Alternative splicing of the *ADAR1* transcript in a region that functions either as a 5'-UTR or an ORF

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ABSTRACT

The ADAR enzymes mediate the hydrolytic deamination of adenosines in specific RNA substrates and thereby diversify both the transcriptome and the proteome in metazoan species. Three promoters drive the transcription from the *ADAR1* gene yielding the *ADAR1-A*, *-B*, and *-C* transcripts, which, in turn, lead to the production of two protein isoforms, namely, iADAR1 and cADAR1. In this study, we establish the presence of a previously unidentified alternative intron within the 5'-end of the common second exon of mRNAs encoding ADAR1 in primate species—a region that can function either as a 5'-UTR or an ORF. In addition, it is shown that the relative expression of the three promoter-specific *ADAR1* transcripts is tissue specific and that the novel intron is excised from all transcripts, but at different relative levels indicating a specific regulation of the alternative splicing. Finally, possible functional consequences of the splicing are investigated. From these studies, we conclude that the alternatively spliced *ADAR1-A* transcript is immune to nonsense-mediated decay although it is a potential substrate. Moreover, this transcript is associated with translating ribosomes, which suggests that a truncated version of iADAR1 is expressed.

Keywords: ADAR; alternative splicing; retained intron

INTRODUCTION

The adenosine deaminases acting on RNA (ADAR) proteins are RNA-editing enzymes that catalyze the deamination of adenosine in the context of a double-stranded RNA substrate. By this process, the adenosine is converted to the noncanonical nucleotide inosine, which, in most respects, is equivalent to guanosine. As a consequence, features such as codons, splicing, stability, and translation efficiency of the targeted RNA can be altered. Thus, RNA editing by an ADAR enzyme leads to a post-transcriptional modification of the sequence of specific genes, and it provides a mechanism for the cell to recode and fine tune gene expression (for review, see Bass 2002; Maas et al. 2003; Valente and Nishikura 2005).

ADAR enzymes are conserved from *Caenorhabditis elegans* to humans and share a similar overall domain

structure consisting of one to three double-stranded RNA-binding domains (dsRBD) followed by a highly conserved catalytic deaminase domain in the carboxyl terminus. Three different ADAR paralogs have been characterized in mammals—ADAR1, ADAR2, and ADAR3—of which only ADAR1 and ADAR2 are functional editing enzymes (for review, see Bass 2002; Keegan et al. 2004; Valente and Nishikura 2005). Furthermore, by knockout analysis, both ADAR1 and ADAR2 were found to be essential in mice, but the deletion phenotypes are different (Higuchi et al. 2000; Wang et al. 2000; Hartner et al. 2004; Wang et al. 2004).

ADARs do not need accessory protein or RNA factors for the deamination reaction per se since it occurs in vitro with the purified protein and RNA substrate (Hurst et al. 1995; Dabiri et al. 1996; Melcher et al. 1996; Polson et al. 1996). However, several regulatory mechanisms have been shown or envisaged to exist in vivo. One way to regulate the activity of the ADAR proteins temporally and spatially is by alternative splicing of the corresponding mRNAs. Indeed, numerous splice variants with important functional features have been identified for *ADAR2* (Gerber et al. 1997; Lai et al. 1997; Mittaz et al. 1997; Rueter et al. 1999; Slavov and Gardiner 2002; Feng et al. 2006). The expression and splicing patterns of *ADAR1* are similarly complex.

Transcription of the human *ADAR1* gene, which spans ~46 kb on chromosome 1 (1q21), initiates from at least

Abbreviations: ADAR1, adenosine deaminase acting on RNA, type 1; 5'-UTR, 5'-untranslated region; ORF, open reading frame; cADAR1, constitutively expressed ADAR1; iADAR1, interferon induced ADAR1; dsRBD, double-stranded RNA-binding domain; PTC, premature termination codon; NMD, nonsense-mediated decay; RT, reverse transcription.

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three different promoters, leading to mRNAs with mutually exclusive first exons (exons 1A, 1B, and 1C) followed by 14 downstream exons (Fig. 1; Wang et al. 1995; Weier et al. 1995; Liu et al. 1997; George and Samuel 1999a,b; Kawakubo and Samuel 2000).

The promoters driving the transcription of *ADAR1-B* and *-C* (including exons 1B and 1C, respectively) are constitutively active (George and Samuel 1999b; Kawakubo and Samuel 2000). Furthermore, the two transcripts share the same open reading frame (ORF) beginning from a start codon situated 870 nucleotides (nt) into exon 2 (Fig. 1) and ending at a stop codon in exon 15, which leads to identical protein products, namely, the short constitutively expressed isoform cADAR1 (p110).

The promoter that initiates the *ADAR1-A* transcript also has a basal constitutive activity but is strongly stimulated by interferons (Patterson and Samuel 1995; Patterson et al. 1995; Der et al. 1998; George and Samuel 1999a). Due to the presence of an in-frame initiation codon at the 3'-end of exon 1A (Fig. 1), the protein product derived from the *ADAR1-A* transcript contains an amino-terminal extension compared to the cADAR1 isoform, but the two proteins are otherwise identical with three successive dsRBDs and a

catalytic deaminase domain. Owing to its induction by interferon, this large protein isoform is called iADAR1 (p150), and it is believed to be involved in cellular defense against viruses and other pathogens. A recent study has established that the *ADAR1* gene in mouse has a conceptually similar organization as the human counterpart, which includes constitutive and interferon-inducible promoters (George et al. 2005).

Both human and mouse *ADAR1* appear to be ubiquitously expressed in both fetal and adult tissues (Kim et al. 1994; O'Connell et al. 1995; Lai et al. 1997), and the mouse *ADAR1-A* and *-B* transcripts have tissue-specific distributions (George et al. 2005), while the distribution of the human splice variants has not been determined.

We have identified and characterized a novel alternative intron situated within the 5'-end of exon 2 of the *ADAR1* gene in humans and other primates, but not in lower mammalian species. The alternatively excised intron is present in all three promoter-specific *ADAR1* splice variants, and the expression patterns of the various mRNA species were determined in detail in several different tissues. The identity of the first exon will determine whether the intron is positioned in the translated part of the transcript, which

implies that the effect of its excision depends on from which promoter transcription was initiated. Most notably, the excision of the alternative intron from the *ADAR1-A* transcript introduces a premature termination codon (PTC), which lead us to investigate whether this transcript undergoes non-sense-mediated decay (NMD) as part of a regulatory mechanism. However, we find that it escapes NMD and instead is associated with translating ribosomes. Based on this observation, we speculate that an amino-terminal peptide of iADAR1 is expressed in primate species.

RESULTS

Identification of a novel intron in *ADAR1*

It has been observed in several studies that two protein species corresponding to iADAR1 and cADAR1 appear when overexpressing iADAR1 from different vector constructs (Sato et al. 2001; Wong et al. 2001, 2003; Wong and Lazinski 2002; Desterro et al. 2003). To investigate this phenomenon further, we constructed mammalian expression vectors encoding iADAR1

FIGURE 1. Promoter region and promoter-specific splice variants of human *ADAR1*. (A) Schematic overview of the promoter region of the *ADAR1* gene. Exons and introns are depicted as boxes and lines, respectively. The three promoters (P_A , P_B , and P_C) driving the transcription from the *ADAR1* gene are depicted in front of their corresponding first exons. (B) The three promoter-specific transcripts (*ADAR1-A*, *-B*, and *-C*). Approximate sizes (in nucleotides) of the first exons are indicated. The translation initiation codons for iADAR1 (AUG1) and cADAR1 (AUG1') are shown. The 5'-end of the second exon, which is depicted in black, can serve distinct purposes as either a 5'-UTR or as part of the ORF encoding iADAR1 (starting from AUG1 in exon 1A).

and cADAR1 with amino-terminal HA epitope tags and carboxy-terminal Flag epitope tags. As observed by others, the iADAR1 expression vector gives rise to two species corresponding in size to iADAR1 and cADAR1 when expressed in HeLa cells (Fig. 2A, cf. lanes 1 and 7). The shorter form, expressed from the wild-type iADAR1 expression vector, is detected by an anti-Flag antibody, and since this epitope is situated in the extreme carboxyl terminus of iADAR1, the protein must be amino-terminally truncated (Fig. 2A, lane 1). Accordingly, the shorter form is not detected by an anti-HA antibody, which recognizes the amino-terminal HA epitope (data not shown).

Lazinski and coworkers have previously reported that internal deletions in the unique amino terminus of iADAR1 prevented the additional translation of cADAR1 (Wong et al. 2003). We therefore made a series of constructs with deletions in the amino-terminal part of iADAR1 and analyzed the expressed proteins by Western blotting using an anti-Flag antibody (Fig. 2A, lanes 2–6), which confirmed that deletion of the amino acids between positions 100 and 130 eliminates the production of the short protein product (Fig. 2A, cf. lanes 3 and 4). To determine whether the observations on the protein level were due to alterations in the primary sequence of the transcripts, RNA was purified from HeLa cells expressing the iADAR1 deletion constructs, and reverse transcription (RT)-PCR was performed with primers spanning from the 5'-end of the vector-encoded part until immediately downstream from the cADAR1 initiation codon (Fig. 2B). The constructs that generate two protein products (Fig. 2A, lanes 1–3) produce major RT-PCR products of ~650 base pairs (bp), which are shorter than the predicted sizes (Fig. 2C, lanes 1–3). Sequencing shows that the 650-bp products are identical and correspond to RNA transcripts that have been spliced using the 5'-splice site of the vector-encoded SV40 intron and a previously uncharacterized 3'-splice site at position 379 in exon 2 in the *ADAR1* sequence (Fig. 2D). This splicing leads to the removal of the first start codons (both the HA and the iADAR1 AUGs), which makes the initiation codon for cADAR1 the first optimal initiation codon in the transcript. Hence, this heterologous splicing leads to the production of a transcript that encodes cADAR1. Furthermore, we presume that the iADAR1 protein species that are detected originate from less abundant mRNA species, for which the PCR products are not readily detected. In contrast, the constructs that produce a single protein product all give double bands with the sizes expected for transcripts with and without the SV40 intron (Fig. 2C, lanes 4–7), and sequencing confirmed this (data not shown).

In order to confirm that the observed splicing is the main reason for the production of cADAR1 from the construct encoding iADAR1, we asked whether mutation of the 3'-splice site and the polypyrimidine tract in a manner that inhibits the splicing but preserves the protein-coding potential (Fig. 2E) would lead to the expression of iADAR1

FIGURE 2. (Legend on next page)

only. Upon transient transfection of HEK293T cells, the expressed proteins were detected by Western blotting using an anti-Flag antibody, and it is evident that the mutant iADAR1 construct gives rise to iADAR1 protein only (Fig. 2E).

Next, we examined whether the novel 3'-splice site is functional in its natural context. Indeed, the GenBank EST database contains several sequences corresponding to mRNAs that have been spliced between positions 242 and 379 in exon 2. The excised region contains the common features of an intron: putative 3'-splice site; branch-point sequence, and polypyrimidine tract all matching the consensus sequences; and a putative 5'-splice site sequence, AG'GCAAGU, which corresponds to the consensus sequence with the relatively common exception that the second intronic position is a C instead of the consensus U (Fig. 3C; Thanaraj and Clark 2001).

To verify the existence of the novel intron, RNA was extracted from the human cell line HEK293 and analyzed by RT-PCR. The PCR primers were chosen to prime within exon 2, flanking the putative intron (Fig. 3A). Two RT-PCR products corresponding to unspliced and spliced transcripts were observed (Fig. 3B, lanes 1–3). Subsequent sequencing verified the predicted identities of the products. Accordingly, it can be concluded that alternative splicing occurs within exon 2 of the *ADAR1* transcript in HEK293 cells.

FIGURE 2. Heterologous splicing is responsible for the production of two ADAR1 species from pCMV-HA-iADAR1-FLAG. (A) Western blot showing the indicated deletion mutants of iADAR1. Flag- and HA-tagged ADAR1 variants were transiently expressed in HeLa JW36 cells. The overexpressed proteins were detected by anti-Flag antibody, which is specific for the carboxyl terminus of the exogenous proteins. (B) Schematic representation of the 5'-end of the heterologous transcript derived from the pCMV-HA-iADAR-FLAG construct and the primers used for the RT-PCR analysis presented in this figure. (Gray) The vector-encoded part (pCMV-HA); (black) the iADAR1 open reading frame (ORF); (white) the vector-encoded SV40 intron. The initiation codons for iADAR1 (AUG1) and cADAR1 (AUG1') are indicated. Relevant nucleotide positions are denoted relative to the first nucleotide in *ADAR1* exon 2. Amino acid positions in iADAR1 are in brackets below the nucleotide position. (C) Total RNA was analyzed by RT-PCR. Reactions without reverse transcriptase were included as controls for DNA contamination (–RT, lanes 9–16). (*) The position of the band corresponding to the spliced (SV40 intron) version of the transcript encoding cADAR1, which is difficult to distinguish in this figure. (D) The observed splicing is indicated by dashed lines on the schematic drawing of the investigated transcript (depicted as in B). The sequence at the exon–exon junction is shown—(gray) vector sequence; (black) *ADAR1* sequence. The 5'-splice site of the SV40 intron and the 3'-splice site of a previously unidentified alternative intron of ADAR1 are spliced. (E) The coding-conservative mutations introduced in the 3'-splice site and the polypyrimidine tract of the novel intron in pCMV-HA-iADAR1-FLAG are shown next to a Western blot comparing mutant iADAR1 and cADAR1 constructs. Flag- and HA-tagged ADAR1 variants were transiently expressed in HEK293T cells, and the overexpressed proteins were detected by anti-Flag antibody.

The alternatively spliced intron in exon 2 is conserved in primates

To test for conservation of the splicing, RNA from the COS-7 (African green monkey) and N2A (mouse) cell lines was subjected to RT-PCR and compared to the equivalent products from the HEK293 cell line (Fig. 3B, lanes 4–9). The RNA from COS-7 gives rise to two RT-PCR products corresponding to unspliced and spliced transcripts. Significantly, the band corresponding to the spliced transcript from the monkey has a lower mobility than the human counterpart. By sequencing of the DNA fragments, it was found that, although the human 5'-splice site is completely conserved in the COS-7 sequence (Supplemental Fig. S1), another splice site further downstream is used (Fig. 3C). This splice site is presumably preferred due to a single nucleotide change compared to the human sequence (AGGGGCGT→AG'GTGCGT), which means that the intron starts with the more optimal consensus GU.

The RNA from the murine N2A cells only gives rise to a single RT-PCR product corresponding to the unspliced variant (Fig. 3B), indicating that the newly identified intron is not excised to a significant degree in mice. The sequence of the corresponding region revealed that neither the human nor the African green monkey 5'-splice site is conserved in the mouse (Supplemental Fig. S1).

To expand the phylogenetic analysis, the published nucleotide sequences of most of the 5'-end of *ADAR1* exon 2 from chimpanzee, rhesus monkey, cow, dog, mouse, and rat were included in an alignment (Supplemental Fig. S1). Surprisingly, the published mouse sequence differs notably from the N2A sequence (84% and 75% identity at the nucleotide and amino acid levels, respectively). The human 5'-splice site is conserved among the primates, but the rhesus monkey has the same potential for an alternative downstream splice site as the African green monkey (Supplemental Fig. S1). In contrast, none of the potential 5'-splice sites is conserved in cow, dog, mouse, and rat, suggesting that the novel intron in its different versions is a characteristic of the primates.

Promoter-specific splicing and tissue- and cell-type distribution of ADAR1 transcripts

Particular splicing events can be dependent on the specific promoter that drives the expression of the RNA (Cramer et al. 1999; Kadener et al. 2001). Therefore, it was investigated whether the splicing occurs in the *ADAR1* transcripts containing exons 1A, 1B, and 1C, respectively. To this end, PCR primers were designed as shown in Figure 4A, with the forward primer complementary to the specific first exon (1A, 1B, or 1C) and the reverse primer matching either the exon junction (spliced) or the intron (unspliced). The specificity of the primer pairs is confirmed in Supplemental Figure S2A. RNA was extracted from five human cell lines of different tissue origin and analyzed by RT-PCR

FIGURE 3. Verification of alternative splicing of *ADAR1*. (A) Schematic outline of the 5'-end of the *ADAR1* transcripts. (Gray) The alternative first exons; (white) the putative alternative intron. The positions of the primers used for the RT-PCR analysis shown in this figure are indicated by arrows. The sizes of the specific PCR products are shown. Indicated nucleotide positions are relative to the first base in exon 2. For simplicity, the three first exons are depicted as one. (B) Total RNA from the indicated cell lines was analyzed by RT-PCR using either 27 cycles (lanes 1,4,7) or 30 cycles (lanes 2,5,8) in the PCR reactions. Reactions without reverse transcriptase were included as controls for DNA contamination (lanes 3,6,9). (C) The sequence of the novel intron is presented in the context of the sequence of the 5'-end of exon 2 (1586 nt in total). (Gray boxes) The consensus sequences important for splicing. (Box with dashed edges) The downstream 5'-splice site used in green monkey *ADAR1*; the single nucleotide change (G to T) that enables splicing from this position is shown above the human sequence.

using the described primers. As is evident from Supplemental Figure S2B, removal of the intron takes place in all the three tested transcripts and in all the tested human cell lines (Supplemental Fig. S2B).

Next, it was investigated whether the alternative splicing is regulated in a tissue-specific manner. Total RNA from a series of tissues was analyzed by reverse transcription followed by quantitative PCR. The mRNA encoding the transcription factor TAFII30 was included as a control since it is expressed at similar levels in different cell lines (Supplemental Fig. S2B) and in most tissues (with the exception of testis) (Fig. 4B). From this experiment, it can be concluded that *ADAR1-A*, *-B*, and *-C* are expressed in all tested tissues (Fig. 4B), with *ADAR1-B* expressed at higher levels than *ADAR1-A*, which is expressed at higher levels than *ADAR1-C*. The levels of the *ADAR1* transcripts are all very low in skeletal muscle, but otherwise, the expression profiles for the transcript variants are different. *ADAR1-A* is expressed at the highest levels in lung and thymus, whereas *ADAR1-B* is most abundant in both adult and fetal brain. The expression of *ADAR1-C* is at its maximum in lung, small intestine, thymus, spinal cord, and testis.

The spliced variants are also produced in all of the tested tissues. The ratio of spliced versus unspliced transcript is low and relatively constant for *ADAR1-A* (0.07–0.17) and *ADAR1-B* (0.06–0.09), while the ratio for *ADAR1-C* is higher and varies more (from 0.19 in the small intestine to 0.62 in fetal liver). This pattern is generally confirmed in the cell lines investigated (data not shown; Fig. 5; Supplemental Figs. S2–S4). Thus, the alternative splicing in exon 2 is regulated in relation to the specific first exon and promoter. Furthermore, the alternative splicing of *ADAR1-C* is regulated tissue-specifically.

The spliced PTC-containing *ADAR1-A* transcript is not an NMD substrate

Depending on whether the transcript encodes cADAR1 or iADAR1, removal of the novel intron will either alter the 5'-UTR or the reading frame, respectively. In the case of alternatively spliced *ADAR1-A*, a PTC is encountered immediately downstream from the exon–exon junction. In the rhesus and green monkey species, the changed position of the 5'-splice site still gives rise to a PTC upon

splicing, which provides a phylogenetic indication of a possible regulatory function of the alternative splicing.

ADAR2 undergoes an autoregulated alternative splicing event that introduces a PTC in the 5'-end of the mRNA (Rueter et al. 1999). Due to the similarities to the homolog *ADAR1*, it is obvious to speculate that the alternative splicing of *ADAR1* could be controlled by a similar mechanism. However, we found that the splicing of the endogenous *ADAR1* transcripts fails to respond to stable overexpression of either of the ADAR1 protein isoforms in HEK293 FLP-In T-rex cells (Supplemental Fig. S3).

Evidently, human spliced *ADAR1-A* is a potential non-sense-mediated decay (NMD) substrate due to the presence of the PTC. siRNA-mediated knockdown of hUpf1 was employed to examine whether the NMD machinery has any influence on the levels of the various *ADAR1* transcripts and spliced *ADAR1-A* in particular. Efficient knockdown of hUpf1 was demonstrated by Western blotting (Fig. 5A). Furthermore, the functional knockdown of the NMD pathway was confirmed by quantitative RT-PCR analysis of cotransfected β -globin reporters β wt and β ter39 (Lykke-Andersen et al. 2000), where it was established that the latter is an NMD substrate due to the presence of a PTC at codon position 39 (Fig. 5B). The levels of unspliced and alternatively spliced *ADAR1* variants were also assessed by quantitative RT-PCR (Fig. 5C). From this experiment, it is apparent that the levels of both spliced and unspliced *ADAR1* transcripts are unchanged upon depletion of hUpf1.

Both alternatively spliced and unspliced *ADAR1* transcripts are translated

Since the alternatively spliced *ADAR1-A* transcript is not degraded by NMD, we speculated that an amino-terminal iADAR1 peptide of 87 amino acids (first 86 amino acids identical to iADAR1) could be produced from this mRNA. Attempts on immunodetection of the putative peptide by using a polyclonal antibody raised against amino acids 9–240 of iADAR1 (K88 #3) (Patterson and Samuel 1995) failed (data not shown). However, overexpression of tagged versions of the amino-terminal peptide revealed that this antibody does not recognize this part of iADAR1 (data not shown).

To address whether spliced *ADAR1-A* is, in fact, translated, we performed a sucrose gradient fractionation on extracts from HEK293T cells that were either untreated or treated for 2 h with the translational inhibitor puromycin. Total RNA was extracted from each of 10 fractions and analyzed for the presence of the different *ADAR1* transcripts by semiquantitative RT-PCR (Fig. 6). As seen from Figure 6A, the total RNA distribution is altered when cells are treated with puromycin. The observed shift is due to the dissociation of polysomes that migrate to the bottom-most fractions of the sucrose gradient. The distributions of unspliced and spliced *ADAR1-A* are different for the

FIGURE 4. (Legend on next page)

untreated sample. However, both transcripts change distribution and display identical sucrose gradient profiles after puromycin treatment, which indicates that both transcripts are translated (Fig. 6B). The shorter open reading frame of the spliced transcript could be responsible for the difference between the profiles of spliced and unspliced transcripts. Thus, it appears that the spliced *ADAR1-A* mRNA is translated.

The sucrose gradient fractionation also addresses possible differences between the unspliced and spliced *ADAR1-B* and *-C* transcripts with regard to translation, which is relevant since retained introns in the 5'-UTR can block translation of certain transcripts (e.g., *Msl2* from *Drosophila melanogaster*) (Gebauer et al. 1998). When comparing the relative distributions of the investigated mRNA species, it is apparent that both unspliced and alternatively spliced *ADAR1-B* mRNA are associated with the translational machinery at the same relative levels in untreated cells. This is evident since the unspliced and spliced transcripts have identical profiles and they change mobility in the gradient upon treatment with puromycin (Fig. 6B). The same is evident for unspliced and alternatively spliced *ADAR1-C* (Fig. 6B). However, the distributions of *ADAR1-B* and *-C* mRNAs are different, suggesting that they are differently associated with the translational machinery.

DISCUSSION

Identification of a novel intron

The discovery of a heterologous splicing event in ectopically expressed mRNA encoding iADAR1 (Fig. 2) inspired the identification of a previously unidentified intron situated within the 5'-end of exon 2 in primate *ADAR1* transcripts (Fig. 3). The intron is present in all three human

ADAR1 transcript variants that are derived from different promoters, and it was established that all the variants are subject to alternative splicing within exon 2 (Supplemental Fig. S2).

Although the phylogenetic analysis indicated that there is a high degree of conservation at the nucleotide level within the investigated region (Supplemental Fig. S1), it is noteworthy that the discovered intron appears only to be present/functional in the primate species, but not in the rodents and other mammalian species investigated here. Furthermore, we were not able to find any conservation of the intron in the published sequences from frog and fishes (data not shown).

Interestingly, in a study by Kawakubo and Samuel (2000), a significant promoter activity was detected to originate from a region defined by the *ScaI* and *HinfI* restriction enzyme recognition sites in exon 2 of human *ADAR1*. This region (position 216–333 in exon 2) partly overlaps with the 5'-part of the alternative intron described in this study (position 243–378 in exon 2). How these data can be correlated is presently unclear.

Tissue distribution

By analyzing a panel of RNA samples derived from various human tissues, the promoter-specific *ADAR1* transcripts were found to be universally expressed. However, the transcript levels vary considerably between tissues, and the expression patterns are different for the *ADAR1-A*, *-B*, and *-C* transcripts (Fig. 4B). These data are in accordance with previous studies in which the *ADAR1* mRNA (detected as one single species by Northern blotting) was shown to be expressed in all tested fetal and adult human tissues (Kim et al. 1994; O'Connell et al. 1995; Lai et al. 1997). Although the levels of the *ADAR1* transcripts vary between the tissues, the *ADAR1-B* transcript is always the most abundant. It is expressed at ~20-fold higher levels than *ADAR1-A* in adult and fetal brain, which is in clear accordance with observations on mouse *ADAR1-B* (George et al. 2005). However, in the lung, small intestine, spleen, thymus, thyroid, and placenta, the *ADAR1-B* and *ADAR1-A* mRNAs are expressed more equally. *ADAR1-C* is generally expressed at very low levels compared to the other variants (approximately 10- to 250-fold below *ADAR1-B* levels and approximately 4- to 20-fold below *ADAR1-A* levels).

The alternative splice variants that were discovered in this study are usually present at levels that follow the overall quantities of the promoter-specific transcripts in the given tissue. On average, the measured ratios of spliced versus unspliced transcripts are 0.07 and 0.13 for *ADAR1-B* and *-A*, respectively, and the relative levels are roughly consistent in all the investigated tissues (Fig. 4B). However, the alternative splicing within exon 2 of *ADAR1-C* seems to occur at higher relative levels than in *ADAR1-A* and *-B* (the

FIGURE 4. Tissue distribution of *ADAR1* transcripts. (A) Schematic outline of the 5'-end of the unspliced and spliced *ADAR1* transcripts. (Gray) The alternative first exons; (white) the alternative intron. The positions of the primers used for the RT-PCR analysis presented in this figure, Figure 5, and Supplemental Figures S2 and S3 are indicated by arrows. For simplicity, the three first exons and the corresponding primers are depicted as one. The sizes of the specific PCR products are shown. Nucleotides are numbered relative to the first base in exon 2. (B) Quantitative RT-PCR analysis of the tissue distribution of *ADAR1-A*, *-B*, and *-C*. The data are presented in histograms showing the levels of unspliced (dark gray columns) and alternatively spliced (light gray columns) *ADAR1* transcripts on the left axis and right axis, respectively. The ratios of spliced versus unspliced *ADAR1* transcripts are shown below the histograms. The bottom panel shows the data from quantitative RT-PCR analysis of the control transcript *TAFII30*. The numbers on the axes are absolute values reflecting the number of transcripts detected in a cDNA sample that was made from 25 ng of total RNA. Error bars indicate the standard deviation (each sample was measured in triplicate, and each standard curve was determined from 15 reactions).

FIGURE 5. The alternatively spliced *ADAR1-A* transcript is not an NMD substrate. HEK293 cells were double-transfected with siRNAs targeted to TEL/AML (control) or hUpf1. The indicated β -globin reporter constructs were cotransfected in the second transfection. (A) Western blot showing the efficient knockdown of hUpf1 (anti-hUpf1 antibody). hnRNP C1/C2 served as a loading control. (B) The functional shut-down of the NMD-pathway was confirmed by determining the levels of the β -globin reporters by quantitative RT-PCR. The levels of β -globin were normalized to the levels of neomycin mRNA expressed from the pcDNA3' vector. (C) *ADAR1-A*, *-B*, and *-C* transcripts were detected by quantitative RT-PCR. The levels of unspliced (dark gray columns; left axis) and spliced (light gray columns; right axis) *ADAR1* transcripts were normalized to the levels of *TAFII30*. The ratios of spliced to unspliced *ADAR1* transcripts are given below the histograms. Error bars indicate the standard deviation (each sample was measured in triplicate, and each standard curve was determined from 15 reactions; furthermore, measurements on the transcripts were averaged from the three independent cell populations expressing either mock, β wt, or β ter39).

average ratio of spliced versus unspliced *ADAR1-C* transcripts is 0.39 in the examined tissues) and also at varying levels in different tissues. The potential functional implication of this remains to be studied. It is known that the identity and the activity of the promoters can have pronounced effects on alternative splicing (e.g., Cramer et al. 1999), which may also be the explanation for our observation.

Alternatively spliced *ADAR1-A* is translated but evades NMD

There are important differences between the three promoter-specific *ADAR1* transcripts. For *ADAR1-A*, the entire sequence of exon 2 serves as an ORF for iADAR1. In contrast, the intron-containing region of the *ADAR1-B* and *-C* transcripts serves as the 5'-UTR. This difference obviously implies that exclusion of the novel intron can have different consequences. The removal of the 136-nt intron from the *ADAR1-A* transcript changes the reading frame, and a PTC is encountered immediately downstream from the splice junction. Importantly, this feature is conserved for the alternative splicing in African green monkey, where the excision of the 109-nt intron produces a similar frameshift. For the *ADAR1-B* and *-C* transcripts, the splicing leads to a 136-nt shortening of the 5'-UTRs. For both groups of transcripts, the alternative splicing could have regulatory effects. Evidently, spliced human *ADAR1-A* encodes a truncated protein product (87 amino acids; 10 kDa), but is also a potential NMD substrate since it contains a termination codon that is situated more than 50–55 nt upstream of the last exon–exon junction (the >50–55-nt boundary rule) (for review, see Maquat 2004). These characteristics are not unique since recent studies indicate that as many as 35% of all alternative splice variants in the human transcriptome lead to the introduction of PTCs (Green et al. 2003; Lewis et al. 2003).

The mentioned features of the spliced *ADAR1-A* are clearly similar to those of an alternative splicing event that has been demonstrated for the paralog *ADAR2* (Rueter et al. 1999). Since *ADAR2* autoregulates its own

expression via self-editing and subsequent alternative splicing (Rueter et al. 1999; Maas et al. 2001; Feng et al. 2006), it is obvious to speculate that this could also be the case for iADAR1 and possibly also cADAR1. However, careful analysis revealed that the *ADAR1* transcripts are neither controlled by autoregulation nor by NMD (Fig. 5; Supplemental Fig. S3). The latter is in agreement with a recent genome-wide study, which showed that many minor

FIGURE 6. Alternatively spliced *ADAR1-A* is associated with translating ribosomes. Sucrose gradient fractionation of lysates from mock- or puromycin-treated HEK293T cells. (A) Total RNA from the fractions was analyzed by agarose gel electrophoresis followed by ethidium bromide staining (two upper panels). The RNA levels in the samples were determined by OD260 measurements, and the relative distributions in the gradients are plotted (lower panel). (Triangles) Mock-treated fractions; (circles) puromycin-treated fractions. (B) Relative levels of the unspliced and alternatively spliced *ADAR1-A*, -B, and -C transcripts in the different fractions were determined by semiquantitative RT-PCR (see Materials and Methods for details), and the distributions are shown. (Triangles) Mock-treated fractions; (circles) puromycin-treated fractions; (closed triangles and circles) unspliced *ADAR1* transcripts; (open triangles and circles) alternatively spliced *ADAR1* transcripts.

alternative splice variant mRNAs with PTCs are, in fact, not degraded via the NMD pathway (Pan et al. 2006). In the same study, it was found that the majority of alternative splice variants with PTCs are of low abundance and are not conserved between man and mouse. Nonetheless, it is still interesting why these mRNAs are not recognized as NMD substrates. The features that distinguish premature from normal termination codons are still not completely characterized, and recent reports have shown deviations to the “>50–55-nt boundary rule.” For instance, it has been shown that the distance of a termination codon from the poly(A) tail is a crucial determinant of NMD—if this

distance is too large, the stop codon will be recognized as a PTC (Buhler et al. 2006). Additionally, mRNAs containing short ORFs (<18–20 codons) evade NMD (Silva et al. 2006). In the case of the alternatively spliced *ADAR1-A* transcripts studied here, the ORF consists of 87 codons, the PTC is upstream of several exon–exon junctions, and the distance to the poly(A) tail is thousands of nucleotides, which in theory would make it an optimal substrate for NMD. Thus, it is not evident why this transcript is immune to NMD.

Interestingly, judged by the sucrose gradient profiles, the spliced *ADAR1-A* transcript is associated with polysomes

(Fig. 6), which indicates that it is, in fact, translated. However, due to the lack of a specific antibody, it has not been possible to identify a corresponding truncated iADAR1 peptide by Western blotting. The putative expressed peptide does not have any apparent homology with any protein domains with known function, and thus it is difficult to assess whether it could be functionally significant. In a preliminary experiment, we found that overexpression of the peptide is unable to alter the editing efficiency of either iADAR1 or cADAR1 on the intronic position +60 in the GluR2 transcript (data not shown). However, this does not exclude that editing of other targets may be affected.

Importantly, we find that the alternatively spliced *ADAR1-A* mRNA responds similarly as unspliced *ADAR1-A* to induction by interferon α (approximately threefold increase in steady-state levels after 24 h of induction) (Supplemental Fig. S4), which indicates that the putative iADAR1 peptide as well as iADAR1 is induced as part of the interferon response. Future studies are needed to reveal whether the peptide is, in fact, expressed at significant levels and whether it plays a role in the interferon response.

Heterologous splicing in vector-encoded iADAR1 mRNA accounts for the observed ADAR1 protein heterogeneity

As mentioned previously, the finding that expression of vector-encoded iADAR1 mRNA also leads to the production of the cADAR1 isoform has been observed by other groups before (Fig. 2A; Sato et al. 2001; Wong et al. 2001, 2003; Wong and Lazinski 2002; Desterro et al. 2003). Using similar vector constructs, we here show that the observation is not a translational phenomenon but originates from a heterologous splicing event between a vector-encoded 5'-splice site (SV40 intron) and the previously unknown 3'-splice site of the intron in exon 2 of *ADAR1* (Fig. 2D). It is clear that the splicing is the sole reason for the observations in the present study, since coding-conservative mutations of the 3'-splice region (no amino acid changes) give rise to iADAR1 protein only (Fig. 2E). It is reasonable to assume that similar splicing events also are responsible for the production of cADAR1 in the other studies mentioned. In a study from Lazinski and coworkers, the production of the two ADAR1 isoforms was observed from a vector that contains most of the 5'-UTR from exon 1A. The predominant production of cADAR1 was reduced considerably when the exon 1A 5'-UTR sequence was deleted and the iADAR1 initiation codon was placed in a more optimal Kozak context (Wong et al. 2003). Nevertheless, it remains to be established whether this observation is caused by an increase in translation initiation from the iADAR1 start codon and therefore a reduction in translation initiation from the cADAR1 start codon in the same

transcript or by a reduction of an uncharacterized alternative splicing event that excises the first initiation codon. Importantly, the same report points to the existence of the alternative intron described herein, since a deletion of amino acids 76–133 in iADAR (corresponding to nucleotides 211–384 in exon 2, which includes all of the intron) leads to a dramatic increase in the production of the long versus the short isoform (Wong et al. 2003).

MATERIALS AND METHODS

Sequences

The sequences of the splice variants were submitted to GenBank (accession numbers: *ADAR1-A* spliced/unspliced: EF190448/EF190449; *ADAR1-B* spliced/unspliced: EF190451/EF190450; *ADAR1-C* spliced/unspliced: EF190452/EF190453).

Sequences corresponding to nucleotides 133–867 of exon 2 of human *ADAR1* were obtained from African green monkey (COS-7) and mouse (N2A). Total RNA from these species was reverse-transcribed followed by PCR using degenerate primers CARATH GARTTYTNAARGGN and YTCYTDDATYTCNGCCATRTC. The former primer was designed based on the “most upstream” conserved amino acid stretch of seven residues (QIEFLKG) in the known mammalian ADAR1 proteins. The obtained PCR products were inserted into the vector pCR-4-TOPO (Invitrogen), and individual clones were sequenced by standard sequencing procedures. The obtained sequences were also submitted to GenBank: *Chlorocebus aethiops* (COS-7) spliced/unspliced: EF190456/EF190455 and *Mus musculus* (N2A): EF190454.

The alignments presented in Supplemental Figure S1 include the sequences found in this study and published ADAR1 sequences from human (BC038227), chimpanzee (XM_513841), rhesus monkey (XM_001111902), cow (XM_581374), dog (XM_547564), mouse (AF291876), and rat (NM_031006).

Plasmid constructs

The open reading frames of iADAR1, cADAR1, and deletion mutants thereof were amplified using pEGFP(C1)-iADAR1 (Poulsen et al. 2001) as the template. A sequence corresponding to a Flag-tag was incorporated into the 3'-end of the PCR fragments, and the obtained products were inserted into the pCMV-HA (BD biosciences/Clontech) vector by standard cloning procedures.

RT-PCR products obtained from *TAFII30* and the alternative splice variants of *ADAR1-A*, *-B*, and *-C* were inserted into the vector pCR4-TOPO (Invitrogen), and the obtained plasmids were used to make standard curves for quantitative PCRs.

The sequences of all constructs were verified by standard sequencing procedures. All sequences of the primers used in this study are available upon request.

Cell culture and transient transfection

All cell lines were cultured at 37°C and 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and supplemented with penicillin and streptomycin (Invitrogen). HeLa JW36 cells were transiently transfected using a Lipofectamine

(Invitrogen) mixture containing 0.1–0.2 µg of plasmid per cm². Protein and total RNA were isolated 1 d later.

The siRNA-mediated knockdown was carried out by a double-transfection procedure. First, 2×10^5 HEK293 cells were seeded in 3.5-cm wells. One day later, the cells were transfected with 45 pmol of siRNA using siLentFect (Bio-Rad). After 2 d, the cells were re-transfected with 45 pmol of siRNA and 2 µg of the indicated plasmids using Lipofectamine 2000 (Invitrogen). Protein and total RNA were isolated after two additional days. The sequences of the siRNA target sequences for hUpf1 and control (TEL/AML) are AAGAUGCAGUCCGCUCAUU (Mendell et al. 2002) and AAGGAGAAUAGCAGAAUGCAU, respectively.

Reverse transcription

Total RNA was prepared from cell lines using either Trizol reagent (Invitrogen) or RNeasy (QIAGEN) according to the manufacturers' instructions. RNA samples were cleared of DNA contamination by incubation with 5–10 units (for a 3.5-cm dish) of DNase I, FPLCpure (Amersham Biosciences) for 30–60 min at 37°C, and the RNA was recovered by phenol (pH 6.6)/chloroform extraction and ethanol precipitation according to standard protocols. Total RNA from human tissues was obtained from BD biosciences/Clontech (Human Total RNA Master Panel II; Lot nr: 4,020,700).

The reverse-transcription (RT) reactions represented in Figure 2 were carried out as described previously (Mili et al. 2001). All other RT reactions were performed using random hexamer primers (Invitrogen) and Superscript II reverse transcriptase (Invitrogen) on 4 µg (Supplemental Fig. S2), 1 µg (Figs. 3–5; Supplemental Figs. S3, S4), or up to 4 µg (Fig. 6) of DNase I-treated total RNA.

RT-PCR

The nonquantitative RT-PCR reactions presented in Figure 2 and Supplemental Figure S2 were performed according to standard protocols. The PCR products were resolved by agarose gel electrophoresis and visualized by staining with ethidium bromide.

The quantitative RT-PCR reactions represented in Figures 4 and 5 and Supplemental Figures S3 and S4 were carried out on one-twentieth of the total RT sample using a SYBR green Q-PCR kit (Invitrogen). Each sample was analyzed in triplicate for each amplicon, and the absolute amounts of *TAFII30* and *ADAR1* cDNA in each sample were determined by the use of standard curves made from reactions on known amounts of plasmids encoding the analyzed amplicons. The samples were processed and analyzed by use of an MX3005P real-time PCR machine (Stratagene) using the preset conditions with the exception of the annealing temperature, which was set to 60°C instead of 55°C.

The semiquantitative RT-PCR reactions represented in Figure 6 contained forward primers with complementarity to the specific first exon (1A, 1B, or 1C) and a ³²P-5'-end-labeled reverse primer that anneals downstream from the intron. Thus, both the unspliced and spliced versions of a specific *ADAR1* variant were amplified. The PCR products were resolved by denaturing 4% polyacrylamide gel electrophoresis followed by visualization and quantification by PhosphorImager analysis. The quantitative nature of this method was validated by analysis of several RNA dilution series (data not shown), and the reason it is referred to as

semiquantitative is due to the different amounts (up to 4 µg) of input RNA in the RT reaction.

Western blotting

Cell extracts and Western blotting were prepared and carried out by standard procedures. The following antibodies were used for immunoblot analysis: anti-hnRNP C1/C2 (4F4) (Pinol-Roma et al. 1988) and anti-hUpf1 (Lykke-Andersen et al. 2000).

Sucrose gradient fractionation

For the polysome analysis, HEK293T cells were grown under normal growth conditions. Two hours before harvest, the cells were either mock-treated or supplemented with 100 µg/mL puromycin. Immediately before harvest, the cells were incubated with 50 µg/mL cycloheximide and subsequently washed in cold PBS containing 50 µg/mL cycloheximide. The cells were scraped and lysed in RSB100 (10 mM Tris/HCl at pH 7.4; 100 mM NaCl; 2.5 mM MgCl₂) containing 0.5% Triton X-100 and 50 µg/mL cycloheximide. After incubation for 10 min on ice, the nuclei were removed by centrifugation at 12,000g for 5 min. The supernatant (400 µL per 10-cm plate) was layered onto a 3.6-mL 10%–50% sucrose gradient (manually assembled) and centrifuged in an SW60 rotor at 41,300 rpm for 75 min at 4°C. Fractions of 400 µL were collected from the bottom using a capillary tube coupled to a pump. Fractions were adjusted to 0.5% SDS, and RNA was extracted using phenol (pH 6.6)/chloroform and ethanol precipitation according to standard protocols. Subsequently, the RNA concentration in each fraction was determined by measuring OD₂₆₀.

SUPPLEMENTAL DATA

Supplemental Figures S1–S4 are available at www.rna.dk/suppl/rnal.

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Alternative splicing of the *ADAR1* transcript in a region that functions either as a 5'-UTR or an ORF

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