J. Biochem. 124, 451-456 (1998)

Evolution of the Apolipoprotein E Receptor 2 Gene by Exon Loss[1]

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Received for publication, March 31, 1998

The apolipoprotein E receptor 2 (apoER2) gene consists of a mosaic of exons, which may have been assembled by "exon shuffling." Analysis of apoER2 transcripts in several species reveals a lost repeat in the ligand-binding domain of primate apoER2. A pseudo-exon found in the primate apoER2 genes corresponds to the lost repeat but contains a crucial deletion that leads to a translational frameshift. The pseudo-exon sequence in primary transcripts of the human apoER2 gene is shown to be abolished by exon skipping due to two nucleotide substitutions at the 5′-splice donor adjacent to the pseudo-exon. These data suggest the occurrence of exon loss in the evolution of the primate apoER2 gene.

Key words: apoE receptor, exon shuffling, exon skipping, pseudo exon, repetitive sequence.

The exon shuffling hypothesis predicts that the presence of introns permits functional domains encoded by discrete exons to be shuffled between different proteins, thereby allowing proteins to evolve by their assembly as new combinations of preexisting functional units (*1-3*). A vivid example demonstrating the existence of exon shuffling is the gene encoding the low density lipoprotein receptor (LDLR) (*4*). LDLR is a mosaic protein consisting of several functional domains shared among different proteins in the LDLR super-gene family including apolipoprotein E receptor 2 (apoER2) (*5, 6*) and very low density lipoprotein receptor (VLDLR) (*7, 8*). Among the LDLR family members, apoER2 (*5, 6*) and VLDLR (*7, 8*) most closely resemble LDLR with respect to ligand specificity and structural similarity. These three receptors consist of: an amino terminal ligand-binding domain, composed of multiple ligand-binding repeats (*9, 10*); an epidermal growth factor (EGF) precursor homology domain, with three growth factor repeats (*11*); an *O*-linked sugar domain with clustered serine and threonine (*12*); a transmembrane domain; and a cytoplasmic domain with a coated pit signal (*13*).

Whereas LDLR binds with high affinity to both apoE- and apo B100-containing lipoproteins (reviewed in Ref. *14*), apoER2 [which predominates in brain in mammals (*5*)] and VLDLR [which is abundant in skeletal muscle and heart in mammals (*7*)] bind apoE-rich β-migrating VLDL (β-VLDL) with high affinity but with much lower affinity to apo B100-containing LDL. The ligand-binding domain of human apoER2 consists of seven repeats (*5, 6*), as does that of LDLR (*15*), but molecular characterization of the human apoER2 gene reveals that it is much more closely related to VLDLR, which contains eight repeats (*7, 8*). An extra ligand-binding repeat also occurs in the chicken homologue of apoER2, termed LR8B (*16*). Although we have found molecular variants of apoER2 containing three or four repeats, we have not found any apoER2 variants with eight ligand-binding repeats in human tissues (*6*).

As an initial approach to elucidate the mechanism causing the diversity of the ligand-binding repeats of apoER2 in various species, we characterized murine apoER2. Analysis of the murine apoER2 transcripts and gene revealed the presence of an extra exon encoding an additional binding repeat that is not found in the human apoER2 transcripts. Nucleotide sequencing of the intron regions corresponding to the extra exon in human and marmoset genes revealed the presence of a pseudo-exon (*17*) containing a crucial deletion that leads to a translational frameshift. The pseudo-exon sequence in the primary transcripts of the human apoER2 gene is shown to be abolished by exon skipping caused by two nucleotide substitutions at the 5′-splice donor adjacent to the pseudo-exon. In this paper, we describe the occurrence of exon loss in the primate apoER2 genes and discuss the contribution of exon loss in the evolution of genes.

EXPERIMENTAL PROCEDURES

Standard Procedures—Standard molecular biology techniques were performed essentially as described by Sambrook *et al.* (*18*). Genomic and cDNA clones were subcloned into pBluescript vectors and sequenced by the dideoxy chain-termination method (*19*) on an Applied Biosystems model 373A DNA sequencer. Total RNA was prepared using standard guanidinium thiocyanate lysis

[1] This work was supported by the Japan Society for the Promotion of Science Grant RFTF97L00803. The sequence reported in this paper has been submitted to the GeneBank data Bank (D85463).

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Abbreviations: apoE, apolipoprotein E; apoER2, apolipoprotein E receptor 2; EGF, epidermal growth factor; LDL, low density lipoprotein; LDLR, low density lipoprotein receptor; RT-PCR, reverse transcription polymerase chain reaction; VLDLR, very low density lipoprotein receptor; β-VLDL, β-migrating very low density lipoprotein.

buffer and centrifugation over a cesium chloride cushion. Human brain poly(A) RNA was obtained from Clonetech. Marmoset brain RNA was the generous gift of Dr. Teiichirou Koga (Sankyo). A mouse cDNA library was constructed using poly(A) RNA and the cDNA synthesis kit from Pharmacia. By screening of 5×10^5 clones using the entire coding region of the human apoER2 cDNA (*5*), we obtained three positive clones; one representative containing the largest insert (pHJ1) was further characterized. The mouse genomic clone $\lambda 5$ was isolated from a genomic DNA library constructed by cloning of partial Sau3A digests of 129/Sv mouse genomic DNA (*20*). Intron sizes were determined by Southern blotting, restriction mapping, and PCR analysis. DNA fragments carrying exons were identified by restriction mapping and Southern blotting and sequenced. The 4.5-kb DNA fragments containing exons 6 and 7 of the bovine, marmoset, and human apoER2 genes were amplified by PCR using an LA PCR kit Ver. 2 (Takara Syuzou) and a set of primers, oligos S and A (see below), and sequenced. Nucleotide sequence comparison was performed using the FASTA (*21*) and BLAST (*22*) programs.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)—cDNA was synthesized from 1 μg of poly(A) RNA using random hexanucleotide primer and 200 units of Superscript™ (Life Technologies) in 20 μl of standard reverse transcription buffer at 37°C for 1 h. PCR was performed with 2 μl of reverse transcriptase reaction mixture under standard conditions. Amplification was carried out over 33 cycles of: 94°C, 30 s; 60°C, 1 min; 72°C, 2 min. Sequences of the primers were as follows: oligo 82 (5′-GCGGATCCGCTGCTGGGC-3′) and oligo 1191 (5′-TGCTGGGCACGTGCACTCTT-3′) for analysis of the region corresponding to the ligand-binding domain of murine apoER2; and oligo S (5′-GTTCCAGTGTGGGGATGGGA-3′) and oligo A (5′-TCTGGTCCAGGAGCTGGAA-3′) for analysis of repeat 8 containing transcripts in various species.

Exon Trap Analysis—Exon trap plasmids containing exon 6′ and intron-flanking sequences of the mouse apoER2 gene (pmWt) and those corresponding to the human gene (phWt) were constructed in the splicing vector pSPL3 (Life Technologies). *In vitro* mutagenesis was carried out on phWt to produce a series of mutant plasmids, phA to phG. The sequences of these mutant plasmids are shown in Fig. 4A. RT-PCR analysis of total RNA from transfected COS cells was performed using primers SA, dUSD2, and dUSA4 (Life Technologies): primer SA was used to prime the synthesis of cDNA; and primers dUSD2 and dUSA4 were used for PCR.

Lipoprotein-Binding Assay—Mouse apoER2 expression plasmids were constructed in the pRc/CMV vector (Invitrogen) using the standard procedures, and transfected into LDLR lacking *ldl*A-7 cells (*23*) as described (*5*). Rabbit β-VLDL ($d < 1.006$ g/ml) was prepared from the plasma of 1% cholesterol-fed animals (*24*). Binding of ^{125}I-labeled β-VLDL by the transfected LDLR-deficient *ldl*A-7 cells was assayed according to the procedure as described (*25*), except that dextran sulfate was replaced by suramin to release bound β-VLDL (*11*).

RESULTS

Species Diversity of Ligand-Binding Repeats—Previous studies revealed the expression of multiple transcripts encoding apoER2 with repeats 1-3, 1-3 plus 7, and 1-7 in human tissues (*6*). To analyze the diversity of murine apoER2 transcripts, we initially isolated and characterized a murine apoER2 cDNA (designated pHJ1) from a brain cDNA library. The amino acid sequence deduced from the cDNA revealed the presence of an additional ligand-binding repeat (repeat 8) in murine apoER2 (Fig. 1A). Excluding repeat 8 in the ligand-binding domain of murine apoER2, approximately 90% of the amino acids are conserved between human and murine apoER2 (Fig. 1B). In murine apoER2 and chicken LR8B, more than 80% of the amino acids in the EGF precursor homology and cytoplasmic domains are identical [excluding a 59-amino-acid insertion sequence (*5, 6*)]. In contrast, only 61% sequence conservation exists within the ligand-binding domains of the two

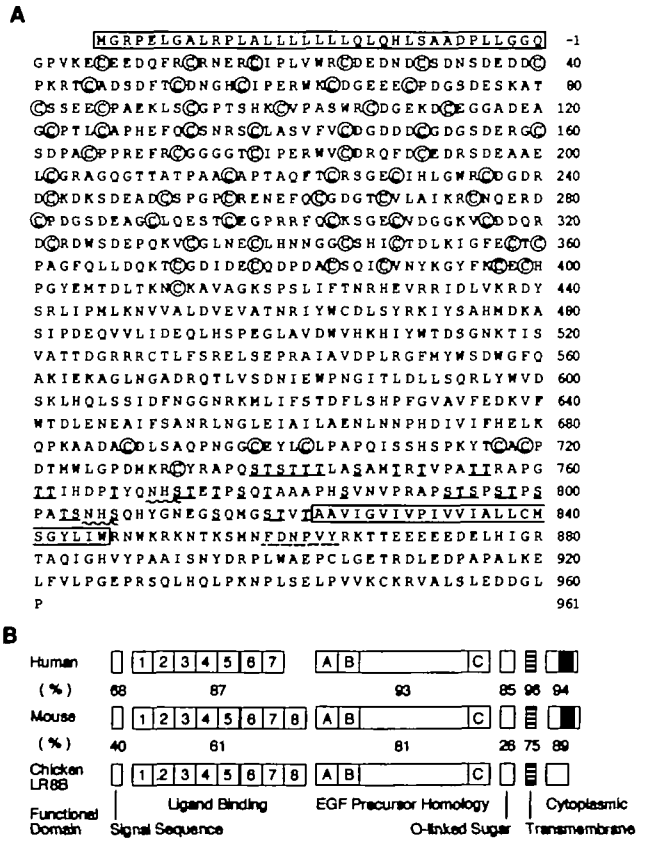

Fig. 1. **Structure of murine apoER2.** (A) The deduced amino acid sequence of murine apoER2. Amino acids are numbered on the right. Residue 1 is the glycine believed to constitute the NH_2-terminus of the mature protein; negative numbers refer to the cleaved signal sequence (boxed, NH_2-terminus). Cysteine residues are circled. Two potential sites of *N*-linked glycosylation (Asn-X-Ser or Asn-X-Thr) are indicated by wavy underlines. Serine and threonine residues in a region that corresponds to the *O*-linked sugar domain of the receptor are underlined. The 24-residue transmembrane segment located toward the COOH-terminus of the protein is boxed. The FDNPXY sequence (*13*) required for clustering of the LDLR in coated pits is indicated by a dotted underline. (B) Schematic comparison of murine apoER2 with human apoER2 and chicken homologue LR8B. The ligand-binding repeats are numbered. The cysteine-rich repeats in the EGF precursor homology domain (growth factor repeats) are lettered A to C. The five functional domains of the three proteins are labeled in the lower portion of the figure. The number of identical residues (expressed as a percentage) in a given domain is indicated between the two relevant proteins.

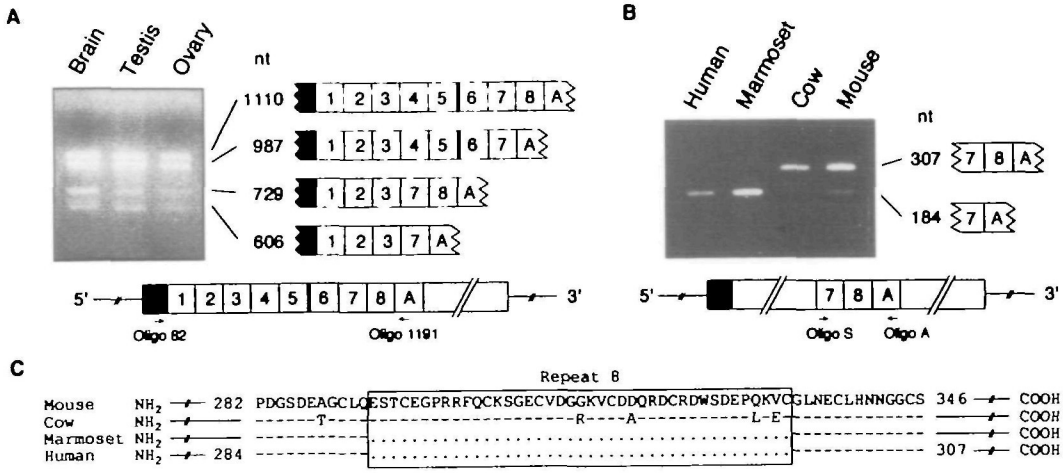

Fig. 2. **Expression of apoER2 variants containing the extra binding repeat.** (A) RT-PCR analysis of the region corresponding to the ligand-binding domain of apoER2 transcripts in mouse tissues. The location of the region corresponding to the ligand-binding domain and oligonucleotide primers used for RT-PCR are shown in the lower portion of the figure. Arrows indicate oligonucleotide primers. Poly(A) RNA (1 μg) from mouse brain, testis, and ovary was used for RT-PCR. The resulting PCR products were separated on a 2% agarose gel and stained with ethidium bromide. (B) Expression of apoER2 transcripts encoding variants containing repeat 8 in human, marmoset, cow, and mouse brains. The location of the region corresponding to repeat 8 and oligonucleotide primers used for RT-PCR are shown in the lower portion of the figure. (C) Amino acid comparison of the region of apoER2 repeat 8 in various species. The amino acid sequences were deduced from the nucleotide sequence of RT-PCR products. The numbers correspond to the mouse and human sequences. Amino acids in repeat 8 are boxed. Amino acid residues identical with those of murine apoER2 are indicated with bars. Dots within each sequence represent gaps which were introduced to maximize homology (*i.e.* repeat 8 is lost from human and marmoset apoER2).

proteins, suggesting that these domains may have evolved at different rates due to different selective constraints. This may be related to their ligand specificity: human and murine apoER2 bind apoE-rich β-VLDL with high affinity (see below); whereas, in chicken, there is no evidence for the presence of apoE (*26, 27*), despite the expression of LR8B in brain (*16*).

RT-PCR analysis of the region corresponding to the ligand-binding domain of murine apoER2 revealed four major amplified fragments with 606, 729, 987, and 1110 nucleotides (nt) in mouse brain, testis and ovary (Fig. 2A). The sequences of these amplified products revealed apoER2 transcripts encoding repeats 1-7, 1-8, 1-3 plus 7, and 1-3 plus 7 and 8, indicating that mice express multiple apoER2 variants containing repeat 8, which is not found in human tissues.

To analyze the species specificity of transcripts containing repeat 8, we carried out RT-PCR of mouse, cow, marmoset, and human brain RNAs. As shown in Fig. 2, B and C, the transcript containing repeat 8 was found in mouse and cow brains but not in those of the primates. These data indicate that apoER2 transcripts containing repeat 8 are present only in nonprimate vertebrates, including cattle, mice, and chickens.

A Lost Exon in the Primate apoER2 Genes—To determine whether the extra ligand-binding repeat (repeat 8) in murine apoER2 is encoded by an extra exon, part of this gene was characterized. By screening a mouse genomic DNA library, we obtained one clone (λ5) that contains an exon encoding repeat 8 (designated exon 6′) of murine apoER2 (Fig. 3A). Excluding this extra exon, exons 7-16 of the mouse apoER2 gene were mapped at positions corresponding exactly with those of the human gene (*6*).

To determine the presence of sequences corresponding to exon 6′ of the mouse apoER2 gene in other species, we

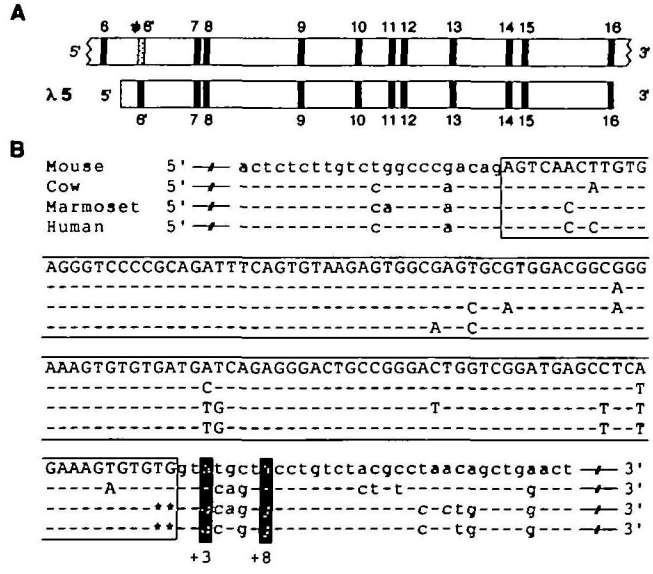

Fig. 3. **Mapping of an exon encoding the extra ligand-binding repeat (repeat 8) in the apoER2 gene.** (A) Schematic comparison of a part of the mouse and human apoER2 genes. A mouse genomic clone λ5 was used to map exons 6′-16 of the mouse gene (lower panel) and schematically compared with exons 6-16 of the human gene (upper panel). Exons are denoted by filled areas and numbers. Introns are indicated by open areas. The exon numbers in the mouse gene correspond to those of the human gene. ψ6′ (stippled) in the human gene indicates a pseudo-exon corresponding exon 6′ of the mouse gene. (B) Nucleotide sequences of exon 6′/pseudo-exon 6′ and flanking introns of the murine, bovine, marmoset, and human apoER2 genes. Exon 6′ and pseudo-exon 6′ sequences are boxed. Missing nucleotides in pseudo-exon 6′ of the human and marmoset apoER2 genes are indicated with asterisks. Nucleotides identical with those of the mouse apoER2 gene are indicated with dashes. The nucleotides at +3 and +8 are boxed in black.

amplified the corresponding regions of the cow, marmoset, and human genes and sequenced them. Although the sequence for mouse exon 6′ is conserved among the species (Fig. 3B), sequence alignment revealed a dinucleotide (TG) deletion at the 3′-end of the corresponding exon sequences in the marmoset and human genes. Also revealed were several nucleotide alterations at the 5′-splice donor of the human and marmoset genes. These data indicate the presence in primates of a lost exon (pseudo-exon 6′) containing a deletion causing a translational frameshift, and they predict the loss of most of the C-terminus half if this pseudo-exon was correctly processed to mRNA.

Exon Skipping of the Deletion-Bearing Exon—To evaluate whether these nucleotide alterations indeed affect correct splicing, exon trap analysis was performed using various plasmid constructs (Fig. 4A). Upon transfection into COS cells, total RNA was isolated and analyzed by RT-PCR using a set of specific primers to detect splicing products. The exon trap analysis with a trapping plasmid (phWt) containing pseudo-exon 6′ and intron-flanking sequences of the human gene revealed amplified products of 529-nt (product 1) and 177-nt (product 3) (Fig. 4B). Product 3 is a vector-derived product and is commonly detected in COS cells transfected with each trapping plasmid. The nucleotide sequence of product 1 revealed that splicing occurred between the vector splice donor (SDv in Fig. 4A) and the 3′-splice acceptor of pseudo-exon 6′, and between the cryptic splice donor (SDc in Fig. 4A) and the vector splice acceptor (SAv in Fig. 4A). In contrast, an exon-trapping plasmid (pmWt) containing exon 6′ and intron-flanking sequences of the mouse gene produced a 300-nt product (product 2) containing the exon 6′ sequence (in addition to the vector-derived product), indicating that the mouse exon 6′ sequence was correctly processed by splicing.

To further define the nucleotide alterations that caused aberrant splicing, mutations were introduced into the exon-trapping plasmid containing the human sequence. Reversion of the missing dinucleotide TG into the wild-type human sequence (phD) had no effect, indicating that the deletion itself has no effect on splicing. Neither G-to-A substitution at position +3 (phA and phE) nor the same substitution at +8 (phB and phF), in the absence or presence of the TG sequence, repaired the aberrant splicing. However, when the Gs at positions +3 and +8 were both replaced by A, correct splicing occurred irrespective of the absence (phC, product 2=298 nt) or presence (phG) of the TG sequence. We therefore conclude that aberrant splicing is caused by the nucleotide substitutions (A to G) at position +3 and +8 of the 5′-splice donor adjacent to pseudo-exon 6′ of the human apoER2 gene.

Binding Affinity of apoER2 Variants—To test whether

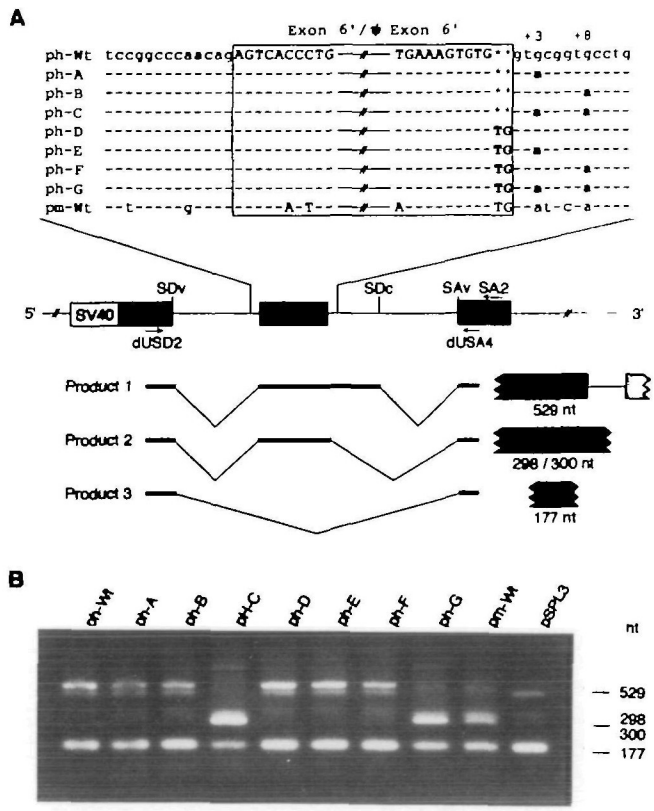

Fig. 4. **Exon trap analysis of the 5′-splice donor adjacent to pseudo-exon 6′ of the human apoER2 gene.** (A) Structure of exon-trapping plasmids. The nucleotide sequences of the test inserts are shown in the upper portion. phWt and pmWt contain human and murine wild-type apoER2 gene sequences, respectively, in an exon-trapping vector, pSPL3. Plasmids phA to phG are a series of mutants made on phWt. Pseudo-exon 6′ and exon 6′ sequences of the human and mouse apoER2 genes are boxed. Missing nucleotides in pseudo-exon 6′ of the human apoER2 gene are indicated with asterisks. Nucleotides identical with those of the human apoER2 gene are indicated with dashes. SV40 is the promoter, exons are boxed in black (insert) and gray (vector), introns are indicated by the thin horizontal line, SDv = vector splice donor, SDc = vector cryptic splice donor, and SAv = vector splice acceptor. dUSD2 and dUSA4 are the positions of the PCR primers. The structure of amplified products 1-3 in panel B is schematically represented in the lower portion. (B) RT-PCR analysis of total RNA from COS cells transfected with different exon-trapping plasmids. The amplified products were separated on a 2% agarose gel and stained with ethidium bromide.

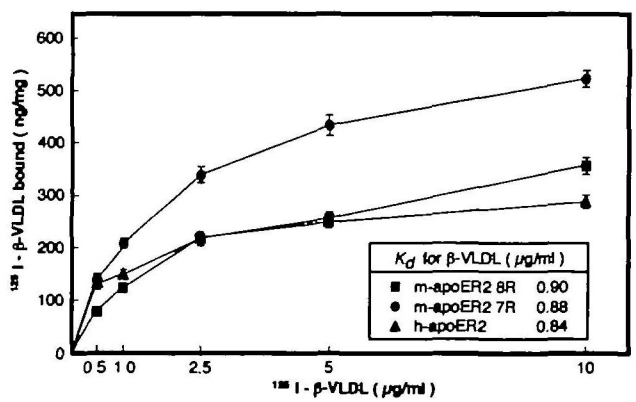

Fig. 5. **Binding of ^{125}I-labeled β-VLDL in *ldl*A-7 cells transfected with ApoER2 expression plasmids.** *ldl*A-7 cells transfected with plasmids encoding murine apoER2 with seven repeats (m-apoER2 7R), with eight repeats (m-apoER2 8R), or human apoER2 (containing seven repeats, h-apoER2) were incubated for 3 h at 4°C with the indicated concentration of ^{125}I-β-VLDL (477 cpm/ng). Surface-bound β-VLDL was then determined using a suramin-release assay (*11*). The specific values were calculated by subtracting the values obtained with pSV2-Neo transfected cells from those obtained with a given plasmid. Each value represents the mean of three incubations. Inset indicates the dissociation constant (K_d) values derived from Scatchard plot analysis.

the extra ligand-binding repeat (repeat 8) encoded by mouse exon 6′ affects ligand-binding affinity, expression plasmids were constructed, introduced into LDLR-lacking *ldl*A-7 cells (*23*), and ligand binding was measured using ^{125}I-labeled β-VLDL. As shown in Fig. 5, cells expressing murine apoER2 with eight repeats (m-apoER2 8R) and with seven repeats (m-apoER2 7R), and human apoER2 containing seven repeats (h-apoER2) bound apoE-rich β-VLDL with similarly high affinity, indicating that the extra binding repeat has essentially no effect on β-VLDL binding.

DISCUSSION

The current study demonstrated the presence of a unique exon skipping that abolishes the expression of an apoER2 mRNA containing a deletion-bearing exon sequence. This exon skipping may be a fail-safe mechanism to abolish the expression of such mutation-bearing sequences, and thus it may be related to the evolution of genes.

Exon skipping is believed to produce various forms of protein from a single gene (*28*). In addition to molecular diversity, this mechanism may enable minimization of the effects of potentially disruptive mutations. Based on the comparison of exon 6′ and its intron-flanking sequences in the murine and bovine apoER2 genes with those corresponding to the human and marmoset genes, several molecular events appear to have occurred during a recent period of evolution of the apoER2 gene: the lost exon of the apoER2 gene is found in the primates. One of the molecular events may have been the deletion of dinucleotides TG at the end of exon 6′ of an ancestral apoER2 gene. This deletion must alter the reading frame of apoER2 mRNA causing premature termination. The other events may have been the nucleotide substitutions occurring at the 5′-splicing donor adjacent to the deletion-bearing exon. These molecular events allow the apoER2 gene to avoid expression of the mutation-bearing sequence by exon skipping. Alternatively, the nucleotide substitutions occurring at the 5′-splicing donor of exon 6′ may have allowed a non-functional mutation to occur later in exon 6′.

The loss of exons assembled by shuffling may be one manifestation of the process of evolution at the gene level. Phylogenetic analysis of the LDLR members in representative species suggest that the vertebrate apoER2, VLDLR, and LDLR genes may have evolved from a common ancestor (data not shown). Interestingly, all the introns in the ligand-binding and growth factor repeat regions of the human apoER2 (*6*), VLDLR (*8*), and LDLR (*4*) genes interrupt codons at the same location, that is, after the first base of the codon. Thus, any or all of the exons encoding the ligand-binding and growth factor repeats could possibly be spliced in or out of the mRNAs without disturbing the reading frame. Together with the present results, this suggests that such a pool of exons may serve as a damage containment strategy enabling exons to be lost if they mutate to a dysfunctional form during evolution. Further characterization and comparison of genetic traits in the introns of various genes over a range of species should clarify this hypothesis.

We thank Dr. Monty Krieger for *ldl*A-7 cell, Drs. Jun-ichi Osuga and Shun Ishibashi for the mouse genomic library, Dr. Teiichirou Koga for marmoset brain RNA, Dr. Ian Gleadall for critical reading of the manuscript, and Kyoko Ogamo and Nami Suzuki for secretarial assistance.

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