Alternative Splicing in the Ligand Binding Domain of Mouse ApoE Receptor-2 Produces Receptor Variants Binding Reelin but Not α2-Macroglobulin*

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LR7/8B and ApoER2 are recently discovered members of the low density lipoprotein (LDL) receptor family. Although structurally different, these two proteins are derived from homologous genes in chicken and man by alternative splicing and contain 7 or 8 LDL receptor ligand-binding repeats. Here we present the cDNA for ApoER2 cloned from mouse brain and describe splice variants in the ligand binding domain of this protein, which are distinct from those present in man and chicken. The cloned cDNA is coding for a receptor with only five LDL receptor ligand-binding repeats, i.e. comprising repeats 1–3, 7, and 8. Reverse transcriptase-polymerase chain reaction analysis of mRNA from murine brain revealed the existence of two additional transcripts. One is lacking repeat 8, and in the other repeat 8 is substituted for by a 13-amino acid insertion with a consensus site for furin cleavage arising from an additional small exon present in the murine gene. None of the transcripts in the mouse, however, contain repeats 4–6. In murine placenta only the form containing repeats 1–3 and 7 and the furin cleavage site is detectable. Analysis of the corresponding region of the murine gene showed the existence of 6 exons coding for a total of 8 ligand binding repeats, with one exon encoding repeats 4–6. Exon trapping experiments demonstrated that this exon is constitutively spliced out in all murine transcripts. Thus, the murine ApoER2 gene codes for receptor variants harboring either 4 or 5 binding repeats only. Recombinant expression of the 5-repeat and 4-repeat variants showed that repeats 1–3, 7, and 8 are sufficient for binding of β-very low density lipoprotein and reelin, but not for recognition of α2-macroglobulin, which binds to the avian homologue of ApoER2 harboring 8 ligand binding repeats.

The low density lipoprotein receptor (LDLR) family consists of a growing number of structurally related composite cell surface receptors with partially overlapping ligand specificity (1, 2). For example, the LDLR harbors structurally and functionally defined modules, corresponding to distinct exons in the gene (3). These modules are as follows: (i) the "type A-binding repeats" (LA repeats) of ~40 residues each, displaying a triple disulfide bond-stabilized and negatively charged surface mediating receptor/ligand interactions; (ii) "type B repeats" (EG repeats), also containing six cysteines each; EG repeats are homologous to regions in the epidermal growth factor precursor; (iii) modules of ~50 residues with a consensus tetrapeptide, Tyr-Trp-Thr-Asp (YWTD); (iv) a so-called "O-linked sugar domain"; (v) a short transmembrane domain of ~20 amino acids; and (vi) the cytoplasmic region with a signal for receptor internalization via coated pits. LR7/8B (4, 5) and its human homologue called ApoER2 (6, 7) belong to the close relatives of the LDLR made up of exactly the same domains in the same order as in the LDLR. The occurrence of distinct splice variants adds yet another level of complexity to this family of proteins. LR7/8B expression in chicken is highly restricted to the brain, where the protein resides in large neurons and Purkinje cells, and in cells constituting brain barrier systems such as the epithelial cells of the choroid plexus and the arachnoid and the endothelium of blood vessels (8). The finding that chicken LR8B acts as receptor for α2-macroglobulin (8) suggests a role in the clearance of α2-macroglobulin-proteinase complexes from the cerebrospinal fluid. Ligand binding studies with two splice variants of human ApoER2 demonstrated high affinity of the receptor to β-VLDL, indicating that in mammals the receptor might be involved in apoE-mediated transport processes in the brain as well (7). This is an interesting aspect because a genetic link between certain alleles of the ApoE gene and the development of late onset Alzheimer's disease has been established (9). However, no differences in the splice variant patterns of ApoER2 between control and Alzheimer's patients exist (10).

Targeted disruption of the apoER2 gene alone or in combination with that for the VLDL receptor gene revealed a key function of both receptors during embryonic brain development (11). Absence of functional ApoER2 and VLDL receptor leads to an inversion of cortical layers and absence of cerebellar foliation. This phenotype is indistinguishable from that seen in

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession number(s) AJ312658 and AJ312659.

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§ The abbreviations used are: LDLR, low density lipoprotein receptor; VLDLR, very LDLR; apo, apolipoprotein; LR7/8B, LDLR relative with 7 or 8 LA repeats; ApoER2, apoE receptor 2; RAP, receptor-associated protein; α2M, α2-macroglobulin; PCR, polymerase chain reaction; LA, type A binding repeats; EG, type B repeats; RT, reverse transcriptase; bp, base pair; RACE, rapid amplification of cDNA ends; HRP, horse-radish peroxidase; GST, glutathione S-transferase.

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animals carrying either a mutation in the \textit{reeler} gene or in the \textit{disabled} gene (for review see Refs. 12–14). Both ApoER2 and VLDL receptor bind reelin, which is secreted by Cajal-Retzius cells (15, 16), and do so apparently together with cadherin-related neuronal receptors (17) and/or $\alpha_3\beta_1$ integrin (18), which may act as co-receptors for reelin to transmit the signal into migrating neurons. Upon reelin stimulation the intracellular adapter protein disabled-1 (Dab1), which interacts with the cytoplasmic domains of apoER2 and VLDLR (19, 20), becomes tyrosine-phosphorylated (21, 22). These data suggest that ApoER2 and VLDL receptor directly relate the extracellular reelin signal into a cellular response via Dab1, leading to the ultimate cell responses required for the correct positioning of newly generated neurons during brain development.

In addition, interaction screens with apoER2 and other members of the receptor family have resulted in the identification of many other candidate proteins interacting with the cytoplasmic domains of the respective receptors, suggesting that they may be part of an intricate network of signaling pathways (23, 24).

Here we present the full-length cDNA and the partial characterization of the mouse \textit{apoER2}. Amino acid sequence numbering starts with the first amino acid in the mature protein after cleavage of the putative signal sequence. The sequence corresponding to the signal peptide is boxed. Cysteine residues are encircled. The transmembrane domain is underlined. The cytoplasmic internalization signal sequence is boxed.

![FIG. 1. Nucleotide and deduced amino acid sequence of the cDNA for murine ApoER2.](http://www.jbc.org/)

Amino acid sequence numbering starts with the first amino acid in the mature protein after cleavage of the putative signal sequence. The sequence corresponding to the signal peptide is boxed. Cysteine residues are encircled. The transmembrane domain is underlined. The cytoplasmic internalization signal sequence is boxed.
A hypothetical cDNA deduced from the murine mouse; a–c, P1 clone was used for PCR amplification with the indicated primer combinations. Amplified products were separated on 1.5% agarose gels (mouse brain and mouse placenta was used for cDNA synthesis with reverse transcriptase. The resulting cDNAs and DNA prepared from a mouse female Balb/c mice, and total chicken brain from female Brown Derco) from 200 mg of frozen tissue (total brain and placenta from mature new sequence exceeding the start codon. 9-GTCCTCGTCGCTGTTGTCCG) and resulted in additional 174 bp of sequence, was done using primer ASR1 (5'-CTGCCGAGAAGTTAAGCTGC; mouse 2, 5'-CCGCTCCTGGTTGCACCGTTTGATG). The second round was performed with primer ASR2 (5'-RACE was done in two successive rounds of PCR provided by the kit. 5'-RACE using the 5'-part and the 3'-part of the ligand binding domain, respectively. The resulting clones were G14AS13 and 8AS-3.5 (see Fig. 3) for this, entire clones were identified by PCR analysis and sequenced. MATERIALS AND METHODS 5'-RACE Cloning of the Complete cDNA for Mouse ApoER2—The 5'-end of the mouse cDNA for apoER2 was cloned by 5'-RACE using the Marathon cDNA amplification kit (CLONTECH) according to the manufacturer’s protocol. First and second strand cDNA was synthesized from 5 μg of poly(A) messenger RNA and the poly(A) synthesis primer provided by the kit. 5'-RACE was done in two successive rounds of PCR amplification, with the sense primer located in an adapter ligated to the cDNA. The antisense primer was located in the known sequence from a partial cDNA clone (4). The first round, which resulted in 334 bp of new sequence, was done using primer ASR1 (5'-CCGCTCCTGGTTGCACCGTTTGATG, 3/4 annealing at 58 °C; chicken 7, 5'-CCAAGCA TTCAGTGTGG; mouse 5, 5'-CTGCCGAGAAGTTAAGCTGC; mouse 2, 5'-CGTGAAGATCAGAGATGGGC, 1/2 annealing at 60 °C; pair 1, 5'-GCCGCGGACCGCAGCTTCCACC, 5'-GCCCTTGATTCGTCAAGGCC; pair 2, 5'-CCGCCGTCCACGCACTCGCC, 5'-CGAGAATGAGTTCCAGTGTGG) located within the 5'-part and the 3'-part of the ligand binding domain, respectively. The resulting clones were G14AS13 and 8AS-3.5 (see Fig. 3A). The rest of the gene covering the ligand binding domain of the receptor was covered by PCR clones derived from the undigested P1 clone with the following primer combinations (see Fig. 3A); 1, 5'-TGCGAGCAGCTTCCACTCACC and 5'-TGCTCCTGGCTGCTTGTGCCG; 2, 5'-CCCTTGTGTTGGAGATGCGTAG and 5'-AGGTGACATGGCGCCCTGCC; 3, 5'-GCCGCGGAGCGGACCTCACC and 5'-GCAGCTTAATTCTTCGGCAGG; 4, 5'-AGCGAGAAGAGCTTGAGG and 5'-TGTCATGAACAAGAAACCATCCGCC; 5, 5'-AGTCTCTAGAAGACAGACCTTCTAGC and 5'-TGTCATGAACAAGAAACCATCCGCC; 6, 5'-AGTGGCGAGCTTCCAGGACCGC and 5'-TGAGCTCAGCTGAGGTGG. PCR products were isolated from the gel using the QIAEX II gel extraction kit (Qiagen) and subcloned into the pCR2.1 vector (Invitrogen). Several clones from each fragment were isolated, and positive clones were identified by PCR analysis and sequenced. Partial Characterization of the Mouse ApoER2 Gene—A mouse ES P1 library (Genome Systems, Inc.) was screened by PCR using the following primer pair: A, 5'-TGCTCGCAATAACCCGCGCCTTGGTCTGTC and B, 5'-ACAGGCTCTTTGCTGCTTTTGGAGCTGAAGAG. PCR conditions are as follows: 2 min initial denaturation at 94 °C, 1 min denaturation at 94 °C, 1 min annealing at 56 °C, 1 min extension at 72 °C for 35 cycles. Using 1 μg of genomic DNA, a 165-bp amplicon derived from exon 9 (EG repeat A) was produced under these conditions. P1 plasmid DNA from positive clones was prepared from 150-ml cultures (Escherichia coli strain NS 3529, Genome Systems, Inc.) using the plasmid MidiKit (Qiagen) according to manufacturer’s instructions including an additional phenol extraction step before loading on the column. P1 DNA was digested with BgII and shotgun-subcloned into the BamHI site of pBluescriptII (aK+). Clones were screened by PCR using two independent primer combinations (pair 1, 5'-GCCGCGGACCGCAGCTTCCACC, 5'-GCCCTTGATTCGTCAAGGCC; pair 2, 5'-CCGCCGTCCACGCACTCGCC, 5'-CGAGAATGAGTTCCAGTGTGG) located within the 5'-part and the 3'-part of the ligand binding domain, respectively. The resulting clones were GI4AS13 and 8AS-3.5 (see Fig. 3A). The gene covering the ligand binding domain of the receptor was covered by PCR clones derived from the undigested P1 clone with the following primer combinations (see Fig. 3A); 1, 5'-TGCGAGCAGCTTCCACTCACC and 5'-TGCTCCTGGCTGCTTGTGCCG; 2, 5'-CCCTTGTGTTGGAGATGCGTAG and 5'-AGGTGACATGGCGCCCTGCC; 3, 5'-GCCGCGGAGCGGACCTCACC and 5'-GCAGCTTAATTCTTCGGCAGG; 4, 5'-AGCGAGAAGAGCTTGAGG and 5'-TGTCATGAACAAGAAACCATCCGCC; 5, 5'-AGTCTCTAGAAGACAGACCTTCTAGC and 5'-TGTCATGAACAAGAAACCATCCGCC; 6, 5'-AGTGGCGAGCTTCCAGGACCGC and 5'-TGAGCTCAGCTGAGGTGG. PCR products were isolated from the gel using the QIAEX II gel extraction kit (Qiagen), subcloned into the pCR2.1 vector (Invitrogen), and sequenced. Exon Trapping Experiments—For the exon trap experiment two constructs were made. One contained the exon coding for repeats 4–6 including 89 bp of the upstream and 563 bp of the downstream intron, respectively (corresponding to genomic clone 5, Fig. 3). For this, entire clone 5 was cloned into filled in SfiI sites of the exon trap vector pet01.
and mRNA was prepared using the Micro-fast track from Invitrogen. 1 grown on a 90-mm plate. 48 h after transfection, cells were harvested, cells using Lipofectin reagent (Life Technologies, Inc.), and cells were sites in the polylinker of pet01. 3

This construct (pEx2, 4–6) was made by cloning the entire genomic harboring two exons, one for repeat 2 and the other for repeats 4–6. (MoBiTec GmbH, Göttingen) resulting in pEx4-6. The second construct containing genomic clone 5 plus clone G14A13B3 into pEx4-6 upstream of clone 5 using

acceptor sites flanking exon 5 are sequences are in uppercase letters.

B, lines are drawn as designation of corresponding structural elements of the receptor. Below

cut out from the pCR2.1 constructs via an internal

full-length cDNAs, fragments containing 4 repeats and 5 repeats were

were assembled by joining a partial cDNA for the mouse protein (4) with

4-6,8 and repeats 1–3 and 7 were purified on a 1% agarose gel, cloned

ucts of the PCR reaction with 5RS1, 5RAS6 containing repeats 1–3, 7,

primers 5RS1 (5’-GGAGCCCCGGGCCGCTATGG) and 5RAS6 (5’

the appropriate PCR products derived from mouse brain cDNA with

were incubated with Dulbecco’s modified Eagle’s medium containing 2% bovine serum albumin, 584 mg/liter glutamine, and 125I-labeled ligand at

expression of chicken LR8B in 293 cells has been described (8). Trans-

incubated with Dulbecco’s modified Eagle’s medium containing 2% bo-

fluency. After three washes with phosphate-buffered saline, cells were

were prepared as described for chicken embryo fibro-

mals fed a 2% cholesterol, 10% corn oil diet for 3 weeks (27) and was

tubes according to the manufacturer’s recommendation (Pierce, catalog number 28601) to specific activities of 300–400 cpm/ng. Labeled

Western blotting were performed as described previously (26). The

glycoprotein complex (αM*), were generated as described (29). Complete

forms were selected by the addition of 500 mg/liter G418 to the

medium (Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.), 10% fetal calf serum, 584 mg/liter glutamine).

Preparation of Cell Extracts, Electrophoresis, and Western Blotting—Total cell extracts from 293 cells expressing LR8B, ApoER2Δ4-6, or ApoER2Δ4-6,8 were prepared as described for chicken embryo fibroblasts (25). Electrophoresis, transfer to nitrocellulose membranes, and

Expression of Chicken LR8B and Mouse ApoER2Δ4-6 and ApoER2Δ4-6.8—Chicken LR8B and mouse apoER2Δ4-6 and apoER2Δ4-6,8 were expressed in the human embryonic kidney cell line 293. The full-length cDNA of mouse apoER2Δ4-6 and apoER2Δ4-6,8 were assembled by joining a partial cDNA for the mouse protein (4) with the appropriate PCR products derived from mouse brain cDNA with primers 5RS1 (5’-GGAGCCCCGGGCCGCTATGG) and 5RAS6 (5’-GCTCATCAATGAGGACCACCC) via an internal Bgl II site. The products of the PCR reaction with 5RS1, 5RAS6 containing repeats 1–3, 7, and 8 and repeats 1–3 and 7 were purified on a 1% agarose gel, cloned into pcR2.1, and sequenced. The partial cDNA (see above) was cloned into the eukaryotic expression vector pCI-Neo. In order to produce the full-length cDNAs, fragments containing 4 repeats and 5 repeats were cut out from the pcR2.1 constructs via an internal Bgl II and the XhoI site (present in the polylinker of pcR2.1) and cloned into the pcI-Neo construct containing the rest of the cDNA. The construct used for expression of chicken LR8B in 293 cells has been described (8). Transfection of the cells was performed using Lipofectin Reagent (Life Technologies, Inc.) according to the manufacturer’s protocol. Stable trans-

forms were expressed in 293 cells as described previously (28). αM was isolated from chicken plasma as described (29). Native αM was radiolabeled using Iodo-Gen pre-coated iodoination tubes according to the manufacturer’s recommendation (Pierce, catalog number 28601) to specific activities of 300–400 cpm/ng. Labeled αM-trypsin complexes (αM*), were generated as described (29). Complete activation of labeled αM by trypsin was monitored by native gel electrophoresis using the Tris borate system described by van Leuven (30). Recombinant human RAP was produced as a glutathione S-transferase (GST) fusion protein using a pGEX 2T-derived (Amersham Pharmacia Biotech) expression plasmid in DH5α bacteria (31). Internalization of 125I-VLDL and 125I-αM* by Rabbit receptor-expressing Cells—Rabbit αM VLDL was prepared from the plasma of animals fed a 2% cholesteryl, 10% corn oil diet for 3 weeks (27) and was radiolabeled with 125I by the iodine monochloride method as described earlier (25). Lipoprotein antibodies are in the cytoplasmic domains of chicken LR8B (8) and mouse ApoER2 (24) are described in the respective references.

Binding and Internalization of βVLDL and αM* by Receptor-expressing Cells—

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FIG. 3. Organization of the 5’-region of the murine ApoER2 gene. A, the organization of the 5’-region of the murine ApoER2 gene is schematically shown. Exons are drawn as lines and introns as triangles. The intron numbers are shown on top. The exons are labeled with the designation of corresponding structural elements of the receptor. Below, P1-derived clones and PCR-derived clones covering the region of the gene are drawn as lines (combinations of numbers and letters refer to the primers described under “Materials and Methods”). The schematic and the clones are not drawn to scale. B, exon/intron organization of the murine ApoER2 gene. Intronic sequences are shown in lowercase letters, and exon acceptor sites are shown in uppercase letters. The exon numbers refer to the numbers used in A. Consensus sequences for the 5’-splice donor and 3’-splice acceptor sites flanking exon 5 are underlined (see text). The corresponding human sequences in introns 4 and 5 are shown below the respective mouse sequences. C, nucleotide and deduced amino acid sequence of the cDNA encoded by exon 5 of the murine ApoER2 gene. Intronic sequences are shown in lowercase letters. Cysteine residues are encircled. Portions of codons interrupted by introns are underlined.
were lysed by addition of 1 ml of 0.1 M NaOH and incubation at 23 °C for 10 min. The radioactivity in the lysate was determined with a γ-counter (COBRA II, Packard Instrument Co.), and the protein concentration was determined.

Kd values were determined by Scatchard analysis and expressed in nM using a Mr 350,000 for bVLDL.

Reelin Binding Studies—293 cells expressing ApoER2 D4-6 and ApoER2 D4-6,8 and mock-transfected cells were washed and resuspended in phosphate buffer and incubated with conditioned media from reelin-expressing 293T cells as described (15). In brief, 293T cells (60–80% confluent) were transfected with 7 μg of the reelin construct pCRL. The following day, the culture medium was replaced with a serum-reduced medium (Opti-MEM). After 2 more days the conditioned medium was collected and used for incubation of ApoER2-expressing cells. For ApoER2 expression, 293 cells were transiently transfected with 10 μg of ApoER2 D4-6 and ApoER2 D4-6,8 constructs. After 48 h the cells were harvested, washed once with 1 ml of phosphate-buffered saline, and incubated with 1 ml of the reelin supernatant at 4 °C for 4 h in the absence or presence of GST-RAP (30 μg/ml), EDTA (20 mM), or β-VLDL (50 μg/ml). CaCl2 was added to a final concentration of 0.5 mM. The cells were washed 2× with 1.5 ml of phosphate-buffered saline and lysed with 50 μl buffer containing 50 mM Tris, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 20 mM sodium fluoride, 1 mM sodium orthovanadate, and proteinase inhibitors. After 30 min lysis on ice, the cell extracts were centrifuged at 13,000 × g for 30 min. 6–10 μl of the supernatant was loaded on a 7.5% gel. Transfer to nitrocellulose was done as described previously (16). The blot was incubated for 3 h with G10 antibody (provided by Dr. Andre Goffinet, Namur Med. School, Namur, Belgium) at a concentration of 1:1000. Goat anti-mouse HRP was used as a secondary antibody at a concentration of 1:10,000. The blots were developed using an ECL kit.

Alternatively, reelin binding to both splice variants of the receptor was tested by a cell-independent assay described by Hiesberger et al. (16). For this assay, cDNAs coding for the ligand binding domains of ApoER2 D4-6 and ApoER2 D4-6,8, respectively, were amplified by PCR using the respective full-length cDNAs as templates and fused to the constant human IgG domain as described (16) resulting in ApoER2 D4-
protein-A Sepharose was then incubated with 500 mM amino acids. Localization of introns are highlighted by filled rectangles. The amino acid sequences of splice variants derived thereof are represented in relation to a schematic representing structural elements of the murine RAP-GST or 20 mM EDTA or medium for 4 h at 4°C. Detection of bound proteins were bound to protein-A Sepharose (30 μl slurry) as described. The amount of receptor-Fc fusion proteins present on the blot were analyzed by Western blotting using an HRP-coupled anti-V5 antibody (Invitrogen).

**RESULTS AND DISCUSSION**

When we reported the cDNA cloning of chicken LR8B, we also presented the amino acid sequence derived from a partial cDNA for the corresponding murine homologue (4). The 5'-end of that cDNA defined the carboxyl-terminal part of the last LA-binding repeat of the mouse receptor. By using this sequence information for RT-PCR experiments, we were able to assign this sequence to repeat number 7 of a mouse variant of LR7/8B that lacks repeat 8 due to differential splicing (5). By using two rounds of 5'-RACE, we now have obtained the full-length cDNA for mouse ApoER2 (Fig. 1). As outlined below, the murine receptor appears to be a highly heterogeneous family of proteins due to multiple alternative splicing events. Therefore we refer to the murine protein as apoER2 as originally suggested for the human protein by Yamamoto and colleagues (6). According to the rule of Van Heijne (33), we assigned the putative furin cleavage consensus sequence.

![Diagram of the region of the murine ApoER2 gene coding for the furin cleavage site.](http://example.com/diagram.png)

**Fig. 5. Partial sequence of the region of the murine ApoER2 gene coding for the furin cleavage site.** Nucleotide sequence and deduced amino acid sequences of splice variants derived thereof are represented in relation to a schematic representing structural elements of the murine ApoER2 protein. Localization of introns are highlighted by filled rectangles. Intronic sequences are shown in lowercase letters and exon sequences in uppercase letters. The putative furin cleavage consensus sequence is underlined. Empty numbered boxes and shaded boxes represent LA repeats and ER repeats A, respectively.

6-Fc and ApoER24-6,8-Fc. Recombinant fusion proteins were expressed in 293 cells, and the secreted proteins (1 ml of cell supernatant) were bound to protein-A Sepharose (30 μl slurry) as described. The protein-A Sepharose was then incubated with 500 μl of conditioned media from reelin-expressing 293T cells in the presence of 50 μg/ml RAP-GST or 20 mM EDTA or medium for 4 h at 4°C. Detection of bound reelin was performed as described (16) using G10 as primary antibody and goat anti-mouse IgG conjugated to HRP as second antibody. The amounts of receptor-Fc fusion proteins present on the blot were analyzed by Western blotting using an HRP-coupled anti-V5 antibody (Invitrogen).

![Diagram of the putative furin cleavage site of the murine ApoER2 gene.](http://example.com/diagram.png)

**Fig. 2. Primers 3 and 4,** respectively. As shown in Fig. 2a, in adult mouse brain 3 distinct variants are present. These transcripts contain in addition to repeats 1–3 the following: (i) either both repeats 7 and 8 (505 bp), (ii) only repeat 7 together with a 13-amino acid insertion defining a consensus furin cleavage site (421 bp), or (iii) repeat 7 only (382 bp). In embryonic brain (day 10), however, the large variant containing repeat 8 is completely missing. This variant appears at day 12 establishing a splice variant pattern indistinguishable from that seen in the adult brain (Fig. 2b). In mouse placenta a single ampiclon with a size of 421 bp is present (Fig. 2b). Upon sequencing, this band turned out to be the transcript coding for the receptor variant containing the insertion with the furin site and lacking repeat 8. If the suggested cleavage at this site does occur, the corresponding placental protein could be a soluble receptor fragment with possibly different functions from those of the full-length variants present in the brain.

By using a primer pair flanking repeats 4–6 (primers 3 and 4), the only ampiclon obtained using mRNA either from adult brain or placenta had a length of 197 bp (Fig. 2c). Upon sequencing, it became evident that the amplified cDNA was identical to the corresponding region of the cDNA shown in Fig. 1 lacking repeats 4–6. As control we performed the same analysis with a chicken-specific primer pair using chicken brain mRNA as template. As seen in Fig. 2f, the only band obtained has a length of 614 bp and is derived from a transcript containing these 3 LA repeats. Since exactly these repeats are encoded for by a single exon in the human gene (7), we tested whether the corresponding exon was lost from the mouse gene or whether it is deleted from the transcripts by alternative splicing.

![Diagram of the putative furin cleavage site of the murine ApoER2 gene.](http://example.com/diagram.png)

**Fig. 3. Partial sequence of the region of the murine ApoER2 gene.** By having obtained the sequence coding for repeats 4–6 from genomic cloning, we were able to design another control experiment for the presence of transcripts containing this exon in brain and placenta, respectively. A primer combination corresponding to sequences within this exon (Fig. 2, primers 5 and 6) were used to amplify corresponding transcripts by RT-PCR. As seen in Fig. 2b, the expected ampiclon with a size of 293 bp was only obtained using the P1 clone as template but not with cDNA derived from either brain or placental mRNA. This experiment
clearly demonstrates that this exon is missing from transcripts in mice. It has been demonstrated that mutations in sequences near splice junctions can affect splicing efficiency (34). Thus, the flanking exon-intron junctions of exon 5 were compared with the consensus sequences for such regions (35). Both splice sites match the consensus in that the 5' donor site contains the

![Figure 6](image_url)
5’-GT, which is essentially invariant, and the 3’-acceptor site contains a CAG which follows a pyrimidine-rich stretch at the 3’-end of the corresponding intron (the respective sequences are underlined in Fig. 3B). Furthermore, the respective intron sequences are completely conserved between the murine and the human gene (Fig. 3B). From the organization of the gene and the sequences of the respective exon/intron junctions, it is not obvious why this exon is spliced out constitutively from the mature transcripts in mice, but only facultatively in man (7). However, in a report where alternate splicing patterns of mature transcripts in mice, but only facultatively in man (7). However, in a report where alternate splicing patterns of mature transcripts in mice, but only facultatively in man (7).

To clarify the situation, we cloned a genomic fragment containing the exon coding for repeat 4–6 and parts of the upstream and downstream introns into the exon trap vector pet01 creating pEx4-6 (Fig. 4A). As internal control we combined this genomic fragment with another fragment harboring the exon coding for repeat 2 (pEx24-6). When transfected into 293 cells (Fig. 4B), pEx4-6 produced a transcript of 246 bp, indistinguishable from that produced by the vector alone. Sequencing of both products confirmed these bands to be derived from the intronic exons present in the vector. pEx2,4-6, however, produced a transcript with a length of 369 bp containing a hybrid cDNA made up of the exons present in the vector and the exon coding for repeat 2. The exon for repeats 4–6 again was missing from the transcript, indicating that this sequence is not recognized as exon in this system.

Another very interesting feature of the murine gene is the fact that the short insertion of 13 amino acids specifying a consensus furin cleavage site, present in some splice variants of ApoER2 (5), is encoded by a separate small exon and is not inserted by the use of an alternative 5’-splice donor site, as might be expected for such a small insertion. The 39-bp exon is located between those for LA repeat 8 and EG repeat A, respectively (Fig. 5). Sequence details of this region are shown in Fig. 5. Since a homologous insertion is also present in variants of human ApoER2 (5), we assume that in the human gene a corresponding exon is present in intron 6 (numbering according to Ref. 7).

To determine if the constitutive loss of repeats 4–6 in the murine receptor which leads to the expression of receptor variants with either 4 or 5 ligand binding repeats results in distinct ligand specificity, we expressed these variants, ApoER2Δ4-6 and ApoER2Δ4-6,8, in 293 cells, measured their affinities for β-VLDL and α2M (α2M*), and compared the results with those obtained with the chicken variant containing the maximum of 8 ligand binding repeats (LR8B). The inset in Fig. 6D represents a Western blot with membrane detergent extracts from 293 cells expressing murine ApoER2Δ4-6,8 (lane 1), ApoER2Δ4-6 (lane 2), and chicken LR8B (lane 3), respectively. Double bands seen for the murine proteins most likely result from differential glycosylation in this cell system. To test for the ability of these receptors to interact with ligands, we used a combined binding and internalization assay (8). As demonstrated in Fig. 6, A–C, all three receptor variants bind β-VLDL with high affinity (Kd value of 53 nM for ApoER2Δ4-6,8; Kd value of 13 nM for ApoER2Δ4-6; and Kd value of 8 nM for LR8B). This is not surprising, since a human variant of ApoER2 harboring only 3 binding repeats has been already shown to bind β-VLDL efficiently (7). Apparently, the affinity for β-VLDL slightly decreases with the successive loss of binding repeats. However, it was very interesting to note that neither mouse ApoER2Δ4-6,8 nor ApoER2Δ4-6 bind α2M* (Fig. 6, D and E), in sharp contrast to LR8B which binds α2M* with high affinity (Kd value of 129 nM) (Fig. 6F, and cf. (8)). The small amount of β-VLDL and α2M* binding to mock-transfected 293 cells is most likely due to low expression of intrinsic LDLR-related protein by these cells (8). From an evolutionary point of view, it is interesting that the loss of 3 binding repeats by constitutive splicing of the corresponding exon in mice compromises the ability of the receptor to bind α2M*. Apparently, a minimum of 8 ligand binding repeats seems necessary for recognition of α2M*, as suggested by the fact that the chicken homologue of
the VLDLR, *i.e.* LR8, contains 8 ligand binding repeats and binds \( \alpha_2 \)-M* with high affinity (8), whereas the LDLR harboring seven repeats does not.

Since it has recently been shown that one of the murine variants (ApoER2-D<sup>4-6</sup>) acts as receptor for reelin (15, 16), and as demonstrated here (Fig. 7, A and B), expression of the 4-repeat and 5-repeat variants in the mouse brain is highly regulated during embryonic development, we determined and compared reelin binding to both receptor variants. Reelin binding was measured with two independent approaches, one using receptor-expressing cells (15) and the other using soluble receptor constructs as described recently (16). As demonstrated in Fig. 7A, cells expressing either ApoER2-D<sup>4-6,8</sup> or ApoER2-D<sup>4-6</sup> bind equal amounts of reelin produced by transfected 293T cells. Binding of reelin to both receptor variants is abolished by EDTA, GST-RAP, and \( \beta \)-VLDL. This result was confirmed by the second assay showing that soluble ApoER2 receptor constructs harboring either 4 or 5 binding repeats bind reelin with equal affinities (Fig. 7B). Again, binding is completely abolished by an excess of GST-RAP or by EDTA.

The complex situation is summarized in Fig. 8, which contains all identified transcripts exhibiting variations in the ligand binding domain of the receptor in different species. In chicken, where the prevalent expression of LR7/8B occurs in brain, only two distinct variants harboring 7 or 8 repeats exist. As analyzed at the mRNA level, the furin cleavage site, which exists in man and mouse, is obviously absent. However, the corresponding region of the chicken gene has not been characterized to date, and it may well be that this exon is constitutively spliced out in chicken transcripts. Therefore, the question whether the corresponding exon in mammals was gained during evolution cannot be answered yet. The mouse, as documented here, produces three major transcripts varying in the ligand binding domain. There are two significant differences to the transcripts in chicken. First, none of the transcripts found contained repeats 4–6, which are present on a single exon (exon 5). Genomic analysis suggests that this exon is constitutively deleted by splicing out in mice. Second, in mice there is an additional small exon following that for LA repeat 8, giving rise to a variant harboring a furin consensus cleavage site at the carboxyl-terminal end of the ligand binding domain. Most interestingly, this variant is the only one detectable in placentas, showing that some of the splice events are tissue-specific.

The situation in man is not as clear yet. Obviously, none of the characterized transcripts contain repeat 8, and analysis of the human gene suggests that the corresponding exon might have been lost (6, 7). However, this exon seems to be present in the human gene, but a mutation in the 5<sup>-</sup>-splice donor site causes this exon to be constitutively skipped (36). In analogy to the mouse gene, a corresponding exon coding for the furin-cleavage site insertion (5) is likely present in the corresponding region of the human gene. In addition to the mouse variants described herein, a receptor transcript containing only repeats 1–3 has been reported to be present in humans (7). As mentioned above, the use of exon 5, coding for repeats 4–6, could not be confirmed in a recent study on normal and Alzheimer brains (10).

So far, it has been shown that all human splice variants are able to bind rabbit \( \beta \)-VLDL with similar affinity (6, 7). Thus, it is not surprising that both murine ApoER2-D<sup>4-6</sup> and ApoER2-D<sup>4-6,8</sup> bind \( \beta \)-VLDL with high affinity. However, the fact that mouse ApoER2 does not bind \( \alpha_2 \)-M* is interesting from an evolutionary point of view considering that the chicken
homologue recognizes α2M*. From a structural point of view the lack of α2M* binding to mouse ApoER2 is not surprising, since the α2M*-receptors found so far harbor a minimum of 8 ligand binding repeats clustered in one domain (LDLR-related protein, chicken LR8, chicken LR8B). Apparently, ApoER2 has lost its ability to bind α2M* but has gained other features during evolution from birds to mammals. These features include the potential to be produced as a soluble receptor fragment, and the ability to interact with certain intracellular adapter proteins like JIP-1 and JIP-2, mediated by an insertion in the cytoplasmic tail of the receptor (24).

Furthermore, this study demonstrates for the first time that both murine ApoER2 variants containing either 4 or 5 LA repeats bind reelin equally well. By taking into account that both murine ApoER2 variants containing either 4 or 5 LA repeats bind reelin equally well. By taking into account that

# Acknowledgments

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Alternative Splicing in the Ligand Binding Domain of Mouse ApoE Receptor-2 Produces Receptor Variants Binding Reelin but Not α2-Macroglobulin

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