is likely that the amine group may sterically hinder cleavage *per se*. This idea is supported by the observation that cleavage at the position adjacent to the GU pair (position 'c', Figure 1F) on the bottom strand of the substrate IIUI/UUGU was disproportionately reduced. A single UI pair in the substrate GGUG/UUIU was inefficient for cleavage, consistent with the observation that multiple IU pairs are required for cleavage (12).

A single U to dU substitution within the cleavage site of the substrate dUB caused cleavage to be abolished at the adjacent scissile bond. In contrast, a dU residue in the substrate dUT had no effect on cleavage of either strand. These data therefore suggest that the OH group may be important mechanistically for cleavage of the RNA, rather than for ribonuclease binding. The OH group may act as the nucleophile for cleavage at the adjacent position.

Cleavage assays using the substrate IIUI-pal revealed that the preferred site of cleavage was between the IU and UI pair within the wild-type sequence IIUI. Although cleavage normally occurs at the adjacent UI pairs on the bottom strand of the IIUI dsRNA, the position of cleavage shifted to occur almost exclusively between the IU and UI pairs within the IIUI sequence on the bottom strand of the palindromic sequence (Figure 4C). This preference for cleavage between an IU and UI pair was confirmed using the COMP dsRNA where cleavage could take place at a sequence containing only UI pairs (4I) or which contained both IU and UI pairs (IIUI). Cleavage again occurred ∼2- to 3-fold more efficiently within the IIUI sequence than at the 4I sequence. Although cleavage of the IIUI-pal and COMP substrates highlighted a preferred

sequence for cleavage, it is important to note that cleavage does occur efficiently at other sequences in the absence of more competitive sites. For example, cleavage occurs efficiently on the bottom strand of the IIUI and 4I substrates (19) where there are adjacent UI pairs. The preference for cleavage between an IU and UI pair in the IIUI sequence may reflect either the optimal binding site for the protein complex involved in cleavage, or the ideal configuration of bases within the active site. Purification of the complete protein complex involved in cleavage will enable further studies to discriminate between these two possibilities. The observation that both strands of an inosine-containing dsRNA are cleaved within a single molecule (Figure 3A) makes it tempting to speculate that a ribonuclease dimer may be responsible for cleavage. This idea is supported by the symmetrical cleavage of the IIUI-pal substrate. Future studies will enable analyses of protein binding and the arrangement of the ribonuclease to be elucidated.

ΔKP was used as a model substrate to analyze editing preferences of ADAR2, ADAR1 and dADAR. An extensive analysis using ADAR2 was initially carried out to verify that editing of ΔKP was consistent with the preferences determined previously using relatively short dsRNA substrates (31). ΔKP was subsequently used as a substrate for hyper-editing by ADAR1 and dADAR. This analysis revealed that each adenosine residue was edited to a different extent by each of the three ADARs (Figure 6D). Of the three enzymes, dADAR edited numerous positions more efficiently than either ADAR1 or ADAR2. This suggests that dADAR may have a broader specificity than either ADAR1 or ADAR2, which is consistent with the idea that dADAR edits multiple RNA targets *in vivo* (32). This study therefore showed that the three ADARs have distinct yet overlapping specificities, even on a long perfect dsRNA. Nevertheless, it is striking that one sequence in ΔKP was edited efficiently by all three ADARs, which gave rise to the preferred cleavage site sequence (5′-IIUI-3′/3′-UUIU-5′). This sequence was also edited by both ADAR1 and ADAR2 when the editing efficiency was low (<10%), and by the deaminase domain of dADAR when <5% editing occurred overall. Efficient editing of the sequence 5′-AAAUA-3′/3′-UUUAU-5′ is in accordance with the neighbor preferences determined both here and previously (15,31). However, the observation that this site appears to be a hot spot for editing suggests that there are other factors contributing to the choice of editing site. It will be interesting to test whether preferential binding of ADARs to this site plays a role in its selection (33). We have thus demonstrated a marked correlation between hyper-editing and subsequent cleavage; we have shown that the preferred cleavage site containing multiple IU and UI pairs would be preferentially created by all three ADARs tested.

The data described here corroborate previous data and provide insight into the determinants of an inosine-containing dsRNA that make it susceptible to cleavage. We have previously described the potential role of TSN in cleavage of hyper-edited dsRNA (12). It is now of interest to identify the complete protein complex necessary for cleavage such that a more precise analysis of dsRNA protein interactions can be undertaken. The insights provided in this work will aid in this future investigation.

REFERENCES

1. Maquat,L.E. and Carmichael,G.G. (2001) Quality control of mRNA function. *Cell*, **104**, 173–176.
2. Samuel,C.E. (1998) Protein-nucleic acid interactions and cellular responses to interferon. *Methods*, **15**, 161–165.
3. Zhou,A., Hassel,B.A. and Silverman,R.H. (1993) Expression cloning of 2-5A-dependent RNase: a uniquely regulated mediator of interferon action. *Cell*, **72**, 753–765.
4. Silverman,R.H. (1997) 2-5A dependent RNase L: a regulated endoribonuclease in the interferon system.. In D'Alessio,G. and Riordan,J.F. (eds), *Ribonucleases—Structures and Functions*. Academic Press, NY, pp. 515–551.
5. Novina,C.D. and Sharp,P.A. (2004) The RNAi revolution. *Nature*, **430**, 161–164.
6. Denli,A.M. and Hannon,G.J. (2003) RNAi: an ever-growing puzzle. *Trends Biochem. Sci*., **28**, 196–201.
7. Bass,B.L. (2002) RNA editing by adenosine deaminases that act on RNA. *Annu. Rev. Biochem*., **71**, 817–846.
8. Scadden,A.D. and Smith,C.W. (2001) RNAi is antagonized by A→I hyper-editing. *EMBO Rep*., **2**, 1107–1111.
9. Knight,S.W. and Bass,B.L. (2002) The role of RNA editing by ADARs in RNAi. *Mol. Cell*, **10**, 809–817.
10. Tonkin,L.A. and Bass,B.L. (2003) Mutations in RNAi rescue aberrant chemotaxis of ADAR mutants. *Science*, **302**, 1725.
11. Yang,W., Wang,Q., Howell,K.L., Lee,J.T., Cho,D.S., Murray,J.M. and Nishikura,K. (2005) ADAR1 RNA deaminase limits short interfering RNA efficacy in mammalian cells. *J. Biol. Chem*., **280**, 3946–3953.
12. Scadden,A.D. (2005) The RISC subunit Tudor-SN binds to hyper-edited double-stranded RNA and promotes its cleavage. *Nature Struct. Mol. Biol*., **12**, 489–496.
13. Palladino,M.J., Keegan,L.P., O'Connell,M.A. and Reenan,R.A. (2000) dADAR, a Drosophila double-stranded RNA-specific adenosine deaminase is highly developmentally regulated and is itself a target for RNA editing. *RNA*, **6**, 1004–1018.
14. Nishikura,K., Yoo,C., Kim,U., Murray,J.M., Estes,P.A., Cash,F.E. and Liebhaber,S.A. (1991) Substrate specificity of the dsRNA unwinding/modifying activity. *EMBO J*., **10**, 3523–3532.
15. Polson,A.G. and Bass,B.L. (1994) Preferential selection of adenosines for modification by double-stranded RNA adenosine deaminase. *EMBO J*., **13**, 5701–5711.
16. Serra,M.J., Smolter,P.E. and Westhof,E. (2004) Pronounced instability of tandem IU base pairs in RNA. *Nucleic Acids Res*., **32**, 1824–1828.
17. Zhang,Z. and Carmichael,G.G. (2001) The fate of dsRNA in the nucleus: a p54(nrb)-containing complex mediates the nuclear retention of promiscuously A-to-I edited RNAs. *Cell*, **106**, 465–475.
18. Wang,Q., Zhang,Z., Blackwell,K. and Carmichael,G.G. (2005) Vigilins bind to promiscuously A-to-I-edited RNAs and are involved in the formation of heterochromatin. *Curr. Biol*., **15**, 384–391.
19. Scadden,A.D. and Smith,C.W. (2001) Specific cleavage of hyper-edited dsRNAs. *EMBO J*., **20**, 4243–4252.
20. Caudy,A.A., Ketting,R.F., Hammond,S.M., Denli,A.M., Bathoorn,A.M., Tops,B.B., Silva,J.M., Myers,M.M., Hannon,G.J. and Plasterk,R.H. (2003) A micrococcal nuclease homologue in RNAi effector complexes. *Nature*, **425**, 411–414.
21. Levanon,E.Y., Eisenberg,E., Yelin,R., Nemzer,S., Hallegger,M., Shemesh,R., Fligelman,Z.Y., Shoshan,A., Pollock,S.R., Sztybel,D. *et al*. (2004) Systematic identification of abundant A-to-I editing sites in the human transcriptome. *Nat. Biotechnol*., **22**, 1001–1005.
22. Luciano,D.J., Mirsky,H., Vendetti,N.J. and Maas,S. (2004) RNA editing of a miRNA precursor. *RNA*, **10**, 1174–1177.
23. Sambrook,J. and Russell,D.W. (2001) *Molecular Cloning: A Laboratory Manual*. 3rd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
24. Smith,C.W.J., Chu,T.T. and Nadal-Ginard,B. (1993) Scanning and competition between AGs are involved in 3′ splice site selection in mammalian introns. *Mol. Cell. Biol*., **13**, 4939–4952.
25. Moore,M.J. and Query,C.C. (1998) *Uses of site-specifically modified RNAs constructed by RNA ligation*. Oxford University Press, Oxford, pp. 75–108.
26. Korner,C.G., Wormington,M., Muckenthaler,M., Schneider,S., Dehlin,E. and Wahle,E. (1998) The deadenylating nuclease (DAN) is involved in poly(A) tail removal during the meiotic maturation of Xenopus oocytes. *EMBO J*., **17**, 5427–5437.
27. O'Connell,M.A., Gerber,A. and Keegan,L.P. (1998) Purification of native and recombinant double-stranded RNA-specific adenosine deaminases. *Methods*, **15**, 51–62.
28. Ring,G.M., O'Connell,M.A. and Keegan,L.P. (2004) Purification and assay of recombinant ADAR proteins expressed in the yeast *Pichia pastoris* or in *Escherichia coli*. *Methods Mol. Biol*., **265**, 219–238.
29. Loakes,D. and Brown,D.M. (1994) 5-Nitroindole as an universal base analogue. *Nucleic Acids Res*., **22**, 4039–4043.
30. Strobel,S.A., Cech,T.R., Usman,N. and Beigelman,L. (1994) The 2,6-diaminopurine riboside.5-methylisocytidine wobble base pair: an isoenergetic substitution for the study of G.U. pairs in RNA. *Biochemistry*, **33**, 13824–13835.
31. Lehmann,K.A. and Bass,B.L. (2000) Double-stranded RNA adenosine deaminases ADAR1 and ADAR2 have overlapping specificities. *Biochemistry*, **39**, 12875–12884.
32. Hoopengardner,B., Bhalla,T., Staber,C. and Reenan,R. (2003) Nervous system targets of RNA editing identified by comparative genomics. *Science*, **301**, 832–836.
33. Klaue,Y., Kallman,A.M., Bonin,M., Nellen,W. and Ohman,M. (2003) Biochemical analysis and scanning force microscopy reveal productive and nonproductive ADAR2 binding to RNA substrates. *RNA*, **9**, 839–846.