Hyperphagia-mediated Obesity in Transgenic Mice Misexpressing the RNA-editing Enzyme ADAR2*

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ADAR2 is a double-stranded RNA-specific adenosine deaminase involved in the editing of mammalian RNAs by the site-specific conversion of adenosine to inosine. To examine the physiologic consequences resulting from ADAR2 misexpression, we have generated mutant mice expressing either wild-type or deaminase-deficient ADAR2 transgenes under the control of the human cytomegalovirus promoter. Transgenic mice expressing either wild-type or inactive ADAR2 isoforms demonstrated adult onset obesity characterized by hyperglycemia, hyperleptinemia, and increased adiposity. Paired feeding analysis revealed that mutant mice on caloric restriction had a growth rate and body composition indistinguishable from wild-type littermates, indicating that the observed obesity predominantly results from hyperphagia rather than a metabolic derangement. The observation that expression of catalytically inactive ADAR2 also is capable of producing an obese phenotype in mutant animals suggests that ADAR2 may possess additional biological activities beyond those required for the site-selective deamination of adenosine or may interfere with the actions of other double-stranded RNA-specific binding proteins in the cell.

The conversion of adenosine to inosine by RNA editing results in subtle alterations in the primary nucleotide sequence of mature mRNAs, thereby producing specific changes in amino acid coding potential that can affect the biological activity of the resulting protein(s) (1). The post-transcriptional conversion of adenosine to inosine is catalyzed by a family of double-stranded RNA (dsRNA)-specific adenosine deaminases referred to as ADARs (adenosine deaminases that act on RNA) (2, 3) that contain variable amino termini, multiple copies of a dsRNA-binding domain (dsRBD) and conserved carboxyl-terminal sequences encoding a catalytic adenosine deaminase domain (3, 4). ADAR2, a member of the ADAR family, has been shown to be involved in the editing of numerous mammalian pre-mRNAs to affect subsequent protein function, including transcripts encoding glutamate-gated ion channels, a voltage-gated potassium channel (K1,1), and the 2C subtype of serotonin receptor (5–7).

Genetically modified mice lacking ADAR2 expression develop progressive seizures and die by postnatal day 21 due to a lack of editing (Q/R site) in transcripts encoding a subunit of the α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid subtype glutamate receptor (GluR-2) (8), demonstrating the critical role that ADAR2 plays in the normal development and function of the central nervous system. Multiple cDNA isoforms of ADAR2 have been identified in rats, mice, and humans resulting from alternate splicing events that affect protein expression and function (9–11). Use of an alternative 3′-splice site near the 5′-end of the ADAR2 coding region produces a −1 frameshift to generate a truncated protein lacking the dsRBDs and catalytic deaminase domain required for editing activity (11). Use of this proximal 3′-acceptor is dependent upon the ability of ADAR2 to edit its own pre-mRNA, thereby providing a unique negative autoregulatory strategy by which ADAR2 can modulate its own expression (11, 12).

To examine whether over- or misexpression of ADAR2 could result in the aberrant editing of ADAR2 target RNAs and subsequent physiologic changes, we generated mutant mouse lines expressing ADAR2 transgenes under the control of the human cytomegalovirus (CMV) promoter. Results from these studies have indicated that expression of transgenes encoding either wild-type or catalytically inactive ADAR2 protein results in maturity onset obesity characterized by hyperphagia and elevated plasma levels of leptin and glucose. The observation that expression of catalytically inactive ADAR2 also is capable of producing an obese phenotype in mutant animals suggests that ADAR2 may possess additional biological activities beyond those required for the site-selective deamination of adenosine or may act as a competitive inhibitor of other dsRNA-specific binding proteins in the cell.

EXPERIMENTAL PROCEDURES

Construction of FLAG-ADAR2b and FLAG-ADAR2b (E396A) Transgenes—A NotI restriction site, an initiation codon, and a FLAG epitope tag (DYKDDDDK) were introduced into the wild-type rat ADAR2b cDNA (11) immediately upstream of
amino acid position 2 in the open reading frame by PCR amplification; subsequently, a NotI-XbaI restriction fragment containing the entire ADAR2b coding region was excised and subcloned into the pRC/CMV eukaryotic expression vector (Invitrogen). A 2.9-kb NruI-XbaI fragment encompassing 660 bp of the human CMV promoter and the ADAR2b coding region was further subcloned into a pBSK-β-based plasmid (Stratagene, La Jolla, CA) that contained a 2.1-kb BamHI-EcoRI fragment with the entire human growth hormone (hGH) gene derived from the p0GH plasmid (13). Introduction of the E396A mutation was performed by PCR-mediated mutagenesis, and the deaminase-deficient transgene was assembled as described for the wild-type FLAG-rADAR2b-hGH construct. For the generation of wild-type and deaminase-deficient ADAR2b transgenes containing the SV40 polyadenylation signal, an initiation codon and three tandem copies of the influenza hemagglutinin tag (YPYDVPDYA) were introduced into the wild-type rat ADAR2b cDNAs immediately upstream of amino acid position 2 in the open reading frame by PCR amplification. Transgenes bearing the hemagglutinin-tagged cDNAs were assembled in a manner identical to that described for FLAG-rADAR2b-hGH transgene except for the presence of a 682-bp BamHI-EcoRI fragment of pcDNA1 (Invitrogen; positions 3120–3801) containing the SV40 polyadenylation signal in place of the hGH-derived sequences. All constructs were confirmed by DNA sequence analysis.

Generation of Transgenic Mice—Transgenes were microinjected into the pronucleus of C57Bl/6j × DBA2 (F1) hybrid embryos (14, 15), and progeny mice were screened for incorporation of the transgene by Southern blotting analysis of genomic DNA. For mice bearing wild-type or deaminase-deficient ADAR2b-hGH transgenes, 20 μg of mouse genomic DNA was digested with ApaI and resolved by electrophoresis on a 0.8% agarose gel. Denatured DNA was transferred to a nylon membrane (Hybond-N+; Amersham Biosciences), baked in a vacuum oven for 2 h at 80 °C, and hybridized to a 32P-labeled DNA probe, as described previously (16), using a random-primed probe (Prime-It II; Stratagene) generated from a 2.1-kb BamHI-EcoRI fragment containing the entire human growth hormone gene (13); the expected 2.6- and 3.7-kb hybridizing fragments were detected in multiple founder animals for each transgene construct (data not shown). For rapid screening of transgenic mice by PCR, genomic DNA was isolated from tail tips of founder mice and subjected to Taq polymerase chain reaction (PCR) amplification analyses with the ABI Prism 7900HT Sequence Detection System (SDS version 2.1; Applied Biosystems). Real-time PCRs and subsequent analyses were performed with the ABI Prism 7900HT Sequence Detection System (SDS version 2.1; Applied Biosystems). Quantification of target gene expression in all samples was normalized to 18S rRNA expression (11); the primers and mixtures of deoxy- and dideoxynucleotides used in each primer-extension assay are indicated in supplemental Table S1.

To quantify levels of ADAR1, neuropeptide Y (NPY), and pro-opiomelanocortin (POMC) mRNA expression, first-strand cDNA was synthesized and amplified using gene-specific primers and assessed using a modified primer extension analysis (11); the primers and mixtures of deoxy- and dideoxynucleotides used in each primer-extension assay are indicated in supplemental Table S1.

Quantification of target gene expression in all samples was normalized to 18S rRNA expression (Ct_target − Ct_18S = ΔCt) as previously described (12).

Western Blotting Analysis—Total cellular lysates were prepared using modified radioimmune precipitation buffer (100 mM NaCl, 1% Igepal CA 630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl, pH 7.6, with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 0.1% aprotinin) as described previously (16); 25 μg of each protein sample were resolved by denaturing polyacrylamide gel electrophoresis (10% SDS-PAGE) and transferred to a nitrocellulose membrane (Hybond-C Super; Amersham Biosciences). The membrane was probed with an affinity-purified ADAR2-specific antisemir raised against amino acids 6–66 of the rat ADAR2 open reading frame (19) and a β-actin-specific antiserum (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and detected with an Alexa Fluor 680-labeled secondary antisemir. Total immunoreactive ADAR2 protein was quantified using the Odyssey infrared imaging system (Li-Cor Biosciences, Lincoln, NE) and normalized to the level of β-actin immunoreactive material in each tissue sample.
ADAR2 Expression and Obesity

Body Composition—Quantification of global body fat in whole animals was performed by magnetic resonance imaging using a modified fast spin echo for the fat suppression difference method (20); the modified fast spin echo sequence was verified with a phantom containing water and olive oil. Because image acquisition was not respiration-gated, data analysis was limited to the hind portion of the mouse to avoid motion artifacts. Fat measurements were made by acquiring data from the implementation of three different forms of a fast spin echo sequence. The first two acquisitions were used primarily for quantification of fat and water: one acquisition with fat suppression pulses centered on the fat resonance and one with no fat suppression pulses applied. A third acquisition was used to check for the possibility of direct saturation of the water peak, whereby the suppression pulses were placed on the opposite side of the water resonance but at the same chemical shift difference as the fat resonance. Fat suppression was achieved with saturation by a series of three 20- to 30-msec Gaussian pulses centered on the fat resonance, followed by an increasing crusher gradient amplitude after each pulse; the nominal suppression bandwidth was 700 Hz. The saturation pulses were placed in front of a fast spin echo imaging sequence, using an echo time of 6 ms and a repetition time of 1 s. Eight echoes were collected, with the first echo being centered on K-space. This was done to minimize both T2 losses and chemical shift artifacts. Slice thickness was 0.5 mm with no slice gap and a field of view of 60; the acquired image matrix was 256 × 128. Each of the three scans took approximately 5 min. All images were acquired with a Varian Inova spectrometer (Varian Associates, Palo Alto, CA) operating at 7 teslas. Fat and water levels were determined by the subtraction of the two corresponding images. Water–fat difference images were analyzed with MATLAB (Mathworks, Inc.). Percentage of fat was defined as \( IFat = 1 - (INS - IS)/(INS) \times 100 \), where \( INS \) represents the intensity from the nonsuppressed image and \( IS \) is the intensity of the suppressed image. The analysis implemented a pixel-by-pixel subtraction, followed by application of a threshold. The effect of direct saturation of water was found not to be a contributing factor in the experiments. This was verified by subtraction of images acquired with no saturation pulses and those images in which saturation pulses were placed at the same frequency as fat but in the opposite frequency direction. These difference images yielded images at the noise level, indicating no measurable effects of direct saturation.

Growth Rate and Plasma Hormone Analysis—Mice were maintained on a 12-h light, 12-h dark cycle in a humidity- and temperature-controlled room with water and standard laboratory chow (mouse chow 5015;Ralston Purina Co., St. Louis, MO) supplied ad libitum. The growth rates of transgenic mice and control littermates were monitored on a weekly basis between 0900 and 1000 h. Blood from ad libitum-fed mice was collected by retro-orbital sinus puncture with heparinized capillary tubes. Blood glucose levels were measured from whole blood using a B-Glucose analyzer and Hemocue strips (Hemocue, Lake Forest, CA); leptin levels were measured by radioimmunoassay using 25 μl of plasma with recombinant mouse leptin as a standard (Linco Research, Inc., St. Charles, MO).

Activity and Metabolic Analysis—Locomotor activity, oxygen consumption (VO2), and carbon dioxide production (VCO2) of mice were measured using a four-chamber Oxymax system with automatic temperature and light controls and equipped with infrared beams crossing the cage to assess animal movement (Columbus Instruments, Columbus, OH). Temperature was maintained at 23 °C, and lights were on from 0600 to 1800 h. System settings included a flow rate of 0.5 liters/min, a sample line purge time of 2 min, and a measurement period of 60 s every 15 min. Mice had ad libitum access to chow and water. The respiratory exchange ratio (RER) was calculated as the ratio of carbon dioxide produced (VCO2) divided by oxygen consumed (VO2) (21).

Feeding Behavior—Cumulative food consumption was assessed for wild-type and transgenic animals using an automated feeding apparatus that measured free feeding behavior by allowing animals ad libitum access to food cups that were mounted on a balance and monitored every 30 s (22). All feeding studies were performed after animals had been acclimated to the apparatus for at least 24 h. Weight-matched transgenic and control littermates were subjected to a paired feeding paradigm (23, 24) in which mice were housed individually and permitted to acclimate to this condition for 3 days before food intake measurements using a normal mouse chow diet. Food intake amounts for ad libitum-fed transgenic and wild-type mice were measured daily starting at 4 weeks of age. Pair feeding was accomplished by measuring the food intake of the ad libitum-fed control animals every 24 h; the following day, individually housed transgenic mice (pair-fed group) were given the average amount of food consumed by the control mice on the previous day. Body weight was monitored on a weekly basis.

To assess the ability of leptin and d-fenfluramine to suppress feeding behavior, mice were housed singly and acclimated for 72 h prior to the onset of feeding studies. Mice were fasted overnight, and ad libitum access to food was provided 30 min after intraperitoneal administration of leptin (2.5 μg/g mouse) in phosphate-buffered saline (25) or d-fenfluramine (3 μg/g mouse) in 0.9% saline (26). Total food consumption was measured for 6 and 10 h after administration of d-fenfluramine and leptin, respectively.

Tissue Culture and Transfection—Human embryonic kidney (HEK293) cells were transiently co-transfected using Fugene 6 (Roche Applied Science) with increasing amounts (5, 10, and 15 μg) of FLAG-rADAR2b-hGH or FLAG-rADAR2b(E396A)-hGH with a constant level of ADAR1 cDNA (5 μg) and a 289-bp 5HT2CR minigene (5 μg) in each transfection (6, 27). The total amount of plasmid DNA in each transfection was kept constant using an empty expression vector plasmid (pRC/CMV; Invitrogen). Cells were harvested 72 h after transfection and total RNA was isolated for quantification of A site editing by primer extension analysis as described above.

Statistical Analysis—Standard statistical analysis (Student's t test, analysis of variance, and χ2) were applied depending upon the type of experiment and performed using GraphPad PRISM (GraphPad Software, San Diego, CA). Values are reported as mean ± S.E., except where indicated. Statistical significance of body weight, cumulative food intake, hypothalamic neuropeptide mRNA expression, ADAR1 mRNA expression, and plasma...
ADAR2 Expression and Obesity

Transgenic Mice Expressing Wild-type and Deaminase-deficient ADAR2b-hGH Demonstrate Maturity Onset Obesity—To determine if overexpression of ADAR2 resulted in either altered editing patterns or phenotypic alterations, we generated transgenic mice in which the cDNA encoding rat ADAR2b was modified with an amino-terminal epitope (FLAG) tag and placed under control of the CMV promoter (Fig. 1A). Since negative autoregulation of ADAR2 protein expression by RNA editing has been shown to occur at the level of pre-mRNA splicing (11, 12), the cDNA encoding an ADAR2 transgene is not subject to such modulation. Due to the broad expression pattern for ADAR2 (3), we chose the CMV promoter, since it previously has been shown to demonstrate efficient expression in numerous cell types (28). The hGH gene was added to the transgene construct as 3′-untranslated information, based upon studies indicating that multiple splicing events can significantly increase the level of transgene expression (14, 15). Transgenic founder animals (C57BL/6 × DBA2 (F1)) were screened by Southern blotting and PCR amplification of genomic DNA (data not shown); four independent founder animals were shown to carry the transgene, and subsequent offspring were viable, fertile, and initially indistinguishable from their wild-type littermates. Total body weight was monitored for the offspring of a single founder animal for 25 weeks, demonstrating a dramatic increase in body weight for both male and female transgenic mice that reached statistical significance at 8 and 18 weeks, respectively, when compared with wild-type littermates (Figs. 1, B and C). Both male and female transgenic mice experienced normal life spans in which they continue to gain weight, reaching as much as 90 and 125 g by 15 months of age, respectively (data not shown). Although the transgene copy number for each mutant line was not determined, nearly identical growth patterns were observed for the offspring of all four independent founder animals, indicating that the altered phenotype was a result of transgene expression rather than the disruption of a specific genomic locus from random transgene integration (data not shown).

To ascertain whether the altered growth rate in mutant mice resulted from transgene-mediated increases in adenosine to inosine conversion for endogenous ADAR substrates, we developed an additional strain of transgenic animals in which the deaminase domain of the ADAR2b transgene was selectively inactivated by mutation of a highly conserved glutamate residue (Glu396) required for proton transfer during catalysis (29). Previous studies of ADAR1 with an analogous glutamate-to-alanine substitution at position 912 (E912A) demonstrated a complete abolition of deaminase activity without affecting sub-

### FIGURE 1. Expression of enzymatically active or inactive isoforms of rat ADAR2 results in obesity in transgenic mice. A, a schematic diagram is presented, indicating the structures of transgenes encoding the rat ADAR2b cDNA driven by the human CMV promoter with either an amino-terminal FLAG or three tandem copies of the hemagglutinin (HA) epitope tag. These transgenes contained either the entire hGH gene (left) or the intron-containing SV40 polyadenylation signal (right) as 3′-untranslated information. The positions of the functional motifs in ADAR2, including the nuclear localization signal (NLS), dsRNA-binding domains, and catalytic adenosine deaminase domain, are indicated. An asterisk denotes the position of a critical glutamate residue that was mutated to an alanine moiety (E396A) to generate a deaminase-deficient transgene product. B, body weight analysis of wild-type mice and mutant animals bearing the enzymatically active ADAR2b-hGH (mean ± S.E., n = 4 for each genotype and gender; p < 0.0001 using two-way analysis of variance) or deaminase-deficient ADAR2b(E396A)-hGH (mean ± S.E., n = 3; p < 0.0001) transgene. C, representative photograph of female ADAR2b-hGH transgenic (88.2 g, left) and wild-type (45.4 g, right) mice at 14 months of age. D, body weight analysis of wild-type mice and mutant animals bearing the enzymatically active ADAR2b-SV40 transgene (mean ± S.D., n = 4 for each genotype and gender; p < 0.0001 using two-way analysis of variance).
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Maturity Onset Obesity in ADAR2 Transgenic Mice Does Not Result from A aberrant Growth Hormone Expression—Although the entire hGH gene was added to ADAR2 constructs as a 3’-untranslated sequence to increase transgene expression (Fig. 1A, left), the dramatic increase in body mass for mice expressing active or deaminase-deficient ADAR2b-hGH transgenes raised questions regarding whether unanticipated hGH expression should be responsible for the observed increase in growth. Previous studies have shown that transgenic mice expressing the rat growth hormone transgene demonstrate an increase in growth rate and body length accompanied by increased lean body mass (31). More recent studies, however, have revealed that although transgenic rats expressing high levels of hGH show increased somatic growth, transgenic rats expressing low circulating hGH demonstrate a leptin-resistant obesity accompanied by hyperglycemia and hyperinsulinemia (24, 32). To eliminate the possibility that low level hGH expression was responsible for the obese phenotype, an ADAR2b transgene was developed containing three copies of an amino-terminal hemagglutinin epitope tag, and the hGH gene was replaced by the intron-containing polyadenylation signal from the SV40 virus (Fig. 1A, right) (33). Analyses of growth rates for four independent mouse lines bearing the wild-type ADAR2b-SV40 transgene revealed a significant increase in growth for male and female transgenic mice, when compared with wild-type littermates, beginning at 27 and 29 weeks of age, respectively (Fig. 1D). The increased rate of growth was significantly less than that observed for mice bearing ADAR2b-hGH transgenes (Fig. 1B), corresponding to a lower level of transgene expression in ADAR2b-SV40 animals (Fig. 3A).

Quantification of ADAR2b Transgene Expression in Mutant Mouse Tissues—Using a ribonuclease (RNase) protection assay specific for the rat transgene, a broad expression pattern was observed in mutant mice in almost all tissues examined; as expected, no transgene-derived transcripts were observed in wild-type animals (Fig. 3A). Transgene-derived ADAR2b mRNA abundance was greatest in skeletal muscle for all three transgenic mouse lines, yet the relative level of transgene expression differed between lines of animals bearing distinct ADAR2b constructs. Ribonuclease protection analysis of mice expressing the ADAR2b-SV40 transgene revealed a pattern of expression similar to that observed for the hGH-containing transgenes (Fig. 3A), yet the level of transgene-derived mRNA in all tissues was 5–20-fold less than that observed for ADAR2b(E396A)-hGH and ADAR2b-hGH mice, respectively (Fig. 3A), correlating with the decreased growth rate for

FIGURE 2. Elimination of ADAR2b editing activity by introduction of an active site (E396A) mutation. An analysis of ADAR2 alternative splicing is shown for total RNA isolated from HEK293 cells transiently transfected with a rat ADAR2 minigene in the presence or absence of wild-type or mutant (E396A) ADAR2b expression plasmids. The migration positions for PCR products derived from minigene-derived transcripts containing (+47) or lacking (−47) the alternatively spliced 47-nucleotide (nt) cassette are indicated.

FIGURE 3. Expression patterns for RNA and protein corresponding to active and deaminase-deficient rat ADAR2b in male transgenic mice. A, ribonuclease protection analysis of RNA from multiple tissues encoding active and deaminase-deficient ADAR2b. The migration positions for predicted RNase protection fragments from the rat transgenes (ADAR2b-hGH, ADAR2b(E396A)-hGH, and ADAR2b-SV40) are indicated; a separate probe for cyclophilin was included as an internal control. B, quantitative Western blotting analysis of total ADAR2 protein expression in control and ADAR2b transgenic mice. The -fold increase in ADAR2 protein expression in transgenic tissues was quantified and compared with the level of ADAR2 expression in tissues from wild-type mice after normalization to an internal β-actin control. RNA and protein samples from transgenic animals are represented with an asterisk.
ADAR2b-SV40 mice (Fig. 1D) compared with transgenic mice bearing ADAR2-hGH alleles (Fig. 1B). Since decreased ADAR2b-SV40 mRNA expression was observed in multiple, independent lines of transgenic mice (data not shown), it is likely that this decreased expression results from use of the SV40 polyadenylation signal rather than the site of transgene integration. Quantitative Western blotting analysis of FLAG-ADAR2b protein expression, using a monoclonal antibody directed against the FLAG epitope, was unsuccessful due to the presence of numerous cross-reactive protein species in mouse tissues (data not shown). As an alternative strategy to assess alterations in ADAR2 protein expression in transgenic animals, total ADAR2 protein was quantified using an antiserum directed against the amino terminus of ADAR2 (19) and normalized to the level of β-actin in each tissue (Fig. 3B). Results from these analyses demonstrated an increase in ADAR2b protein expression in most tissues examined; however, the greatest fold increase in ADAR2b protein for both wild-type and deaminase-deficient mice was observed in skeletal muscle and testis, consistent with observed levels of transgene-derived mRNA expression (Fig. 3A).

Maturity Onset Obesity in ADAR2b-hGH Transgenic Mice

Results from Hyperphagia—The central nervous system influences energy balance and body weight through three mechanisms: 1) effects on behavior, including feeding and physical activity; 2) effects on autonomic nervous system activity, which regulates energy expenditure and other aspects of metabolism; and 3) effects on the neuroendocrine system, including secretion of hormones, such as growth hormone, thyroid, cortisol, insulin, and sex steroids (34, 35). Locomotor activity, metabolic rate, and food intake were determined for wild-type and transgenic animals to further elucidate the physiologic mechanism(s) by which ADAR2b transgene expression affected body weight regulation. Changes in physical activity were assessed using an open field paradigm in which activity was monitored over a 22-h period (0900-0700 h), yet no significant differences in locomotor activity were observed between wild-type and ADAR2b-hGH mice during stages of preobesity (5 weeks) or obesity (32 weeks) for mutant animals (supplemental Fig. S1, A and B). To further address whether the obesity was associated with altered metabolism, indirect calorimetry was used for measurements of heat production, O₂ consumption, and CO₂ production to calculate the respiratory exchange ratio (RER) (36), a measurement of nutrient partitioning of fat and carbohydrates. No significant differences in heat production or RER for transgenic and wild-type animals were observed at 5 weeks of age, yet an increase in RER (p < 0.01) was seen for the obese state (supplemental Fig. S1, C and D), indicating a decreased ability of transgenic mice to use fat as an energy source.

To determine whether the increased body mass observed for ADAR2b transgenic mice resulted from alterations in feeding behavior, cumulative food consumption for age-matched (13 weeks) transgenic (37.9 ± 1.1 g) and wild-type (32.1 ± 3.9 g) littermates was assessed. Wild-type and transgenic animals showed no differences in daily feeding patterns with increased bouts of food consumption beginning at the onset of the dark cycle, yet transgenic animals consumed ~50% more food per day than their wild-type littermates (Fig. 4A). Since alterations

FIGURE 4. Feeding analysis of control and ADAR2-hGH transgenic mice reveals a hyperphagia-dependent obesity. A, cumulative food intake for male wild-type and ADAR2b-hGH transgenic mice, with ad libitum access to food, was monitored for 48 h (mean ± S.D.; n = 4, p < 0.0001). Subjective day and night during the 12-h light-dark cycle are indicated with white and black rectangles, respectively. B and C, body weight analysis of wild-type mice and transgenic (ADAR2b-hGH) mice on an ad libitum- or pair-fed regimen (mean ± S.D.; n = 3 for males, n = 6 for females; p < 0.05). Transgenic mice on the restricted diet were limited to the mean food consumption of control (ad libitum-fed) mice from the previous day.
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TABLE 1
Quantitation of body fat and plasma hormone levels for control and ADAR2b-hGH transgenic mice
All values are represented as mean ± S.E. Control and transgenic mice at comparable ages were compared using the paired Student’s t test.

| Genotype          | Gender  | n    | Age  | Feeding protocol | Body weight | Glucose | Leptin | Body fat |
|-------------------|---------|------|------|------------------|-------------|---------|--------|----------|
| Wild type         | Male    | 7    | 5    | Ad libitum       | 18.1 ± 0.5  | 162 ± 7 | 8.1 ± 2.5 | 2.0 ± 0.1 |
|                   | Female  | 6    | 5    | Ad libitum       | 15.7 ± 0.9  | 152 ± 18 | 8.9 ± 0.7 | 2.2 ± 0.1 |
| ADAR2b-hGH        | Male    | 5    | 40   | Ad libitum       | 40.2 ± 1.9  | 150 ± 8 | 35.8 ± 9.4 | 4.1 ± 0.9 |
|                   | Female  | 5    | 40   | Ad libitum       | 16.7 ± 2.7  | 152 ± 23 | 11.8 ± 4.3 | 2.8 ± 0.3 |

* p < 0.001.
** p < 0.01.
*** p < 0.05.

in feeding behavior for animals of even slightly different weights could result from the dominance of anabolic pathways to increase food intake and energy storage in order to maintain adipose mass (37), paired feeding studies were also performed in which weight-matched control and transgenic animals were given ad libitum access to food or the transgenic mice were limited to the mean food consumption of control animals (Fig. 4, B and C). Although individually housed mice with unrestricted food access demonstrated growth rates similar to that previously observed in Fig. 1B, transgenic mice under caloric restriction demonstrated growth rates that were not significantly different from ad libitum-fed control animals. These results suggest that the obesity observed in ADAR2b transgenic animals is largely mediated by a positive energy balance associated with alterations in central feeding behavior rather than major changes in energy expenditure.

Recent studies have demonstrated that alterations in plasma glucose levels can modulate ADAR2 expression levels in the endocrine pancreas and cultured β-cell lines (38). To determine whether endogenous ADAR2 expression is similarly altered in the hypothalamus in response to an unrestricted or food-deprived (20 h) state, wild-type animals were assessed for changes in hypothalamic ADAR2 protein expression by quantitative Western blotting analysis (supplemental Fig. S2) using antisera directed against ADAR2 and β-actin (19). Results from these studies indicated that there were no significant differences between ADAR2 protein expression in the fed versus fasted state, suggesting either that hypothalamic ADAR2 is not regulated in response to feeding status or that it does not play a role in acute regulation of feeding under normal circumstances.

Alterations in Body Composition and Plasma Chemistry in ADAR2b-hGH Transgenic Mice—To image and quantify the distribution and percentage of body fat, we employed an MRI-based strategy for control and transgenic mice subjected to both ad libitum and paired feeding paradigms (39, 40). Results from this analysis revealed that control and preobese transgenic mice had comparable levels of body fat at 5 weeks of age (Table 1), yet transgenic animals showed a dramatic increase in adiposity for intra-abdominal, subcutaneous, and total abdominal fat when compared with the control littersmates at 40 weeks of age (Fig. 5, top and middle). Transgenic mice on the calorie-restricted diet had body fat levels indistinguishable from those of control mice on an ad libitum diet (Table 1 and Fig. 5, bottom), further indicating the role of hyperphagia in mediating the obesity of ADAR2b-hGH transgenic mice. Quantification of body fat for obese animals using this MRI-based strategy was highly variable (Table 1), presumably due to magnetization transfer (MT) between unsuppressed water and suppressed fat, thereby lowering the water intensity in the fat-suppressed images (41). Nevertheless, the MRI images clearly demon-
stratified a significant difference between obese transgenic mice under the ad libitum feeding regimen versus wild-type and pair-fed transgenic animals. In addition to increased adiposity, mice expressing the ADAR2b-hGH transgene demonstrated a significant elevation in plasma leptin and glucose concentrations, whereas paired feeding of mutant animals reduced all of these values to control levels (Table 1).

**Alterations in Hypothalamic Feeding Pathways in ADAR2b-hGH Transgenic Mice**—Previous studies have demonstrated that the expression of many hypothalamic molecules modulating feeding behavior, including syndecan-3, agouti-related peptide, POMC, and NPY, are altered in the obese state and can be regulated in response to feeding status (42). To further define the known hypothalamic feeding pathways that are affected in ADAR2b-hGH animals, we employed a quantitative reverse transcription-PCR-based strategy to examine changes in the expression of mRNAs encoding NPY and POMC (Fig. 6A). Although no differences in relative NPY or POMC mRNA expression were observed between wild-type mice and transgenic animals prior to the onset of obesity, a significant increase and decrease in the relative expression of NPY and POMC mRNA, respectively, was observed in age-matched (30-week) transgenic versus control mice, consistent with the hyperphagia observed in mutant animals from cumulative and paired feeding studies (Fig. 4).

In addition to alterations in the expression of orexigenic peptides such as NPY and agouti-related peptide in several models of rodent obesity (43, 44), disruption of melanocortin signaling has also been shown to result in the ectopic expression of NPY in the dorsomedial nucleus of the hypothalamus that may represent a downstream target of POMC neurons in their effects on feeding and metabolism (45, 46). In situ hybridization analysis using a 35S-labeled antisense riboprobe for prepro-NPY revealed specific labeling in both the arcuate (ARC) and dorsomedial (DMH) nuclei of the hypothalamus in ADAR2b-hGH transgenic animals (Fig. 6B), similar to the pattern observed in melanocortin-4 receptor (MC-4)-null and lethal yellow (Ay) mutant mice (45), yet such labeling in the dorsomedial hypothalamus was not observed in wild-type mice. These observations are consistent with a decrease in melanocortin signaling that could be mediated by the observed decrease in POMC-derived α-melanotropin expression (Fig. 6A).

**Leptin and d-Fenfluramine Suppression of Feeding Is Intact in ADAR2b-hGH Transgenic Mice**—Given that the onset of obesity in male ADAR2b transgenic animals was detectable by as early as 8 weeks of age (Fig. 1B), hyperphagia must have preceded the observed increases in body mass when plasma leptin levels were normal (Table 1). By 12 weeks of age, however, transgenic mice were both hyperphagic and hyperleptinemic (data not shown), suggesting that these animals were functionally leptin-resistant. To determine if the onset of hyperphagia for preobese ADAR2b transgenic animals (6 weeks of age) resulted from defects in leptin transport or signaling, cumulative food intake was assessed after intraperitoneal administration of leptin (2.5 μg/g body weight) (Fig. 7, left). Peripheral leptin administration resulted in a comparable reduction in food intake for wild-type and transgenic mice compared with vehicle-injected controls (49.2 and 52.5% reduction, respec-
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tively), suggesting that defects in the transport of leptin across the blood-brain barrier or in diffusion from circumventricular organs (such as the median eminence) were not responsible for the increased food consumption of young, nonobese ADAR2b-expressing animals. It should be noted that the food intake of transgenic mice was significantly less than that of control animals during these studies, possibly resulting from the transient stress associated with the individual housing of animals (47) in the experimental design.

The serotonergic system also has been extensively implicated in the control of feeding behavior (48), and the indirect serotonin receptor agonist and appetite suppressant, \(d\)-fenfluramine (\(d\)-FEN), has been shown to inhibit food intake and lead to weight loss through activation of melanocortin pathways in the arcuate nucleus (49). Observations that transcripts encoding 5-HT2cR undergo up to five RNA editing events to decrease the efficacy of receptor-G protein interactions and that mice lacking the 5-HT2cR demonstrate age-dependent obesity, hyperinsulinemia, and hyperglycemia (25, 26, 50) suggested that a reduction in serotonergic signaling could be responsible for the hyperphagia and increased adiposity in ADAR2b-expressing transgenic animals. To determine whether the ability of \(d\)-FEN to suppress food intake was altered in ADAR2b-hGH transgenic mice (5 weeks of age), control and transgenic animals were given either \(d\)-FEN (3 \(\mu\)g/g body weight) or 0.9% saline by intraperitoneal injection. Administration of \(d\)-FEN resulted in a 53.8% decrease in food intake for control mice and a 47.3% reduction in cumulative food consumption for transgenic animals, indicating that the observed hyperphagia could not be directly explained by alterations in serotonergic signaling (Fig. 7, right). As observed in the leptin studies, individually housed transgenic animals ate significantly less than when housed in groups.

Expression of Wild-type or Deaminase-deficient ADAR2 Can Affect Adenosine to Inosine Editing—Previous in vitro studies have suggested that ADAR1 and ADAR2 can affect the site selectivity of one another, presumably due to sequence-independent competition for binding duplex RNA (51), suggesting that the obese phenotype observed in deaminase-deficient ADAR2b (E396A) transgenic mice could result from indirect alterations in the editing of ADAR1 targets or interference with the actions of other dsRNA-binding proteins. To further determine whether expression of the deaminase-deficient transgene could affect ADAR1-selective editing, we took advantage of an in vitro heterologous expression system. Human embryonic kidney (HEK293) cells were cotransfected with cDNAs encoding ADAR1 and wild-type or mutant ADAR2b, along with a 289-base pair minigene substrate derived from the 5-HT2cR gene (6) (Fig. 8). Total RNA from transfected HEK293 cells was isolated and assessed for editing at the A site of minigene-derived 5-HT2cR transcripts, representing a position preferentially modified by ADAR1 (6, 8). Results from this analysis indicated that both ADAR1 and ADAR2b were capable of editing the A site, although ADAR1 appeared to be more efficient, yet cells transfected with the ADAR2bE396A cDNA demonstrated background levels of editing identical to control cells transfected with the 5-HT2cR minigene alone (Fig. 8). Cotransfection of ADAR1 with increasing amounts of ADAR2b resulted in a dose-dependent inhibition of A site editing, consistent with previous observations that ADAR1 and ADAR2 can compete for site-selective deamination (51). Cotransfection of ADAR1 with the deaminase-deficient ADAR2b mutant also resulted in the inhibition of A site editing, although the E396A mutant was more potent than wild-type ADAR2b, presumably acting solely as an inhibitor of ADAR1, with no intrinsic editing activity of its own.

Observations that the 5-HT2c receptor is involved in the modulation of feeding behavior (25, 50) and that pre-mRNA transcripts encoding this receptor are modified by adenosine to inosine conversion to affect receptor signaling (6, 52, 53) have made changes in the editing of 5-HT2cR transcripts an attractive target for explaining, at least in part, the hyperphagia and adult onset obesity observed in ADAR2b transgenic mice. Analysis of 5-HT2cR editing patterns in transcripts derived from whole brain RNA (data not shown) or hypothalamus (Fig. 9A), however, did not reveal any significant alterations in site-selective editing. To determine if the expression of the ADAR2b transgene resulted in an alteration of editing for other ADAR substrates, we quantified the extent of adenosine to inosine modification for RNAs encoding the GluR-2 and GluR-5 subunits of ionotropic glutamate receptors (54, 55), the K\(_5\),\(_\)1,\(_\)1 subunit of the K\(_5\) voltage-gated potassium channel (7), and an autoediting site within ADAR2 pre-mRNA (11, 12). Since the expression of most identified ADAR substrates is restricted to the nervous system (8), we focused upon the editing status of ADAR2 targets isolated from whole brain RNA in wild-type and ADAR2b transgenic mice using a modified primer extension analysis (6, 11) (supplemental Table S1). No significant changes in the editing of any ADAR substrates were observed either prior to or after the onset of the obesity (Fig. 9B), suggesting that the level of transgene expression in the brain may have been insufficient to further increase the extent of adenosine to inosine conversion in a variety of ADAR2 targets. Initial comparisons of ADAR2 protein expression in wild-type and ADAR2b-hGH transgenic mice revealed only a 20% increase in...
increase in ADAR1 expression. Quantitative reverse transcription-PCR analysis, however, revealed no differences in ADAR1 mRNA levels between wild-type and transgenic animals using RNA isolated from cortex, olfactory bulb, and hypothalamus either before (5 weeks) or after the onset of obesity (30 weeks) (supplemental Fig. S4). Alternatively, the absence of editing changes in mutant animals could result from transgene-mediated inhibition of endogenous ADAR2 expression by increasing the editing of ADAR2 pre-mRNAs (−1 site) to produce a transcript that does not encode a functional ADAR2 protein (+47 isoform) (11, 12). Although no significant changes in editing were observed for the −1 site within ADAR2 pre-mRNA transcripts isolated from whole mouse brain (Fig. 9B), we focused further upon alternative splicing patterns for endogenous ADAR2 transcripts in the olfactory bulb, where the transgene expression was greater than in all other brain regions examined (supplemental Fig. S3). Results from this analysis revealed only a slight change in the extent of ADAR2 alternative splicing for wild-type and mutant mice with the +47 mRNA isoform, representing 76.7 ± 0.2 and 81.2 ± 1.1% ($p < 0.05; n = 3$) of total ADAR2 mRNAs, respectively.

**DISCUSSION**

Widespread expression of ADAR2b in transgenic mice resulted in maturity onset obesity in both male and female animals (Figs. 1 and 3). Although plasma analysis of preobese transgenic animals showed normal levels of glucose and leptin when compared with control littermates, hyperglycemia and hyperleptinemia appeared to develop secondary to the obese state, similar to that observed for 5HT2C−/− null mice (25) and for syndecan-1 transgenic mice in which the CMV promoter/enhancer was used to drive transgene expression (42). Analysis of cumulative food intake showed that ADAR2 transgenic mice consumed significantly more food than control littermates (Fig. 4A), with paired feeding studies further indicating that hyperphagia was largely responsible for the observed phenotypic alterations (Fig. 4, B and C, and Table 1). Chronic hyperphagia has been reported previously for 5-HT2CR null mice, leading to late onset obesity associated with partial leptin resistance and hyperleptinemia (25, 26) and for syndecan-1 mice that develop hyperglycemia, hyperinsulinemia, and hyperleptinemia in the postobese state (42). Since obesity also results from a lack of physical activity and/or metabolic defects, we also compared the physical activity and metabolic activity of transgenic and wild-type animals, demonstrating that transgenic mice had a decreased ability to utilize fatty acids relative to carbohydrates (56) with no differences in physical activity (supplemental Fig. S1). Interestingly, pair-fed transgenic mice were significantly hyperactive (data not shown), presumably resulting from hyperphagia-mediated foraging behavior in the face of limited food availability (57).

Leptin and serotonin signaling contribute substantially to the regulation of feeding behavior and energy expenditure (34, 58, 59). Defective leptin signaling has been associated with the development of both hyperphagia and obesity (60–62), whereas 5-HT2C agonists not only reduce feeding when acutely administered to rats or mice but also can reduce body weight when administered chronically to obese animals (63,
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64). Intraperitoneal administration of leptin or D-fenfluramine, prior to the onset of obesity, resulted in comparable reductions in food intake for control and ADAR2b-hGH transgenic animals (Fig. 7), suggesting that functional alterations in serotonin or leptin responsiveness do not underlie the hyperphagia that precedes significant differences in body mass between wild-type and transgenic mice. However, previous studies of 5-HT2cR-null animals have indicated that mutant and wild-type mice had an equivalent hypophagic response when leptin was provided at 5 μg/g body weight, whereas mutant mice administered a lower dose (2.5 μg/g body weight) had diminished inhibition of food consumption compared with control mice of similar age (26). Although the dose of leptin and D-fenfluramine used for our analyses was based upon previous studies demonstrating a difference between control and 5-HT2cR-null animals (26, 50), the absence of a differential response for ADAR2b transgenic mice could result from differences in leptin or serotonin sensitivity that may be revealed only by more detailed concentration-response curves.

Editing of RNA transcripts encoding the 5-HT2cR can generate receptor isoforms with a reduced efficacy for G-protein coupling and reduced constitutive activity (6, 52, 65–67). Since mice expressing 5-HT2cR receptors with sufficiently reduced coupling efficiency could phenocopy the obese characteristics 5-HT2cR-null animals, an alteration in the editing of 5-HT2cR RNAs represents an attractive molecular mechanism by which the expression of an ADAR2b-hGH transgene could result in hyperphagia. Examinations of editing patterns in whole brain (Fig. 9B) or hypothalamus (Fig. 9A) showed no alteration in the extent of editing for transgenic mice, consistent with the small increase in whole brain ADAR2 expression (1.2-fold) in ADAR2b-hGH mice (Fig. 3B). The absence of changes in editing patterns could result from a low level of transgene expression or maximal levels of ADAR2-dependent activity that are unaffected by additional ADAR2 expression, or alternatively, physiologically relevant changes in editing may have occurred in only a small percentage of neurons in the central nervous system.

Although our original hypothesis proposed that phenotypic alterations in mice expressing elevated levels of ADAR2 would result from increased editing of ADAR2 substrates, expression of the catalytically inactive (E396A) form of ADAR2b also resulted in obesity (Fig. 1B), thus raising the possibility that low level hGH expression might underlie this phenotype, as previously described for transgenic rats (24, 32). However, when the hGH-encoding 3‘-untranslated region of the transgene was replaced by sequences encoding the SV40 polyadenylation signal (Fig. 1A), transgenic mice continued to demonstrate increased growth and adiposity, thereby indicating that the obese phenotype did not result from “leaky” hGH expression prior to the onset of obesity. Analysis of transgene-derived RNA levels in ADAR2b-SV40 transgenic mice demonstrated dramatically lower transgene expression when compared with mice bearing transgenes with the hGH gene as 3‘-untranslated region (Fig. 3A), consistent with the delayed onset of obesity observed in these animals (Fig. 1D). Unfortunately, decreased ADAR2b-SV40 expression was observed in all tissues, making it impossible to correlate the decreased transgene expression in any single tissue with the delayed appearance of obesity.

Recent studies from our laboratory have demonstrated that the ability of ADAR2 to edit its own pre-mRNA represents a negative autoregulatory strategy to modulate ADAR2 protein expression (12). Genetically modified mice in which ADAR2 autoediting was ablated demonstrated a significant increase in ADAR2 protein expression in multiple tissues, including a 2.4-fold increase in whole brain, as compared with the 1.2-fold increase observed in ADAR2b transgenic mice (Fig. 3B). Unlike the absence of editing changes observed in ADAR2b mutant animals, however (Fig. 9), ADAR2 autoediting-deficient animals demonstrated significant increases and decreases in editing at multiple sites that are selectively modified by ADAR2 and ADAR1, respectively (12). Despite the increase in ADAR2 expression and changes in adenosine to inosine conversion, mutant animals lacking ADAR2 autoregulation did not demonstrate any obvious phenotypic alterations, indicating that the obesity observed in ADAR2b transgenic mice may result from CMV-driven misexpression of the transgene outside cells normally expressing ADAR2. Furthermore, since expression of the deaminase-deficient FLAG-ADAR2b (E396A) transgene also resulted in hyperphagia-mediated obesity (Fig. 1B), it is likely that the observed phenotype results from a novel activity of ADAR2 or its ability to interfere with the function of other members of a large family of dsRNA-binding proteins (68, 69).

Molecular recognition of dsRNA is a key event for numerous biological pathways, including the trafficking, localization, editing, and maturation of cellular RNA, translation regulation, the interferon-mediated antiviral response, and RNA interference (68, 70, 71). Co-crystallization studies of the dsRBD from the Xenopus Xrlnb protein and its dsRNA target have indicated that there are few contacts between the dsRBD and the functional groups of the bases within the narrow major groove of the A-form RNA duplex, suggesting little sequence-dependent binding specificity (69, 72). A lack of sequence-specific binding for members of the dsRBD-containing family suggests that the obese phenotype observed in transgenic mice may not result from the editing activity of FLAG-ADAR2b but rather from its ability to bind to a wide variety of dsRNA substrates, thus competing with the functions of other dsRNA-binding proteins within the cell. Consistent with this hypothesis, recent studies have demonstrated that ADAR1 and ADAR2 can avidly bind short interfering RNA without editing the short interfering RNA target, thereby limiting the efficacy of RNA interference in mammalian cells (73). ADAR2 also has been shown to interfere with the site-selective editing of ADAR1 (51), thereby providing mechanisms by which the catalytically inactive ADAR2b isoform (E396A) could serve as an inhibitor to interfere with the site-selective editing of endogenous ADAR1 or ADAR2. Proteins containing dsRBDS play numerous and varied roles in modulating cellular function (68); however, our current lack of information regarding their biological activities, RNA targets, and protein partners suggests that much needs to be done to determine the roles that dsRBD-containing proteins may play in regulating feeding behavior and energy balance.

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