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Neuron-specific Splicing of the Alzheimer Amyloid Precursor Protein Gene in a Mini-gene System

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Several forms of Alzheimer amyloid precursor protein (APP) mRNA are generated by alternative splicing. Among them, the APP695 mRNA skipping the exon 7 and 8 is expressed specifically in neurons, suggesting that this alternative splicing is regulated in a neuron-specific manner. As the first step for investigating the mechanism of the neuron-specific splicing, a mini-gene system was developed, in which mini-APP genes consisting of the exon 6, 7, 8, 9 and their flanking regions were introduced into neuronal and nonneuronal cultured cell lines to see their expression profiles. In the system the exon 7 and 8 of the mini-gene were significantly skipped in the neuronal cell, and the deletion study indicated that *cis*-acting elements for skipping the exons existed in the corresponding skipped-exon and its flanking regions. A small deletion upstream of the exon 8 suppressed the skipping of the exon 8 in the neuronal cell, suggesting that one of the regulatory sequence(s) for the exon skipping exists in a small region upstream of the skipped exon.

Alzheimer's disease (AD) is the most common neurodegenerative disorder presenting dementia in adulthood. Its pathological hallmark is deposition of amyloid fibrils in the brain, and a major constituent of the amyloid is ß/A4-proten (1,2), derived from a larger precursor protein(s) (Alzheimer amyloid precursor protein, APP) (3).

The APP gene is a large one consisted of 19 exons (4) and several forms of APP mRNAs are generated from a primary transcript by alternative splicing. Six isoforms, APP695, APP751, APP770, APP714, APP563 and L-APP, have been found so far (5-10). The former three are the major species, and the APP695 mRNA lacking the exon 7 and 8 is specifically found in neurons, while APP751 and APP770 mRNAs are ubiquitously expressed (6,7,9,11). Thus, the alternative splicing generating the APP695 mRNA is regulated in a neuron-specific manner. There have been several reports that the expression of APP751 and/or 770 mRNAs were enhanced in AD or aged brains (12,13). These suggested that APP695 should have some neuron-specific function(s) and that the aberrant APP expression in the brains may be related to the pathogenesis of AD

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and/or aging of the brains. However, little is known about the regulatory mechanism of the neuron-specific splicing of the APP mRNA.

As the first step for understanding the regulatory mechanism, we investigated the neuron-specific splicing of the APP mRNAs using mini-APP genes in cultured cell lines. Our results suggest that at least one of the *cis*-acting elements for the exon-skipping exists in an intron region upstream of the exon 8.

MATERIALS AND METHODS

Cell cultures

A human neuroblastoma cell line, LA-N-5, (14) and a human glioma cell line, U251, (15) were grown in Dulbecco's modified Eagle medium (Gibco, NY) supplemented with 10 % fetal bovine serum (Irvine Scientific, CA).

Mini-gene constructs

Individual mini-genes were constructed by ligating the following DNA fragments containing each exon and its flanking introns as shown in Fig. 1B:

exon 6, the 1.0 kb *Pst*I-*Sac*I fragment
exon 7, the 0.7 kb *Sph*I-*Pst*I or 2.2 kb *BamH*I-*Pst*I fragment
exon 8, the 0.9 kb *Hinc*II-*BamH*I fragment
exon 9, the 1.0 kb *EcoR*I-*BamH*I fragment

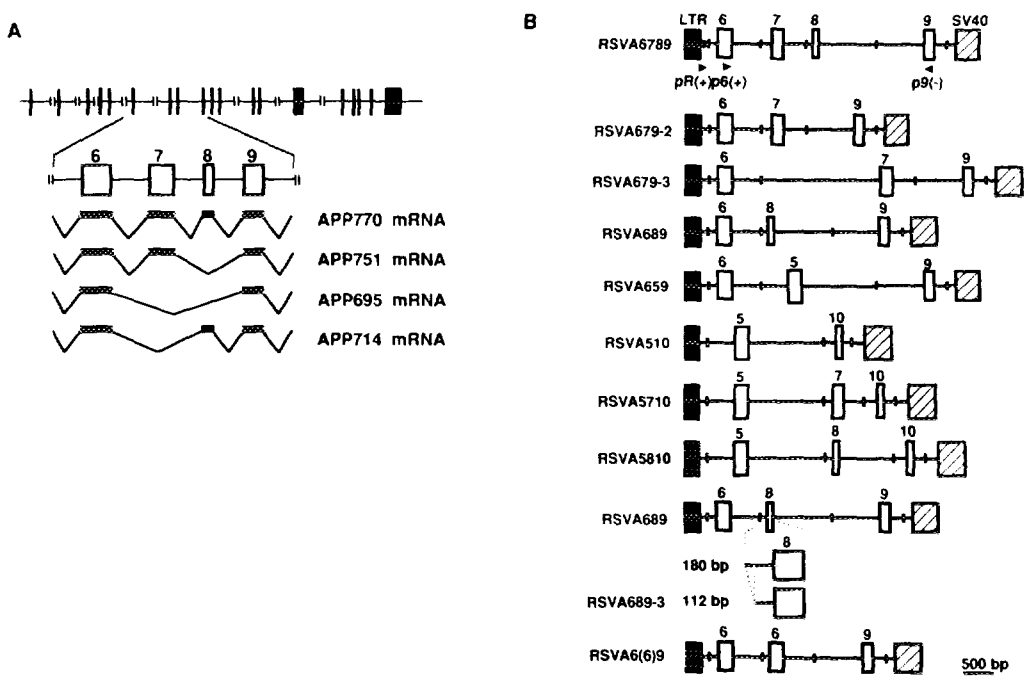

Fig. 1. The APP and mini-APP genes. (**A**) Schematic representation of the human APP gene (4) and four alternatively spliced forms of APP mRNA. Boxes and lines indicate exons and introns, respectively. (**B**) Constructs of mini-APP genes. Closed boxes, open boxes, lines and shaded boxes indicate RSV-LTR promoter, APP exons, introns and SV40 poly(A) signal, respectively. Numbers above open boxes show individual exons. Only the modified regions are shown with magnification in the lower two constructs. Numbers beside lines indicate the lengths of the intron upstream of the exon 8. The closed circle and arrowheads on the construct RSVA6789 indicate a transcription initiation site and the positions of PCR primers (pR(+), p6(+) and p9(-)), respectively.

exon 5, the 1.6 kb *Pst*I-*Pst*I fragment
exon 10, the 0.3 kb fragment produced by PCR.

When cohesive ends were not available, the ends of fragments were made blunt by T4 DNA polymerase to generate compatible sites. These ligated fragments were inserted into the unique *Hind*III site in pRSVi vector containing Rous sarcoma virus (RSV) LTR promoter and poly(A) signal of SV40 small t antigen (16). According to the included exons and their orders, the resultant plasmids were named as indicated in Fig. 1B. RSVA689-3 was constructed by deleting an intron region upstream of the exon 8 in RSVA689 by Exonuclease III and S1 nuclease. The position of the deletion was precisely determined by sequencing. RSVA6(6)9 was done by replacing the exon 8 and its downstream intron by the exon 6 and its downstream region in RSVA689.

Transfections and RNA analysis

LA-N-5 and U251 cells were transfected with the APP mini-gene plasmid DNAs by the calcium phosphate method (17). The cells were collected after 48 h, and total RNA was extracted by the acid guanidium phenol chloroform method (18). The mRNAs were analyzed using reverse transcription and polymerase chain reaction (RT-PCR) principally as described before (19). Oligonucleotides used for PCR were as follows:

pR(+)	5' ATTCACCACATTGGTGTGCA 3'	(nucleotides 122-103, ref. 20)
p6(+)	5' AGGAACCCTACGAAGAAGCC 3'	(nucleotides 776-795, ref. 3,7)
p9(-)	5' ATTCTCTCTCGGTGCTTGGC 3'	(nucleotides 1217-1198)
p5(+)	5' TTGTGTGTTGCCCACTGGCT 3'	(nucleotides 551-570)
p10(-)	5' CTGGATAACTGCCTTCTTAT 3'	(nucleotides 1240-1259).

Oligonucleotides used for hybridization probes were as follows:

p7(-)	5' ACATCCGCCGTAAAAGAAT 3'	(nucleotides 972-954)
p8(-)	5' AGTCTTGAGTAAACTTTGGG 3'	(nucleotides 1056-1037)
p91(-)	5' AGGTGTCTCGAGATACTT 3'	(nucleotides 1146-1129).

(+) and (-) indicate sense and antisense sequences, respectively.
cDNA was synthesized using 1 μg total RNA, 10 pmol antisense primer, 10 units of reverse transcriptase RAV2 (Takara, Kyoto, Japan) and each dNTP at 400 μM in 50 μl 2 x reaction buffer at 42 °C for 30 min. 1x reaction buffer consists of 50 mM KCl, 10 mM Tris HCl (pH8.3), 1.5 mM $MgCl_2$ and 0.01 % gelatin. The reverse transcription was terminated by heating to 94 °C for 5 min. The sample was added with 10 pmol sense primer and 1 unit of Taq polymerase (Perkin Elmer Cetus, CT). Primer p6(+) or pR(+) was used for the endogenous APP mRNA or mini-APP mRNAs, respectively. For mini-genes of RSVA5710, RSVA5810 and RSVA510, dimethylsulfoxide (DMSO) was added to the reaction buffer at 5 % finally (21). Samples were subjected to 15 or 20 cycles of amplification (denaturation at 94 °C for 1 min, annealing at 55 °C for 2 min and extension at 72 °C for 1 min) in 100 μl 1x reaction buffer. After addition of 0.5 pmol ^{32}P-labelled sense primer, the sample was further amplified once with extension at 72 °C for 5 min. For mini-genes of RSVA5710, RSVA5810 and RSVA510, ^{32}P-labelled p5(+) was used. Each sample was electrophoresed on a 5 % native polyacrylamide gel and radioactivities of amplified mRNA bands were measured by the Image Analyzer Fujix BAS2000A (Fuji Film, Tokyo, Japan). Amplified bands were confirmed by sequencing or Southern blot hybridization.

RESULTS AND DISCUSSION

Endogenous APP mRNAs in cultured cell lines

We at first investigated the expression profile of APP mRNAs in the cell lines used for the present study. Endogenous APP mRNAs were amplified by PCR of 15 cycles using a primer pair of p6(+) and p9(-). We previously showed that the APP mRNAs could be proportionally amplified during 15 cycles of PCR under our conditions (19). The APP695, APP751 and APP770 mRNAs were detected as the amplified fragments of 217, 385 and 442 bp, respectively (Fig. 2A). Based on the results of Fig. 2A, the ratios of APP695 : APP751 : APP 770 were estimated to be 4.1 : 2.7 : 1 in the neuroblastoma LA-N-5, and 0.3 : 0.9 : 1 in the glioma U251,

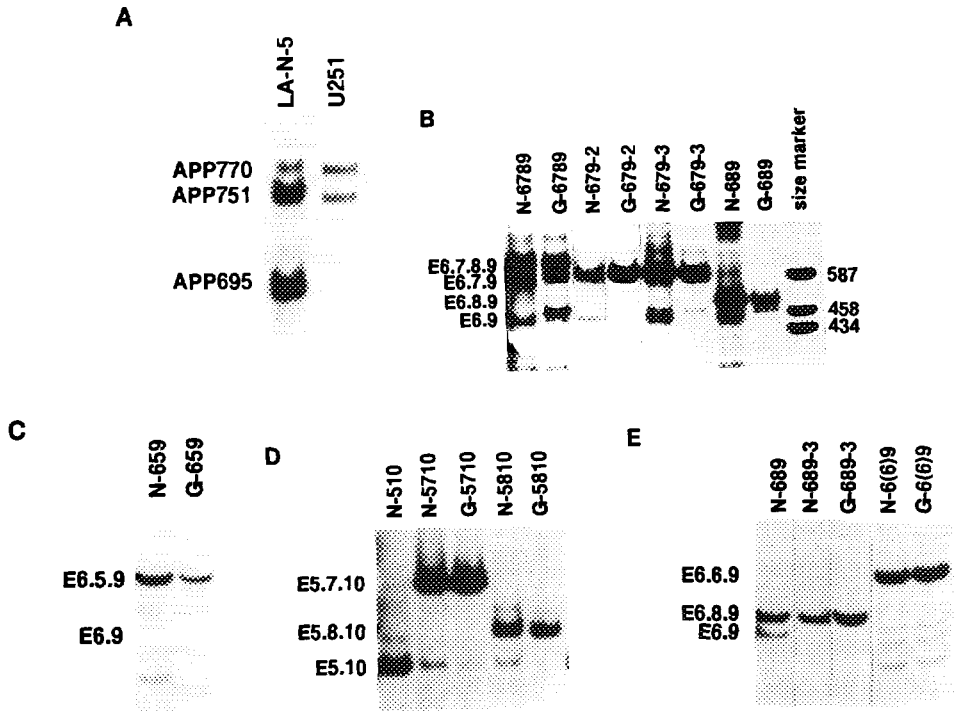

Fig. 2. RT-PCR analysis of APP and mini-APP mRNAs in cell lines. (**A**) Endogenous APP mRNA expression. (**B, C, D, E**) Mini-APP mRNAs expression. N and G indicate the neuroblastoma, LA-N-5, and the glioma, U251, respectively. Numbers added to N or G indicate corresponding mini-APP genes. The positions of mini-APP mRNAs were indicated on the left side. *Hae*III digested pUC19 plasmid DNA was used as size marker. (**B**) RSVA6789, RSVA679-2, RSVA679-3 and RSVA689. (**C**) RSVA659. (**D**) RSVA5710 and RSVA5810. (**E**) RSVA689--3 and RSVA6(6)9.

respectively. The results indicated that these cell lines still retained the features of original cell types and can be used for studying the mechanism of the neuron-specific splicing of the APP gene.

Amplification of mini-APP mRNAs

To investigate the regulatory mechanisms for the neuron-specific skipping of the exon 7 and 8, we constructed the mini-genes shown in Fig. 1B, and introduced them into the cell lines described above. For the specific detection of the mini-APP mRNAs, primer pR(+) was synthesized according to the sequence 11 bp downstream of the transcription initiation site in the pRSVi vector (20). By RT-PCR using the primer pair of pR(+) and p9(-), it was confirmed that only mini-APP mRNAs were amplified in transfected cells (Fig. 2B) and no band was detected in non-transfected cells (data not shown). The sequencing of the PCR amplified product of the mRNA containing exon 6, 9 (E6.9) from RSVA69 indicated that the splicing between exon 6 and 9 took place exactly between the donor and the acceptor sites. The sizes of amplification products of mini-APP mRNAs containing the exon 6, 7, 8, 9 (E6.7.8.9), the exon 6, 7, 9 (E6.7.9) and the exon 6, 8, 9 (E6.8.9) were 685, 628 and 517 bp, respectively, as expected from the size of

E6.9 (460 bp). For the amplification of the mRNAs from RSVA510, RSVA5710 and RSVA5810, DMSO was found to be required. This may be due to the secondary structure formation of the template during PCR. Furthermore, RT-PCR using pR(+) and p10(-) was found to amplify several unexpected bands (data not shown). This was thought to be caused by the extra splicing between the vector and the intron sequences upstream of the exon 5, so a radiolabelled internal primer p5(+) was used in place of pR(+) for the detection of the mRNAs from the mini-genes containing the exon 5. The sizes of amplified mRNAs containing the exon 5, 7, 10 (E5.7.10), the exon 5, 8, 10 (E5.8.10) and the exon 5, 10 (E5.10) were 355, 244 and 187 bp, respectively (Fig. 2C).

Splicing pattern of mini-APP mRNA

Using the RT-PCR detection system described above, we investigated the splicing pattern of the mini-APP mRNAs in the cell lines. In the neuroblastoma transfected with RSVA6789, three forms of mRNA, E6.7.8.9, E6.7.9 and E6.9, were detected (Fig. 2B), indicating that the skipping of the exon 7 and 8 of the mini-APP gene did take place in the neuroblastoma. In the glioma with RSVA6789, E6.7.8.9 and E6.7.9 were detected but E6.9 was undetectable. The band just above the position of E6.9 was not E6.8.9 because it did not hybridize with the probe specific to the exon 8. The origin of the band has been unknown. The results indicate that the exon 7 of the mini-gene was skipped efficiently in the neuroblastoma. Thus, the mini-APP gene could mimic the neuron-specific splicing of the APP pre-mRNA in the cultured cell line.

We next modified the mini-APP gene to investigate the *cis*-acting elements for the alternative splicing. The modified constructs are summarized in Fig. 1B. In the neuroblastoma, RSVA679-2 generated both E6.7.9 and E6.9, although the latter was only 10 % of the product (Fig. 2B and Table 1). The intron upstream of the exon 7 in RSVA679-2 was 191 bp long. When the construct containing the intron of about 1.7 kb (RSVA679-3) was used, E6.9 was generated more efficiently (Fig. 2B and Table 1). In the glioma, RSVA679-2 and RSVA679-3 rarely generated E6.9. Similarly, RSVA689 generated the significant amount of E6.9 in the neuroblastoma, but rarely in the glioma (Fig. 2B). When the exon 7 or 8 was replaced by the exon 5 (RSVA659),

Table 1. Proportions of alternatively-spliced APP mRNAs in neuroblastoma and glioma cell lines

	LA-N-5				U251			
	E6.7.8.9	E6.7.9	E6.8.9	E6.9	E6.7.8.9	E6.7.9	E6.8.9	E6.9
RSVA6789	41.5	53.8	n. d.	4.7	75.7	24.3	n. d.	n. d.
RSVA679-2	-	90.0	-	10.0	-	99.5	-	0.5
RSVA679-3	-	84.7	-	15.3	-	98.7	-	1.3
RSVA689	-	-	71.0	29.0	-	-	98.0	2.0
	E5.7.10	E5.8.10	E5.10		E5.7.10	E5.8.10	E5.10	
RSVA5710	94.0	-	6.0		97.4	-	2.6	
RSVA5810	-	91.3	8.7		-	96.6	3.4	

n.d., not detectable.

only E6.5.9 was expressed and E6.9 could not be detected in both cell lines (Fig. 2C). These results indicated that the exon 7 and 8 were skipped independently and the *cis*-acting element(s) for skipping each exon in neuronal cells should be in each corresponding exon and its flanking regions. When the exon 6 and 9 were replaced by the exon 5 and 10, respectively, E5.10 skipping the internal exon was detected in both RSVA5710 and RSVA5810 (Fig. 2D and Table 1), suggesting that the sequences in the neighboring exons (exon 6 and 9) and their flanking regions are not essential for the skipping of the exon 7 and 8.

In general, the exon 8 was skipped more efficiently than the exon 7. To localize the regulatory region for the exon 8 skipping, we constructed RSVA689-3 by deleting 68 bp from the 5' flanking region of the exon 8. RSVA689-3 was found to generate only E6.8.9 (Fig. 2E), suggesting that the *cis*-acting elements exist in the region between 181 and 114 bp upstream of the exon 8. To see whether the -181 to -114 region is sufficient for the exon skipping, RSVA6(6)9 was then constructed by replacing the exon 8 and its downstream intron in RSVA689 by the exon 6 and its downstream intron. RSVA6(6)9 could not generate E6.9 (Fig. 2E), indicating that not only the -181 to -114 region but also the exon 8 itself and/or its downstream region are required for the skipping of the exon 8.

In conclusion, the present study demonstrated that the mini-APP gene system can be used for identifying the regulatory elements for the neuron-specific alternative splicing of the APP gene. Our initial deletion studies revealed that the elements should exist in the exon 7, 8 and their flanking regions. Further fine deletion and mutagenesis studies may clarify the *cis*-acting elements for the alternative splicing.

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