Expression of Interferon-inducible RNA Adenosine Deaminase ADAR1 during Pathogen Infection and Mouse Embryo Development Involves Tissue-selective Promoter Utilization and Alternative Splicing*

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ADAR1 (adenosine deaminase acting on RNA) is widely expressed in adult mammals and has a critical role during embryogenesis. Two size forms of ADAR1 are known that possess adenosine-to-inosine editing activity: an interferon (IFN)-inducible ~150-kDa protein and a constitutively expressed N-terminally truncated ~110-kDa protein. We defined the structure of the 5'-flanking region of the mouse Adar1 gene, and we show here that mouse Adar1 transcripts possess alternative exon 1 structures (1A, 1B, and 1C) that initiate from unique promoters and are spliced to a common exon 2 junction. Exon 1A-containing transcripts encoding p150 were expressed in all tissues examined from adult mice (brain, cecum, heart, kidney, liver, lung, spleen, and Peyer's patches) and were elevated most significantly in liver but remained lowest in brain following oral infection with Salmonella. Exon 1B-containing RNA was most abundant in brain and was not increased in any tissue examined following infection. Exon 1C-containing RNA was very scarce. Exon 1A, but not exon 1B or 1C, expression was increased in fibroblast L cells treated with IFN, and a consensus ISRE element was present in the promoter driving exon 1A expression. Exon 1B, but not 1A, was detectable in embryonic day 10.5 embryos and was abundantly expressed in embryonic day 15 embryos. Furthermore, the ADAR1 p110 protein isoform was detected in embryonic tissue, whereas both p110 and the inducible p150 proteins were found in IFN-treated L cells. Finally, the presence of alternative exon 7a correlated with exon 1B-containing RNA, and alternative exon 7b correlated with exon 1A-containing RNA. These results establish that multiple promoters drive the expression of the Adar1 gene in adult mice, that the IFN inducible promoter and exon 1A-containing RNA are primarily responsible for the increased ADAR1 observed in Salmonella-infected mice, and that the constitutive exon 1B-containing transcript and encoded p110 protein product are abundantly expressed both in adult brain and during embryogenesis.

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Adenosine (A) to inosine (I) RNA editing is a post-transcriptional process by which an RNA sequence is modified by C-6 deamination of one or more A's, leading to the formation of transcripts whose sequence differs from that found in the genome (1–4). Such A-to-I editing represents a form of genetic recoding, because inosine is recognized as guanosine by decoding ribosomes and by transcribing polymerases. Enzymes that catalyze A-to-I editing are known as ADARs (adenosine deaminases that act on RNA) (5). ADARs deaminate RNA substrates with double-stranded structure. In fact, the enzymatic activity of ADARs was discovered as an RNA duplex unwinding activity (6, 7).

A-to-I editing is of broad biologic significance. Among the best characterized cellular and viral RNAs that undergo A-to-I editing with high selectivity at one or a few positions are transcripts encoding glutamate receptor (GluR) channels, the serotonin-2C receptor, and hepatitis delta virus RNA (8, 9). RNA editing of the neurotransmitter receptor transcript subfractions is specified by imperfect duplex RNA structures, and the editing leads to selective amino acid substitutions in the encoded receptor proteins that alter either the conduction properties in the case of the GluR ion channels or G-protein coupling function in the case of the serotonin-2C receptor (1, 8). Editing of the viral hepatitis delta virus RNA allows for the production of two forms of delta antigen from a single open reading frame; conversion of an amber UAG translation termination codon to a UIG tryptophan codon permits the synthesis of a C-terminally extended protein (9, 10). In addition to the highly selective A-to-I deamination, A-to-I (G) hypermutation has also been observed for several negative-stranded RNA animal virus genomes during lytic and permissive infection, as exemplified by the measles virus (11). However, transcripts encoded by polyoma virus, a double-stranded DNA virus, also are edited late in infection (12). Although the cellular GluR and serotonin-2C receptor substrates were identified serendipitously, more recently several thousand candidate A-to-I editing sites were discovered by a computational search of the human transcriptome using large numbers of expressed sequences (13). These newly identified sites, together with a more limited number found using a cloning strategy for I-containing RNAs in Caenorhabditis elegans (14), occur primarily in noncoding regions of RNA and often in Alu repeats (13).

The abbreviations used are: A, adenosine; I, inosine; IFN, interferon; RT, reverse transcription; RACE, rapid amplification of cDNA ends; Ea, embryonic day; GluR, glutamate receptor; BAC, bacterial artificial chromosome; ORF, open reading frame; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ADAR1, adenosine deaminase 1 acting on RNA.
Two mammalian genes, Adar1 and Adar2, encode catalytically active RNA adenosine deaminases that efficiently edit synthetic and naturally occurring substrates with double-stranded RNA character (1–4). The ADAR1 and ADAR2 enzymes display different activities for the different editing sites in the GluR-B and serotonin-2C receptor transcript RNAs (9, 15–20). ADAR1 and ADAR2 both possess multiple copies of a canonical double-stranded RNA-binding motif that are located in the central region of the proteins: three double-stranded RNA-binding motif copies in ADAR1 and two in ADAR2 (3, 4). The double-stranded RNA-binding motifs of ADARs are distinct from the deaminase catalytic domain, which is positioned in the C-terminal region of the ADAR proteins (4, 21, 22). Although the double-stranded RNA-binding motifs are presumed to play a role in the binding of substrate RNAs, they might also play a role in the regulation of enzymatic activity. For example, adenovirus VA RNA, a small and highly structured viral gene product transcribed by cellular RNA polymerase III, efficiently antagonizes ADAR deaminase activity (23).

The regulation of ADAR1 protein expression in cells is a complex process. Two differently sized ADAR1 enzyme isoforms, p150 and p110, are known (24). The larger form of ADAR1, p150, is an interferon (IFN)-inducible protein that is found in both the cytoplasm and nucleus of human cells (24, 25). The inducible p150 isoform of ADAR1 possesses, in addition to the multiple RNA-binding motifs (21, 24), two copies of a Z-DNA-binding motif (Za and Zb) in the N-terminal region of the protein (24, 26, 27). The constitutive p110 isoform, which lacks the N-terminal 295 amino acids that include the Z-binding motif, is found predominantly if not exclusively in the nucleus (24, 28). For the ~50-kb human Adar1 gene, alternative promoters including one inducible by IFNs and at least two that are constitutively active, give rise to transcripts containing alternative exon 1 structures with the capacity to encode either the constitutive p110 protein or the inducible p150 protein (29–31). Additional ADAR protein multiplicity is achieved by alternative splicing involving downstream exons, in the cases of both ADAR1 and ADAR2 (32–34). For ADAR1, the two major downstream exon splice variants observed involve alternative exon 7 structures (32). The full physiologic significance of the internal exon 7a and 7b splice variants of ADAR1 is not known; one possibility may relate to the substrate selectivity of the enzymes (18, 19, 22, 32).

The mammalian Adar1 gene is ubiquitously expressed in most tissues examined (24, 35–37). Furthermore, there is an essential requirement for ADAR1 during embryogenesis. Mouse embryos homozygous null for the Adar1 gene die between E11 and E12.5, and they are characterized by a rapidly disintegrating liver structure and widespread apoptosis in embryonic tissue (39, 40). By contrast to the embryonic lethality of the Adar1 null, mice homozygous null for the Adar2 gene die several weeks after birth from a neurological syndrome, but they appear normal when the unedited CAG codon at the exon 11 Q/R ADAR2 editing site of GluR is replaced with the edited CIG codon in transgenic mice (38). In addition to the role of ADAR1 in embryo development possibly beyond the nervous system, ADAR1 also appears to play a role in innate immune responses. Increased ADAR1 transcript levels are seen in mice orally infected with Salmonella at doses that typically result in death 7–8 days after infection (37). Increased ADAR1 expression is also seen in mice treated with endotoxin, which leads to systemic acute inflammation (41). However, little is known with regard to the isoforms of ADAR1 expressed during embryogenesis or in uninfected versus bacteria-infected adult mice. Likewise, the mechanism by which ADAR1 gene expression is regulated during microbial infection is poorly understood.

In this study, we delineated the structure of the mouse Adar1 gene 5′-flanking region, which allowed us to undertake whole animal studies of ADAR1 expression in the mouse model. We found that in mice multiple alternative promoters drive the expression of ADAR1 transcripts that possess alternative exon 1 and 7 structures. We established that the alternative exon 1A-containing transcripts that encode the p150 ADAR1 isoform also contain exon 7b and that these transcripts are up-regulated in a number of tissues from adult mice following bacterial infection, most strikingly in liver. We found that alternative exon 1B/exon 7a-containing transcripts that encode the p110 ADAR1 isoform are abundantly expressed in the developing embryo and also in the brain of adult mice, and we detected the p110 protein both in embryonic tissue and uninfected adult tissue with antibody prepared against recombinant ADAR1. These results reveal a complex organization and regulation of Adar1 in mammals and provide novel insights regarding the function of the different isoforms of ADAR1 during embryogenesis and adult life that indicate a broad biological importance of ADAR1.

**EXPERIMENTAL PROCEDURES**

Isolation and Characterization of ADAR1 Genomic Clones—A mouse genomic library in the λ phage vector EMBL-3SP6(hT) (Clontech) was screened initially by filter hybridization using random primed 32P-labeled restriction fragment probes from the mouse ADAR1 cDNA as probes. A specific ~0.8-kb genomic DNA probe that included contiguous exon 2 and intron I sequence then was used to obtain λ phage clones carrying genomic sequence 5′ to exon 2, including λ 52. Subsequently, the mouse bacterial artificial chromosome (BAC) genomic library CichiJF7 (California Institute of Technology) was screened for BAC clones carrying the mouse Adar1 gene, yielding clones BAC 229 and BAC 232 (42). Genomic inserts were characterized by restriction mapping and Southern blot analysis (43). Restriction fragments from the λ 52 and BAC 232 genomic clones were then subcloned into the pBluescript plasmid for detailed restriction mapping and DNA sequence analysis.

**Determination of the 5′-cDNA Region**—The 5′-region of the mouse Adar1 cDNA was obtained by the 5′-rapid amplification of cDNA ends (RACE) procedure (44) by using the Marathon-Ready cDNA system (Clontech) according to the manufacturer’s recommendations. A library of adaptor-ligated cDNA prepared from mouse brain was used to amplify the 5′-end of the mouse Adar1 cDNA. The first round PCR was performed with an Adar1 exon 2 minus sense primer (E2 minus 842) corresponding to nucleotides 842–822 (5′-CAATGCTAATGACGCT- GAGGT-3′) of exon 2 (numbered with the 5′-end of exon 2 designated as nucleotide 1) and the plus-sense adaptor primer AP1 supplied by Clontech. PCR was then performed with the Clontech primer AP2 and either the original minus primer or a new mouse Adar1 cDNA-specific minus primer (E2 minus 646) primer (5′-GCTCTAGGAAATTCTGAGT-3′) corresponding to nucleotides 646–627 of exon 2. The 5′-RACE PCR products were cloned into pBlueScript plasmid and sequenced, and the results were compared with the DNA sequences obtained for the genomic clones.

Sequence Analysis—Plasmid subclones were sequenced by the Sanger dye terminonucleotide procedure (45) by using T7 Sequenase version 2.0 from U.S. Biochemical Corp. Sequences were analyzed with University of Wisconsin Genetics Computer Group programs on a Silicon Graphics IRIS 4D/340VGX computer. The sequences obtained have been deposited in the GenBank database and assigned accession numbers AY488121–AY488123 and AY508855–AY508858.

**Southern Gel Blot Analysis**—Southern gel blot analyses (46) of λ phage and BAC genomic clones for the Adar1 gene were performed as described previously (24, 29). λ and BAC genomic clone DNAs were digested with restriction endonucleases, fractionated on agarose gels, and transferred to Hybond-N filter membranes (Amersham Biosciences) for hybridization analysis (43). The filters were probed with 32P-random labeled cDNA fragments or 32P end-labeled specific single-nucleotide probes corresponding to alternative exon 1 regions of mouse ADAR1 cDNA.

**Northern Gel Blot Analysis**—Northern gel blot analyses of RNA isolated from mouse L fibroblast cells and from mouse tissue samples were performed as described previously (24, 37). RNA was isolated from cultured L cells, either left untreated or treated with IFN, and from organs of mice either uninfected or infected with Salmonella, by

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RESULTS

Isolation and Characterization of 5'-RACE Clones of Mouse ADAR1—To define the 5'-terminal region of mouse ADAR1 mRNA transcripts, 5'-RACE was carried out using nested primers and an adapter-ligated cDNA library prepared from mouse brain. The ADAR1 primer E2 minus 842 (corresponding to antisense nucleotides 842–822 of exon 2) and the plus adapter primer AP1 first were used together, followed secondly by the nested primer pair ADAR1 E2 minus 646 (corresponding to antisense nucleotides 646–627 of exon 2) and plus adapter primer AP2. The resultant 5'-RACE cDNAs were subcloned into pBluescript SK plasmid and sequenced. Three classes of ADAR1 5'-RACE cDNA clones were identified on the basis of the unique 5'-sequences that diverged from each other exactly at the exon 1-exon 2 junction (Fig. 1).

One form of mouse ADAR1 5'-RACE cDNA clone possessed the 5'-sequence identified earlier (12, 40) as exon 1 and hence was designated herein as exon 1A. An AUG codon was present in the mouse exon 1A, which initiated the long reading frame predicted from the mouse ADAR1 cDNA to encode a protein of ~130 kDa (12), similar to what was found for the human ADAR1 exon 1A-containing RNA (29, 32). The mouse ADAR1 exon 1A is 44 nucleotides in length, and except for the presence of the AUG initiation codon, there was no extensive sequence homology between the human and mouse exon 1A sequences. The second class of 5'-RACE cDNA clone identified was designated as exon 1B; this class was the most abundant of the three classes of 5'-RACE clones obtained from a mouse brain library. The longest 5'-RACE-1B cDNA clone extended 106 nucleotides upstream of the exon 2 junction (Fig. 1). The third and least abundant kind of unique 5'-RACE cDNA obtained was called exon 1C, which extended 31 nucleotides upstream of the exon 2 junction (Fig. 1). Neither exon 1B nor exon 1C sequences contained an AUG translation initiation codon. Therefore, initiation of exon 1B- and 1C-containing mRNA translation would occur at an AUG located in exon 2 that corresponds to amino acid position Met249 (of the long ORF beginning at AUG1 in exon 1A), consequently reducing the size of the encoded protein from the observed ~150 kDa (exon 1A start) to ~110 kDa (exon 1B or 1C start).

Genomic Organization of Alternative Exon 1 Structures—Overlapping λ phage and BAC clones containing the mouse Adar1 gene were isolated (42) and characterized by restriction mapping and Southern blot analyses to map the positions of exons 1A, 1B, and 1C. A composite map for the Adar1 gene 5'-region was determined (Fig. 2A). An exon 1A-specific probe hybridized to a ~5.3-kb SacI fragment from λ 52 genomic DNA, from BAC 232 genomic DNA (and also BAC 229 DNA, which is not shown), and from genomic DNA isolated from mouse L fibroblast cells (Fig. 2B). The precise exon 1A-intron 1 junction

**Fig. 1.** Nucleotide sequence of mouse Adar1 5'-RACE-derived cDNA clones. The three 5'-cDNA sequences diverge from each other beginning at the exon 2 junction. The unique 5'-sequences are designated alternative exon 1A, alternative exon 1B, and alternative exon 1C. The underlined sequences correspond to the exon 1-specific oligonucleotide primers; the ATG that specifies the translation start of p150 in exon 1A is shown in bold, uppercase letters. Exon 2 sequence is shown in bold, lowercase letters. The numbers at the right correspond to the nucleotide number +1 corresponding to the 5'-nucleotide of the respective alternative exon 1.
was determined by sequencing plasmid subclones of the Sacl fragment. Probes targeted specifically to the 5'-region of exon 2 also hybridized to the 5.3-kb Sacl fragment (data not shown). Further sequencing of plasmid subclones precisely positioned exon 2 at 4.2 kb downstream of exon 1A (Fig. 2A). The sequences obtained from the 5.3-kb Sacl fragment containing exon 1A, intron I, and the 5'-region of exon 2 were identical for genomic DNA from four sources: mouse L fibroblast cells, BAC clone 229, BAC clone 232, and ϕIV-H11032.

An exon 1B-specific probe hybridized to a ~6.7-kb Sacl fragment of genomic DNA from mouse L fibroblast cells and from BAC clones 229 and 232 but did not hybridize to any fragment from the λ 52 phage clone (Fig. 2C; data not shown). An exon 1C-specific probe also hybridized to this Sacl fragment (data not shown). Further subcloning of the Sacl fragment revealed that both exon 1B and exon 1C are located in a 2.3-kb Xbal genomic fragment. Precise positioning of exons 1B and 1C was accomplished by Southern blot and sequence analyses of overlapping genomic subclones (Fig. 2A). The sizes and sequences of the genomic fragments containing exons 1B and 1C were identical for DNA from three sources: mouse L cell genomic DNA, BAC 229 DNA, and BAC 232 DNA. Exon 1C was positioned ~11.2 kb upstream from exon 1A, whereas exon 1B was located just ~300 bp upstream of exon 1C (Fig. 2A).

**Effect of Interferon Treatment on Expression of Alternative Exon 1-containing ADAR1 Transcripts in Mouse Fibroblasts—**

The effect of IFN treatment on the expression levels of ADAR1 mRNA species containing exon 1A, exon 1B, and exon 1C was examined by semiquantitative RT-PCR to distinguish between exon 1A-, exon 1B-, and exon 1C-containing transcripts (Fig. 3). cDNAs prepared using RNA isolated from mouse L cells, either left untreated or IFN-treated, were used as templates for PCR amplification. Primers specific for the alternative exon 1 structures were used; E1A plus 19, E1B plus 72, or E1C plus 8 was paired with the E2 minus 646 primer for detection of transcripts possessing the exon 1A-exon 2 junction, exon 1B-exon 2 junction, or exon 1C-exon 2 junction, respectively (Fig. 3A). Amplification of templates was analyzed after increasing cycle number, ranging from 20 to 35, by gel electrophoresis to resolve the products that were quantified using a Bio-Rad VersaDoc imaging system and Quantity One software.

The amount of exon 1A-exon 2-specific product obtained was low with template cDNA prepared with RNA from untreated relative to IFN-treated mouse L cells (Fig. 3B, top panel). After IFN treatment, a severalfold increase of the exon 1A-containing product was observed, whereas no increase was seen for the exon 1B-exon 2 product (Fig. 3B). In some experiments, such as

![Figure 2](http://www.jbc.org/)

**FIG. 2. Genomic organization of the mouse Adar1 alternative exon 1 structures.** A, physical map showing organization of the exons and introns within the 5'-region of the mouse Adar1 gene established from λ phage (λ 52) and BAC (BAC 229 and BAC 232) genomic clones. Exon 1A is positioned ~4.2 kb upstream of exon 2, exon 1B is ~15.8 kb upstream of exon 2, and exon 1C is ~15.4 kb upstream of exon 2. The positions of restriction enzyme sites are shown for EcoRI (E), HindIII (H), PstI (P), Sacl (S), Sall (S), XbaI (X), and XhoI. Southern blot analyses were performed using probes specific for exon 1A (B) and exon 1B (C).

![Figure 3](http://www.jbc.org/)

**FIG. 3. Effect of interferon treatment on expression of exon 1A, 1B, and 1C-containing ADAR1 mRNA transcripts in mouse L fibroblast cells.** A, schematic diagram showing the locations of the alternative exons 1A (red), 1B (yellow), and 1C (green) and exon 2, with exons denoted by boxes and introns denoted by triangles. The exon-specific oligomer primers used for RT-PCR are indicated by arrows. B, RNA was isolated from L cells treated with IFN-α/β for 24 h or left untreated and then analyzed by RT-PCR as described under "Experimental Procedures." PCRs were carried out using primer pairs specific to transcripts containing exon 1A (exon 1A plus 19 and 32P-labeled exon 2 minus 646), exon 1B (exon 1B plus 72 and 32P-labeled exon 2 minus 646), or exon 1C (exon 1C plus 8 and 32P-labeled exon 2 minus 646) or GAPDH plus and minus primers as indicated. PCR products were analyzed by gel electrophoresis after the indicated number of amplification cycles. cDNA templates were prepared by random oligomer priming using RNA from either untreated or IFN-α/β/ treated L cells.
the one shown, IFN treatment caused a modest reduction in the expression level of exon 1B containing ADAR1 RNA. Exon 1C-containing ADAR1 transcripts were not readily detected in cultured mouse L cells, either left untreated or IFN-treated (Fig. 3B). As a control, the same cDNA preparations used to examine ADAR1 transcript levels were also examined using glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-specific primers. The amount of GAPDH product was similar for cDNA samples prepared from untreated as compared with IFN-treated L cells (Fig. 3B, bottom panel). The observed sizes of the ADAR1 and GAPDH products were consistent with the sizes predicted from their cDNA sequences.

Northern gel blot analyses with hybridization probes specific to either exon 1A or exon 1B were performed to estimate the size of the corresponding ADAR1 transcripts (Fig. 4). The exon 1A-specific probe detected a single major transcript of ~6.5 kb with RNA isolated from mouse L cells. The amount of this exon 1A-containing ADAR1 RNA was significantly elevated after IFN-α/β treatment (Fig. 4, lanes 1 and 2). By contrast, an abundant ~6.5-kb transcript was detected with the exon 1B-specific probe, and this transcript was present in comparable amounts in untreated and interferon-treated cells (Fig. 4, lanes 3 and 4). The integrity and amounts of GAPDH RNA and 18 and 28 S ribosomal RNAs were comparable between the RNA samples prepared from untreated and IFN-α/β-treated cells (Fig. 3B and data not shown).

Differential Expression of Alternative Exon 1-containing ADAR1 Transcripts in Tissues from Uninfected and Infected Mice—We next examined the expression of alternative exon 1-containing ADAR1 transcripts in several tissues from adult uninfected Balb/c mice and from mice stimulated to produce cytokines including IFN by oral infection with Salmonella (37, 51). RNA preparations isolated from liver, brain, lung, cecum, heart, spleen, Peyer’s patches, and kidney were analyzed by RT-PCR using primer pairs specific for exon 1A-exon 2, exon 1B-exon 2, and exon 1C-exon 2 (Fig. 3A). The results are shown in Fig. 5. Quantification showed that although differences between individual mice can occur, exon 1A-containing transcripts were readily detected in all tissues examined and were increased in amount after Salmonella infection. Exon 1A-containing ADAR1 RNA was most abundant in the liver of infected animals and was least abundant in the brain of uninfected mice (Fig. 5). Exon 1B-containing tran-

![Fig. 4. Northern gel blot analysis of ADAR1 mRNA expression in untreated and interferon-treated mouse L fibroblast cells. Poly(A)+ RNA was isolated from L cells treated with IFN-α/β for 24 h (+) or left untreated (−), fractionated by formaldehyde agarose gel electrophoresis (~1.5 μg), transferred to nylon membrane, UV cross-linked, and then hybridized with 32P end-labeled exon-specific probes as follows: exon 1A (1A) oligonucleotide probe, minus oligonucleotide E1A (lanes 1 and 2); exon 1B (1B) oligonucleotide probe, minus oligonucleotide E1B (lanes 3 and 4). The positions of the 18 and 28 S ribosomal RNA are indicated.](http://www.jbc.org/)

![Fig. 5. ADAR1 transcripts containing alternative exon 1 structures are differentially expressed in Balb/c mice following bacterial infection. RNA was isolated from tissue samples taken from uninfected Balb/c mice (diamonds) or from mice 6 days after infection with wild-type Salmonella (circles). For measurement of alternative exon 1 transcript amounts, the RNA samples were analyzed by RT-PCR as described under “Experimental Procedures” and Fig. 3 using primer pairs specific to transcripts containing exon 1A (red; exon 1A plus 19 and exon 2-minus 646), exon 1B (yellow; exon 1B plus 72 and exon 2 minus 646), or exon 1C (green; exon 1C plus 8 and exon 2 minus 646) or GAPDH plus and minus primers as indicated. The transcript levels were normalized between samples by using primer pairs for the constitutively expressed GAPDH transcript as the standard. The number associated with each symbol corresponds to the identification (ID) number of the mouse from which the specified tissues (liver, brain, lung, cecum, heart, spleen, Peyer’s patches, and kidney) were isolated.](http://www.jbc.org/)
scripts were most abundant in the brain compared with all other tissues where the levels typically were quite low. The level of exon 1B RNA was not affected significantly by *Salmonella* infection (Fig. 5). Exon 1C-containing transcripts were extremely low in all of the tissues analyzed, and they remained low even after infection (Fig. 5). However, nested PCR using two different exon 2 primers (E2 minus 842 and then E2 minus 646) paired with the specific exon 1C primer detected exon 1C transcripts in the brain and also the heart but not in other tissues (data not shown).

When Northern gel blot hybridization analysis was carried out using an exon 2 hybridization probe common to all known ADAR1 transcripts, a single major transcript of ~6.5 kb was observed in brain that was comparable in amount between uninfected and *Salmonella*-infected mice (Fig. 6). Consistent with earlier observations, a similarly sized ~6.5-kb RNA was seen in liver that was abundantly expressed following infection, and in cultured L cells following IFN treatment, as well as a smaller and less abundant transcript that was also increased in liver by infection and L cells by IFN treatment (Fig. 6).

**Expression of ADAR1 in Mouse Embryonic Tissue**—Homozygosity for different null alleles of *Adar1* deleted in exons 2–13, 7–9, or 12–15 causes an embryonic lethal phenotype prior to E12.5 (39, 40). With the knowledge that at least three unique ADAR1 transcripts possessing alternative exon 1A, 1B, and 1C structures are present in adult mouse tissues (Figs. 1 and 3), we performed RT-PCR analysis with embryonic tissue to gain insight into the identity of ADAR1 transcripts synthesized in developing wild-type embryos. When RNA was isolated from pooled embryos as early as day E10.5, the constitutively expressed exon 1B-containing ADAR1 transcript was readily detected, but the inducible exon 1A-containing transcript was not seen (Fig. 7A). The brains and livers from two adult female mice (M1 and M2), from which the embryos were obtained, were also analyzed. Exon 1B-containing transcripts represented the major form found in the brain, whereas exon 1A-containing transcripts were the major form found in the liver (Fig. 7A). Examination of later stage embryos permitted the analysis of RNA isolated separately from the head and the torso regions, as shown for E15 (Fig. 7B). Exon 1B- and exon 1A-containing transcripts were both present at E15, but the level of exon 1B was much greater than exon 1A, the latter of which was relatively more prevalent in RNA from the torso than the head region (Fig. 7B).

Two sizes of forms of the ADAR1 protein are known, p110 and p150 (4, 24). The p150 protein isoform is IFN-inducible and encoded by exon 1A-containing mRNAs, and the p110 isoform is constitutively expressed and could be encoded by either exon 1B- or exon 1C-containing transcripts. Western immunoblot analysis of tissue extracts prepared from embryos at E10 and E15 were performed to further assess ADAR1 expression at the protein level (Fig. 8) as compared with the RNA level (Fig. 7). The p110 constitutively expressed isoform of ADAR1 was readily detected in tissue extracts prepared from whole E10 embryos (lane 1) and from the head region of E15 embryos (lane 4). The mobility of the p110 protein present in embryonic tissue was comparable with the constitutively expressed p110 protein present in L cell extracts (lanes 1, 2, 5, and 6). No signal was detectable corresponding to the inducible p150 isoform of ADAR1 protein when either whole E10 or head region E15 embryonic tissues were analyzed (lanes 1 and 5). However, a low level of p150 protein was detectable in extracts prepared from the torso region of E15 embryos (lanes 2 and 3) from two different mice (M3 and M4).

**Differential Expression of Alternative Exon 7 Splice Variants in Embryonic and Adult Tissues**—Two alternative exon 7 splice site variants of the ADAR 1 protein, designated ADAR1–7a and ADAR1–7b, have been detected in human (32), rat (19), and mouse (37) cells and tissues, but their relationship to the alternative exon 1 mRNA structures is unclear. RT-PCR and sequence analyses of subcloned products from mouse brain and J774.1 macrophage cDNA libraries established that the mouse exon 7b was a 5′-splice site variant that results in a 78-nucleotide deletion from the 3′-region of exon 7a (GenBank™ accession numbersAY509125 andAY509126), as summarized by the schematic shown in Fig. 9A. Both exon 7 splice variants of ADAR1, exon 7a and exon 7b, were expressed in developing embryos at E14.5 (Fig. 9B). By contrast, in adult mouse brain the form of exon 7 predominantly if not exclusively seen was 7a.
animal is more complex than most known interferon-regulated genes. Although we isolated the human ADAR1 cDNA in a screen for IFN-inducible genes (24) and established that human ADAR1 transcription is IFN-inducible (29, 52), both human and mouse ADAR1 transcripts clearly show a significant basal expression level in the absence of IFN treatment in cell culture. Our results provide a mechanistic understanding for the significant basal transcription of the ~6.5-kb ADAR1 RNA seen in the absence of IFN treatment, as well as for the increased expression of the similarly sized ADAR1 transcript seen following IFN treatment. We find that mouse ADAR1 mRNAs possess three alternative exon 1 structures spliced in an identical manner to a shared exon 2. The alternative exon 1A, exon 1B, and exon 1C transcripts initiate from different ADAR1 promoters that drive expression of the single-copy Adar1 gene present on mouse chromosome 3F2 (42). Exon 1A was inducible both by IFN and by infection, whereas exons 1B and 1C were constitutively expressed and not significantly affected by IFN treatment or microbial infection. The fact that the ADAR1 5' -RACE cDNA and the ADAR1 genomic DNA sequences were identical for the alternative exon 1A, 1B, and 1C sequences suggests that no editing occurs in these regions of the ADAR1 exonic sequences. Interestingly, the A-to-I editing enzyme ADAR2 modulates its own expression by editing its own RNA in a manner that affects the ADAR2 transcript alternative splicing pattern (50).

Elucidation of the organization of the mouse Adar1 gene provided us with the detailed molecular information necessary to undertake subsequent functional analyses of ADAR1 expression under physiologically important conditions in the whole animal, studies that could not be easily performed in the human system. We gained a mechanistic understanding of the expression of ADAR1 in uninfected adult mice, the variance in expression between individual animals under laboratory conditions, and finally how the ADAR1 expression profile is altered in a tissue-selective manner in response to infection with a microbial pathogen.

In uninfected mice, we found that exon 1A transcripts were expressed in all the tissues tested (liver, spleen, Peyer’s patches, kidney, heart, lung, and cecum) except for brain where the exon 1A-containing RNA was very scarce. When mice were infected with Salmonella by the natural oral route, exon 1A transcript levels increased, most significantly and abundantly in the liver and Peyer’s patches. By contrast, in brain where the exon 1B-containing transcripts constituted the major if not exclusive form of ADAR1 mRNA in uninfected mice, they remained the predominant transcript form even following infection. The levels of exon 1B RNA were not further increased in brain, or in any other tissue, by microbial infection. The low level of exon 1A-containing RNA expression seen in the brain compared with other tissues of uninfected animals, and the fact that the exon 1A RNA levels remained low in the brain even after infection implies that the integrity of the blood-brain barrier likely shields the brain from the inducer of exon 1A RNA transcription and that this presumed barrier was not compromised during Salmonella infection.

The functional role of type I IFN-regulated genes such as ADAR1 in protection versus pathogenesis in bacterial infections is not yet resolved. We found that infection with wild-type Salmonella induced a robust induction of type I IFN-stimulated genes when death was imminent, whereas infection with an avirulent Dam− vaccine candidate Salmonella strain showed a reduced expression of the same set of IFN-stimulated genes (37). Likewise, type I IFN production was found to enhance susceptibility to Listeria monocytogenes infection (54). Thus, although IFN plays an important protective role in the

DISCUSSION

Here we demonstrate that the mouse Adar1 gene specifies multiple transcripts that possess unique exon 1 structures spliced to a shared exon 2 and that these alternative transcripts are differentially expressed during embryogenesis and in adult tissues. Most importantly, we establish that under a condition of natural physiologic stress, microbial infection, the expression of mouse ADAR1 transcripts is altered in a tissue-selective manner in adult mice. Several important points emerge from these findings.

Our results reveal that the physical organization of the mouse Adar1 gene and its regulated expression in the whole
Interferon-inducible ADAR1 RNA-specific Adenosine Deaminase

innate immune response to viral infection (4), at least under certain conditions of microbial infection (51) a robust type I IFN response including ADAR1 induction is associated with enhanced pathogenesis (37, 54). We now know that the elevation in ADAR1 expression observed during bacterial infection is the result of increased expression of exon 1A-containing ADAR1 transcripts. Interestingly, it has been reported that A-to-I editing by ADAR1 is involved in systemic acute inflammation and that editing activity and ADAR1 expression is elevated by endotoxin treatment as well as IFN (41).

The organization of the mouse Adar1 gene 5′-flanking region is summarized in the Fig. 3A schematic diagram. The position of the alternative exon 1A, exon 1B, and exon 1C structures relative to exon 2 were determined initially by Southern blot hybridization analyses and then confirmed more precisely by directly sequencing genomic subclones and subsequent comparison of genomic and 5′-RACE CDNA sequences. Because the expression of exon 1A-containing transcripts was increased by IFN treatment of cultured cells and also by infection of mice, genomic DNA subclones of the 5′-flanking region upstream of exon 1A were analyzed for sequence and also for promoter activity in transient transfection assays. The 5′-flanking region of exon 1A drove luciferase reporter expression in an IFN-activity in transient transfection assays. The 5′-flanking region of exon 1A drove luciferase reporter expression in an IFN-inducible manner in transiently transfected cells, and the genomic sequence (GenBankTM accession number AY488121) that displayed this IFN-inducible promoter activity possessed a consensus copy of the IFN-stimulated response element known as ISRE.2 Type I IFN-mediated transcriptional activation of gene expression is best understood in the context of the ISRE DNA element and the heteromeric transcription factor ISGF3 assembled from STAT1, STAT2, and IRF9 (4, 55). The appreciable basal expression of mouse ADAR1 observed in the absence of IFN treatment or infection in cell culture and whole animals is unlike many other well characterized IFN-regulated genes such as Mx or p56 that characteristically display very low expression levels in the absence of IFN or infection (4, 56, 57). We now have a firm mechanistic understanding of the basis of the significant Adar1 basal transcription seen under normal physiologic conditions. Multiple promoters and alternative exon 1 structures are utilized. Among the more than 100 IFN-regulated genes (57), the only one other than ADAR1 that utilizes this type of regulation of which we are aware is the transcription factor CIITA. Like ADAR1, multiple promoters and alternative exon 1 splicing to a shared exon 2 are utilized to generate transcripts that encode the constitutively expressed form of CIITA (4, 58).

The three alternative exon 1-containing transcripts predict the synthesis of two different sized forms of mouse ADAR1 proteins: one larger version and the other an N-terminally truncated smaller version synthesized in the same ORF. The deduced coding of the inducible exon 1A-containing transcript is the 1152-amino acid protein, or a protein of 1126 amino acids if the exon 1A-containing transcript possesses the alternative exon 7b (which is quite likely at least for most of the mature RNA transcript present in liver). By contrast, the exon 1B- and exon 1C-containing RNAs would both specify the truncated but in-frame 903-amino acid ADAR1 protein. A polyclonal antibody, prepared using a central coding region of recombinant ADAR1 that is common between the inducible and basal transcripts, recognized two mouse ADAR1 proteins: a smaller constitutively expressed protein and a larger inducible protein isoform. A conceptually similar gene organization and expression profile is seen for the human ADAR1 gene, where two sized forms of the ADAR1 proteins are seen in cultured human cells: one (p150) IFN-inducible and the other (p110) constitutively expressed (24, 29, 32).

Exon 1A includes an initiator methionine codon, AUG1, that begins the 1152-amino acid ORF. The next methionine codon present in the long ORF is nearly 250 amino acid residues downstream, at AUG249 within the unusually large exon 2 (Fig. 3A). Because the alternative exons 1B and 1C both lack an AUG codon, translation initiation of 1B- and 1C-containing transcripts is predicted to begin at AUG249 in exon 2. Only three methionine codons are present in the −0.8 kb of exonic sequence upstream of AUG249, and all are out-of-frame relative to the long 1152-residue ORF; they begin very short ORFs, 24, 4, and 2 residues. The nucleotides flanking AUG249 at the −3 and +4 positions relative to the A of the AUG are both purines, GacAUGG, consistent with an especially strong start codon, whereas a pyrimidine is present at the +4 flanking position of AUG1 (AcAUGt) that would presumably weaken the translation start site and allow for the possibility of leaky scanning (53). The two differently sized mouse ADAR1 proteins detected by Western immunoblot assay of extracts prepared from mouse cell lines and tissues can be explained on the basis of the transcript forms described above in which different AUGs are utilized to initiate the same ORF.

It is known from mouse Adar1 gene disruption studies that ADAR1 is required in embryogenesis. ADAR1 deficiency leads to stress-induced apoptosis and liver disintegration in the embryo, resulting in death between E11.5 and E12.5 (39, 40). The alternative exon 1 form of ADAR1 expressed in embryonic tissue is not known, nor is the identity of the RNA substrates that are presumably edited that protect against apoptosis and embryonic lethality. We observed that the constitutively expressed exon 1B-containing ADAR1 message was detectable at embryonic day 10.5 and was abundantly expressed by E15, whereas the inducible exon 1A-containing transcript form was not detectable at E10.5, and only very low amounts were seen at E15. ADAR1 mRNA that possesses the 5′ exon 1B would encode the p110-sized form of the ADAR1 protein that initiates translation at AUG249 present in exon 2. Transcripts with the 5′ exon 1A, however, would encode the larger N-terminally extended p150 ADAR1 protein that initiates at AUG1 in exon 1A, which is in-frame with AUG249 (our sequences obtained herein, and submitted to GenBankTM with accession numbers AY508955, AY508956, and AY508958; see also Refs. 3 and 4). Furthermore, we were able to detect the p110 ADAR1 protein in wild-type embryonic tissue both at E10 and at E15 by Western analysis, consistent with the identification of exon 1B-containing transcripts at these times. However, the p150 protein was not seen at E10 and only variably and in low amounts at E15 in wild-type embryos, which likewise correlates with the extremely low if not undetectable expression of exon 1A transcripts at these times. The polyclonal antibody that we used in the Western assays was prepared against recombinant mouse ADAR1 protein expressed in E. coli, and this antibody recognized both sizes of forms of mouse ADAR1, the constitutive p110 protein and the inducible p150 protein, present in extracts prepared from IFN-treated mouse L fibroblast cells.

The simplest explanation for the embryonic lethality reported to occur by E12.5 in homozygous null Adar1 mouse embryos (39, 40) is that the p110 form of the ADAR1 protein is necessary for normal embryogenesis. This explanation is consistent with ADAR1 expression patterns and with the phenotype of mouse gene disruptions in the IFN response pathway. In Adar1 wild-type mouse embryos, ADAR1 p110 protein and exon 1B RNA expression were detectable at E10.5, but p150 protein and exon 1A RNA were not found as shown herein.

2 C. X. George, E. G. Short, and C. E. Samuel, unpublished observations.
Furthermore, p150 expression was inducible by IFN, and it is known that IFN responsiveness of gene expression is dependent upon receptor-mediated Jak1 kinase signal transduction for transcriptional activation (4). Although ADAR1 deficiency results in embryonic lethality between E11.5 and E12.5 (39, 40), neither Jak1 deficiency nor IFN receptor deficiency is known that IFN responsiveness of gene expression is dependent upon receptor-mediated Jak1 kinase signal transduction for transcriptional activation (4).

Studies with homozygous null mice reveal that deficiency in Jak1 results in perinatal lethality (59), whereas knock-out mice deficient in both IFN receptors are viable but especially susceptible to viral infection (60). However, it is intriguing that the null phenotype of mouse Adar1 manifests a rapidly disintegrating liver structure and increased apoptosis in the liver, because we found that in adult mice the major form of ADAR1 expressed in liver was the inducible exon 1A-containing transcript encoding p150 and that the levels of the exon 1B-containing RNA encoding p110 were much lower. Possibly additional genetic knock-outs in which the inducible promoter p1 and that the levels of the exon 1B-containing tran-
Expression of Interferon-inducible RNA Adenosine Deaminase ADAR1 during Pathogen Infection and Mouse Embryo Development Involves Tissue-selective Promoter Utilization and Alternative Splicing

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