Identification of cis-Elements Regulating Exon 15 Splicing of the Amyloid Precursor Protein Pre-mRNA

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Alternative splicing of exon 15 of the amyloid precursor protein (APP) pre-mRNA generates two APP isoform groups APPex15b (containing exon 15) and L-APP (without exon 15), which show a cell-specific distribution in non-neuronal cells and neurons of rat. Both APP isoforms differ in regard to functional properties like post-translational modification, APP secretion, and proteolytic production of Aβ peptide from APP molecules. Since Aβ generation is an important factor in the development of Alzheimer’s disease, one could anticipate that these major APP isoforms might contribute differentially to the mechanisms underlying neurodegeneration in Alzheimer’s disease. In this study, we established an APP minigene system in a murine cell system to identify cis-acting elements controlling exon 15 recognition. A 12.5-kilobase pair genomic fragment of the murine APP gene contained all cis-regulatory elements to reproduce the splicing pattern of the endogenous APP transcripts. By using this approach, two intronic cis-elements flanking exon 15 were identified that block the inclusion of exon 15 in APP transcripts of non-neuronal cells. Point mutation analysis of these intronic regions indicated that pyrimidine-rich sequences are involved in the splice repressor function. Finally, grafting experiments demonstrated that these regulatory regions cell-specifically enhance the blockage of a chimeric exon in the non-neuronal splicing system.

Amyloid plaques represent characteristic neuropathological lesions in brains of patients with Alzheimer’s disease. These amyloid deposits are mainly composed of a short, highly hydrophobic peptide (40–42 amino acid residues) termed Aβ (1, 2). Aβ is derived by proteolytic processing of the amyloid precursor protein (APP), which is expressed abundantly in almost every cell type including neurons (3, 4). The human APP gene is located on chromosome 21 (5, 6); it spans ~300 kb, and the coding region is distributed over 18 exons (7). The APP pre-mRNA molecules are targets for post-transcriptional processing by alternative splicing of exons 7 (6, 8, 9), 8 (9), and 15 (10) generating eight different isoforms of a type I transmembrane glycoprotein ranging in length from 677 to 770 amino acids.

Alternative splicing of APP exon 15 was first discovered in peripheral mononuclear leukocytes and activated microglial cells (10). Therefore, isoforms lacking exon 15 were denoted as leukocyte-derived APP (L-APP). Detailed expression studies revealed a ubiquitous distribution of L-APP transcripts in rat tissues (11). In peripheral tissues L-APP mRNA molecules constitute between 25 and 72% of total APP transcripts. In contrast, the expression levels of L-APP in the central nervous system, including hippocampus, cerebellum, and cerebral cortex, is low (<10%), and no significant L-APP amounts were detected in cultured primary rat neurons. These findings suggested that exon 15 is recognized by a cell type-specific mechanism in rat.

Exclusion of 18 amino acids encoded by exon 15 has important consequences for the functional properties of the corresponding APP isoforms. Fusion of exon 14 with exon 16 creates a new post-translational modification motif ENEGSG. This motif is recognized by xylosyltransferase that attaches a chondroitin sulfate glycosaminoglycan chain via xylose to the serine residue of the ENEGSG sequence. Chondroitin sulfate glycosaminoglycan chain-modified L-APP is denoted as appican (12, 13). The biological function of appican is not known, but it may be similar to the function of other chondroitin sulfate proteoglycans in biological processes as wound healing, cell adhesion, neurite outgrowth, and axon guidance (14–16). Evidence for appican function in mediating cell adhesion came from expression studies on appican in rat C6 glioma cells. Appican-expressing cells attach more avidly to substrate surface than untransformed cells (17). In former cells appican is enriched in the extracellular matrix and displays adhesion-promoting properties for neuronal cells (17). Furthermore, alternative splicing of exon 15 regulates the basolateral and apical secretion of APP and L-APP isoforms in polarized Madin-Darby canine kidney II cells by either creating a new apical or destroying a basolateral sorting signal in L-APP molecules, respectively (18). In context of Alzheimer’s disease pathogenesis it is relevant that exclusion of the region encoded by exon 15 from the mature APP also affects its processing to the Aβ peptide by decreasing the relative amount of generated Aβ (18).

In order to get some insight into the splicing regulation of exon 15, in the present study we performed a molecular anal-
ysis of this splicing process. We demonstrate that the cell type-specific recognition of exon 15 in non-neuronal and neuronal cells is conserved between mouse and rat. To characterize the regulatory requirements for the cell type-specific usage of exon 15, an APP minigene expression system was established in murine cell lines NIH3T3 and P19. By using this approach we were able to identify two intronic regions flanking exon 15, which we refer to as “upstream control region” (UCR) and “downstream control region” (DCR). Finally, we present evidence that both intronic regions play a key role for the cell type-specific exclusion of exon 15 in APP mRNA molecules in non-neuronal cells.

MATERIALS AND METHODS

Animals and Tissue Preparation—All tissues were prepared from 2- to 3-month-old female NMRI mice, provided by the animal facility of Center for Molecular Biology, Heidelberg, Germany. The animals were killed by cervical dislocation, and the tissues were immediately removed, washed in ice-cold phosphate-buffered saline, and quickly frozen in liquid nitrogen.

Cell Culture Conditions—The following murine cell lines NIH3T3, P19, N2a, and AT20 were cultured in Dulbecco’s modified Eagle’s medium plus 10% fetal calf serum (Sigma and Boehringer Ingelheim) as adherent monolayers on 10-cm tissue culture dishes at 37 °C under 5% CO2 atmosphere.

Isolation of Genomic Clones—The genomic clone APP C12 (Fig. 2A) was isolated from a Lambda Fix II 129 SVJ mouse genomic library. The genomic clone L12 (Fig. 2A) was isolated from a Lambda Fix II 129 SVJ mouse genomic library (Stratagene) using an α-32P-labeled human APP cDNA probe encoding exons 14–17.

Minigene Constructs—All clones were created by using standard methods (19). For cloning the APP minigene MG1, two DNA fragments of the genomic APP clone C12 were used (Fig. 2A) as follows: fragment I, SacI site of exon 14 to XhoI site of intron 14 (–5.5 kb), and fragment II, XhoI site of intron 14 to EcoRI site of exon 16 (–6.9 kb) (Fig. 2A). The recipient vector pSKE1416X for fragment I and II was created by inserting a mouse APP cDNA fragment (exons 14–16) that was amplified by PCR using the primer pair aE14EcoRV (5′-ACGGTATTATACTATGGAGCTTC-3′) and aE16XhoI (5′-AGACCTCCTGACGATGGCG-3′) into the EcoRI/HindIII sites of pSKMG1. The E16 XhoI site of pSKMG1 was inserted into the EcoRI/XhoI site of the genomic APP clone L12 producing the pSK1516X plasmid. Afterward fragment I was inserted into SacI and XhoI site of pSK1516X producing the APP minigene pSKMG1. The EcoRV/XhoI fragment of pSKMG1 was inserted into the SacI/XhoI site of the mammalian expression vector pZeoSV (Invitrogen) generating the APP minigene MG1. All cloning steps were controlled by DNA sequencing. MG2 was constructed by deleting a 2.8-kb NcoI DNA fragment from pSK1516X, followed by a religation and exchanging the wild type XbaI/XhoI fragment of MG1 against the shortened one of pSK1516X.

Deletions constructs A1 to A6 and B1 to B8 were generated by PCR cloning, amplifying 5′- or 3′-truncated PacI/AluII fragments (Figs. 4 and 5). Minigene constructs δUCR, δDCR, δACUR, δUCR, δDCR, δCR1, δCR2, δCR3, and PM34.1 were produced by using a recombinant PCR approach. Mutagenized PacI/AluII fragments of the genomic APP region were inserted in the parental APP minigene MG2. Point mutations PM13, PM14, PM15, PM23, PM25, and PM35 were introduced into the PacI/AluII fragment of MG2 by a modified protocol of the megaprimer PCR method (20). Instead of Taq DNA polymerase we used Fd2 DNA polymerase (Stratagene), and therefore no Klenow treatment of newly synthesized megaprimer was necessary. All introduced mutations were confirmed by sequencing. MG4 was generated by PCR using the primer pair aE15EcoRV (5′-ACGGTTATTATGCTCGAAGTGTTTC-3′) and aE16XhoI (5′-AGACACTCCTCGAGCTCGAATCCG-3′); bold-face nucleotides indicate restriction sites) in the EcoRV and XhoI site of a Bluescript (SK+ ϕ)] plasmid. Finally, an XbaI polylinker was introduced between the SacI (exon 14) and EcoRI (exon 16) sites of the APP cDNA fragment. Fragments were cloned into the EcoRI and XhoI site of the pSKE1416X generating the pSK1516X plasmid. Afterward fragment I was inserted into SacI and XhoI site of pSK1516X producing the APP minigene pSKMG1. The EcoRV/XhoI fragment of pSKMG1 was inserted into the SacI/XhoI site of the mammalian expression vector pZeoSV (Invitrogen) generating the APP minigene MG1. All cloning steps were controlled by DNA sequencing. MG2 was constructed by deleting a 2.8-kb NcoI DNA fragment from pSK1516X, followed by a religation and exchanging the wild type XbaI/XhoI fragment of MG1 against the shortened one of pSK1516X.

RESULTS

APP mRNA Isoforms without Exon 15 (L-APP) Are Ubiquitously Expressed in Murine Tissues, Including Brain, but Not in Neurons—A previous report on the APP expression established the ubiquitous distribution of L-APP in rat tissues with the exception of primary cultured neurons (11). Since in the present study we used the mouse system to identify cis-acting elements involved in the splicing regulation of exon 15, we first examined the expression pattern of exon 15-containing (APPα15) and lacking (L-APP) APP mRNA isoforms in a number of organs of three adult female mice. To assay the relative amounts of APPα15 and L-APP in these murine tissues, cDNA was prepared and served as a template in a radioactive PCR using a primer pair flanking exon 15 (Fig. 1A). After separation of the radioactively labeled PCR products by denaturing polyacrylamide gel electrophoresis, two major bands of 420 and 366 base pairs were visible in the autoradiogram (Fig. 1B), corresponding to MG-APP ex15/MG-L-APP, respectively. The identity of these bands was confirmed by comparison with the mobility of DNA fragments amplified from corresponding plasmid standards and sequencing (data not shown). APPα15 and L-APP were present in all tested tissues (Fig. 1B, lanes 1–14). A quantitative determination of the relative L-APP amounts by phosphorimaging revealed that the relative L-APP portions in the non-neuronal tissues varied between 45% (adrenal gland) and 65% (lung) with the mean value of 55% (Table I). In contrast, the relative intensities of the L-APP bands in cDNA preparations from...
chose the following murine cell lines: NIH3T3 (fibroblastoma), neuronal and neuronal L-APP expression pattern. For this, we skipping of exon 15 appears to be a very rare event during murine brain exclusively by cells of non-neuronal origin, and splicing is regulated in a cell type-specific manner.

PCR assay (reverse-transcribed without SuperScript the half of a RNA preparation from a primary cell culture sample were measured by the quantitative RT-PCR assay described using the primer pair sm1805 and am2225 (33P-labeled). To check the RNA of each tissue sample and the complete total RNA preparation of 14 examined mouse tissues (lanes 1–14), two primary brain cell cultures (lanes 16 and 17), and four murine cell lines (lanes 19–22) are shown. 2 μg of total DNA of each tissue sample and the complete total RNA preparation of primary cell cultures were subjected to a reverse transcription reaction, and resulting cDNAs were amplified for 24 cycles in a PCR reaction using the primer pair sm1805 and am2225 (32P-labeled). To check the specificity of amplified PCR signals, 2 μg of total RNA of one tissue or the half of a RNA preparation from a primary cell culture sample were reverse-transcribed without SuperScript and subjected to the RT-PCR assay (lanes 15 and 18).

cerebellum or cortex were markedly weaker (Fig. 1B, lanes 13 and 14; Table I) consisting of 7 or 8%, respectively, and suggesting a predominant production of APPex15 isoforms in the central nervous system. To clarify which cell types in the central nervous system contribute to L-APP production, primary cultures of microglial cells derived from newborn mice were cultured, and the relative APPex15 and L-APP amounts were measured by the quantitative RT-PCR assay described above. As shown in Fig. 1C, murine primary neurons do not synthesize detectable amounts of L-APP, whereas in microglial cells both APPex15 and L-APP are expressed, the latter accounting for more than a half of the total APP mRNA (Table I; Fig. 1C, compare lanes 16 and 17). Thus, L-APP is produced in murine brain exclusively by cells of non-neuronal origin, and skipping of exon 15 appears to be a very rare event during post-transcriptional processing of APP pre-mRNA transcripts in neurons. These results are consistent with the data reported by Sandbrink et al. (11) for rat tissues and suggest that exon 15 splicing is regulated in a cell type-specific manner.

By using our quantitative RT-PCR assays, we next searched for a murine cell culture model that mimics the in vivo non-neuronal and neuronal L-APP expression pattern. For this, we chose the following murine cell lines: NIH3T3 (fibroblastoma), AtT20 (pituitary gland), N2a (neuroblastoma), and P19 (embryonic carcinoma). Analysis of the APP splicing pattern by phosphorimaging revealed that APPex15 isoforms were expressed by all cell lines tested and constituted between 43 (NIH3T3) and 99% (P19) of the total APP message (Fig. 1D, lanes 19–22; Table I). Conversely, L-APP constituted 14 or 57% in the non-neuronal cell lines AtT20 or NIH3T3, respectively, whereas only minor amounts of these APP isoforms were expressed in the two cell lines N2a (<5%) and P19 (<1%). Thus, the P19 cell line appeared to be most suitable for studying exon 15 splicing in a neuronal context since the very low or absent L-APP expression is a characteristic feature of neurons. The NIH3T3 cell line was chosen for the following experiments as representative for cells with the non-neuronal L-APP splicing pattern.

A Minigene Spanning APP Exons 14–16 Mimics the Alternative Splicing Pattern of APP Exon 15 in NIH3T3 and P19 Cells—To examine the regulation of the alternative splicing of APP exon 15 in more detail, a minigene construct encompassing the exons 14–16 of the murine APP gene was generated. For this, a mouse genomic library was screened with a fragment of the human APP cDNA (exons 14–17). A hybridizing λ clone termed L12 contained a genomic fragment of 18 kb including the exons 14–17 of the murine APP gene (Fig. 2A).

Since cis-acting elements involved in the regulation of alternative splicing are often located in intronic sequences proximal to an alternatively used exon or in regions of upstream and downstream exons of neighboring sequences (21–24), the genomic regions adjacent to exons 14–16 were determined and compared with the corresponding regions in the human APP gene (Fig. 2B). The sequence alignment revealed a high degree of conservation in the intronic areas upstream and downstream of exon 15 (80 or 60% similarity, respectively), suggesting the presence of functionally important regulatory cis-elements.

To generate an APP minigene vector a 12.5-kb fragment

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**Table I**

**Table I** Quantification of APPex15 and L-APP mRNA isoforms in peripheral tissues, neuronal tissues, primary cultured brain cells, and cell lines of murine origin.

| Sample               | L-APP | APPex15 | No. of samples |
|----------------------|-------|---------|----------------|
| Non-neuronal tissue  |       |         |                |
| Gullet               | 47 ± 1| 53 ± 1  | 3              |
| Lung                 | 65 ± 2| 35 ± 2  | 2              |
| Heart                | 61 ± 1| 39 ± 1  | 2              |
| Thymus               | 57 ± 5| 43 ± 5  | 2              |
| Adrenal gland        | 45 ± 11| 55 ± 11| 3              |
| Kidney               | 50 ± 5| 50 ± 5  | 2              |
| Spleen               | 64 ± 2| 36 ± 2  | 3              |
| Liver                | 57 ± 5| 43 ± 5  | 2              |
| Stomach              | 53 ± 3| 47 ± 3  | 3              |
| Small intestine      | 50 ± 3| 50 ± 3  | 3              |
| Rectum               | 53 ± 3| 47 ± 3  | 3              |
| Ovary                | 51 ± 2| 49 ± 2  | 3              |
| Neuronal tissue      |       |         |                |
| Cerebellum           | 8 ± 2 | 92 ± 2  | 3              |
| Cortex               | 7 ± 2 | 93 ± 2  | 2              |
| Primary cultured brain cells |       | |                |
| Primary neurons      | <1    | >99     | 7              |
| Primary microglia    | 61 ± 7| 39 ± 7  | 14             |
| Cell lines           |       |         |                |
| NIH3T3               | 57 ± 4| 43 ± 4  | 10             |
| AtT20                | 14 ± 1| 86 ± 1  | 3              |
| N2a                  | 5 ± 2 | 95 ± 2  | 13             |
| P19                  | 1 ± 1 | 99 ± 1  | 6              |
spanning exons 14–16 was inserted into the multiple cloning site of the mammalian expression vector pZeoSV as described under “Materials and Methods” (Fig. 2A). To examine whether the minigene-derived mRNA can be efficiently expressed in cells and whether the minigene-encoded exon 15 undergoes alternative splicing, the APP minigene MG1 was ectopically expressed in NIH3T3 (non-neuronal splicing system) and P19 cells (neuronal splicing system). The minigene splicing pattern was monitored by a quantitative RT-PCR assay, using vector-specific primer pairs in a PCR reaction with 26 cycles (Fig. 2A). Concomitantly, the endogenously produced APP transcripts were detected by using the quantitative RT-PCR assay described in “Materials and Methods” (Fig. 2A). By using both primer pairs in two separate RT-PCRs, we thus were able to monitor and discriminate between the endogenously and the ectopically expressed APP isoforms. As shown in Fig. 3A, in addition to the endogenous APP mRNA fragments, two minigene-derived splice products were visible as bands of 447 and 501 bp, and these bands were not present in cDNA preparations from “mock”-transfected control cells (Fig. 3A, lanes 1 and 4 versus 3 and 6). In analogy to the APP mRNA isoforms generated from the endogenous APP gene, we refer to the 447-bp fragment as MG-L-APP (minigene-derived APP mRNA isoforms without exon 15) and to the 501-bp fragment as MG-APPex15 (minigene-derived APP mRNA isoforms with exon 15). The identity of the bands representing MG-L-APP and MG-APPex15 was confirmed by comparison of their electrophoretical mobilities with those of corresponding bands derived by PCR amplification of a plasmid standard (Fig. 3A, lanes 1 and 4 versus 7) and by sequencing (data not shown). A quantitative analysis of the minigene-derived PCR products demonstrated that exon 15 inclusion occurred to 41 ± 3% (n = 11) in NIH3T3 cells and to 86 ± 5% (n = 11) in P19 cells (Fig. 3B). The ectopic expression of the APP minigene did not influence the processing of the endogenous APP mRNA as revealed by a quantitative analysis of endogenous APP mRNA levels in transfected and non-transfected cells (Fig. 3B). This has also been confirmed for NIH3T3 and P19 cells stably transfected with the APP minigene (data not shown). Further control experiments were performed to exclude effects on the splicing regulation due to different transfection efficiencies. Control transfections with a pZeoSVgal reporter construct and subsequent staining of fixed cells with

**FIG. 2.** Organization of the genomic region exons 14–17 of mouse APP locus. A, maps of the murine APP locus and the APP minigene MG1. On top the genomic clone L12, containing an ~18-kb APP DNA fragment, is shown (constitutive exons, open boxes; alternatively spliced exon 15, gray; introns, black solid lines). Only restriction sites involved in cloning of APP minigenes are indicated (compare “Materials and Methods”) and are not necessarily unique. The approximate scale is given by the 1-kb bar on the top. In MG1, the genomic fragment spanning exons 14–16 (~12.5 kb), marked by the connecting solid lines between L12 and MG1, was set under the control of a SV40 early promoter (SV40 prom, black box) and an SV40 polyadenylation signal (SV40 3′A, black box) of the mammalian expression vector pZeo SV (Invitrogen). Vector sequences are not drawn in scale. Small black arrows show the localization of the minigene-specific primer pair spZEO1 and apZEO2 used in the quantitative RT-PCR assay to determine the splicing pattern of the minigene-derived mRNA molecules. Below, expected minigene splicing pattern in non-neuronal and neuronal cells is diagrammed (vector-specific sequences, black; constitutive exons, white; and alternative exon, gray), showing the two amplified PCR products either skipping (MG-L-APP) or including exon 15 (MG-APPex15). B, alignment of genomic sequences flanking the mouse and human exon 15 (exonic sequence, capital letters; intronic sequence, small letters; exon 15, gray box; identical nucleotides, vertical bars). The depicted sequences were aligned by using the method of J. Hein (Lasergene 2.0). Constitutive splicing signals: 3′-SS, 3′-splice site; 5′-SS, 5′-splice site; * putative branch points sharing four or more nucleotides with branch site consensus sequence. The intronic sequences of murine APP gene have been submitted to the GenBank™ Data Bank with accession numbers AF199003, AF199004, and AF199005.
5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) confirmed similar transfection rates in NIH3T3 and P19 cells (data not shown). In summary, the genomic APP sequence spanning exons 14–16 contained all cis-acting elements necessary for cell type-specific recognition of exon 15 in the minigene pre-mRNA molecules. Since the removal of a 2.8-kb fragment in intron 15 of the APP minigene did not affect the regulation of exon 15 splicing, we used this shortened minigene version, denoted as MG2, as parental construct in all subsequent experiments.

Identification of an Inhibitory cis-Element, Located 69 nt Upstream of the Exon 15 3’-splice site, Denoted as Upstream Control Region (UCR)—Alignment of human and mouse intronic sequences flanking exon 15 of the APP gene revealed the highest level of conservation in the 3’-terminal region of intron 14. This was reflected by 80% nucleotide sequence similarity within −500 bp upstream of exon 15 3’-splice site (3’-SS) and suggested the presence of conserved cis-acting elements. To examine this more precisely, deletions increasing in length (between 65 and 371 bp) were introduced in this intronic area, and the splicing pattern of these mutagenized APP minigenes was determined in NIH3T3 and P19 cells (Fig. 4A, A1–A6). Removal of up to 303 bp led to a moderate increase of the relative MG-APPex15 level between 56 ± 3 and 65 ± 2% in NIH3T3 cells (Fig. 4A, A1–A4). In contrast, the largest deletions present in minigenes A5 and A6 caused a drastic increase of the relative MG-APPex15 amounts to 90 ± 4% (n = 5) and 84 ± 2% (n = 6), respectively, compared with 52 ± 6% (n = 6) MG-APPex15 in control cells expressing the intact APP-derived MG2 (Fig. 4A). None of the 3’-terminal deletions in intron 14 significantly influenced the recognition of exon 15 in P19 cells (Fig. 4A). To confirm the specificity of this effect, the potential regulatory region located 69–135 nt upstream of exon

![Fig. 3. Quantitation of endogenous and minigene-derived APP mRNA isoforms by RT-PCR. A, an autoradiogram of the endogenous and minigene-derived APP splicing pattern in NIH3T3 and P19 cells transiently transfected with minigene MG1 and pZeo SV is shown. 48 h after transfection the mRNA splicing pattern of endogenous and minigene-derived APP was analyzed by RT-PCR. Amplified PCR products are indicated: MG-APPex15 (minigene-derived APP with exon 15), MG-L-APP (minigene-derived APP without exon 15), APPex15 (endogenous APP with exon 15), and L-APP (endogenous APP without exon 15). On top of each lane the analyzed splicing pattern and the transfected constructs are depicted: APP-PCR, endogenous APP transcripts; MG-APP, minigene-derived APP mRNA molecules; MG1, APP m, mock control; K, RT control. S, PCR-standard. On buttons of the autoradiogram cell lines are indicated, NIH3T3 (lanes 1–3), P19 (lanes 4–6), and PCR standard (lane T). B, quantitation of exon 15-containing isoforms. Percent exon 15 inclusion was analyzed by a PhosphorImager using the following calculation ((counts in exon 15 including product) + (counts in exon 15 excluding product)/100% × (counts in exon 15 including product)). White, gray, and dark gray bars represent the mean ± S.E. of at least three independent experiments (each transfection was performed in duplicate).]

![Fig. 4. Mapping of the “upstream control region.” A, left panel, structure of wild type and truncated minigenes. On top the genomic map of wild type minigene MG2, carrying a 2.8-kb deletion between the indicated NcoI sites, is shown. Below the genomic region of MG2 spanning exon 15 (gray box) to the PacI (418 nt upstream) is enlarged. The numbers at the 5’-end of the truncated clones A1–A6 mark the beginning of the deletion. Right panel, quantitative analysis of minigene splicing pattern in NIH3T3 (dark gray) and P19 cells (gray) using RT-PCR, followed by a PhosphorImager analysis (bars, mean ± S.E. of percent exon 15 inclusion), measured in two independent transfection experiments with double or triple samples. The two vertical dashed lines mark the degree of exon 15 inclusion in NIH3T3 and P19 cells, observed for the wild type minigene MG2. The p values of mutant constructs versus the parental minigene MG2 were determined by Student’s t test; * p < 0.05; ** p < 0.01; and *** p < 0.001. B, left panel, maps of minigenes to analyze sequence-specific effects. DUCR contains a total deletion of nucleotides 135 to 69. RCU has an inverted copy of nucleotides 135 to 69 (black box with white arrow). Right panel, quantitative determination of percent exon 15 inclusion in minigene-derived transcripts. At least three independent experiments in duplicate were performed and analyzed by PhosphorImager. In the case of DUCR all measured values (NIH3T3, n = 16; P19; n = 13) during this study were included in this diagram. For the control construct MG2, the corresponding values that were determined in one experiment together with DUCR and/or RCU were used in this graphical blot. The symbols in this diagram are the same as used in A. Below two autoradiograms of representative gels are depicted, showing the splicing pattern of the minigenes marked above.

15 3’-SS was removed (Fig. 4B, DUCR), or the orientation of this area was turned around (Fig. 4B, RCU). Expression of both constructs in NIH3T3 cells resulted in significantly elevated MG-APPex15 levels (Fig. 4B, DUCR, 79 ± 3%, n = 16, lane 2; RCU, 85 ± 1%, n = 7, lane 1) in comparison to wild type-transfected control cells (Fig. 4B, MG2, 52 ± 5%, n = 15, lane 3). In contrast, destruction or misorientation of this potential regulatory region did not give rise to a markedly altered minigene splicing pattern in the neuronal splicing system (Fig. 4B,
Since the sequence downstream of exon 15 is also conserved (63% similarity between human and mouse sequence within −500 bp downstream of exon 15), a deletion scan of the 5’-terminal portion of intron 15, extending from the 5’-splice site (5’-SS) of exon 15 to the NcoI restriction site of intron 15, was performed (Fig. 5A). For this purpose, a set of deletion constructs was generated by removing stepwise fragments of ~400 to 1300 bp length in the 5’-portion of intron 15 (Fig. 5A, B1–B8). Splicing pattern analysis of these minigenes in NIH3T3 cells showed that the removal of large parts in the 5’-terminal sequences of intron 15 only caused a moderate increase of the relative MG-APPex15 amount between 60 ± 3 and 63 ± 3% in comparison to 52 ± 2% MG-APPex15 wild type-transfected cells (Fig. 5A, B1–B6 versus MG2). In contrast, deletions of approximately 1.1 or 1.2 kb, respectively, led to an activation of exon 15 splicing in NIH3T3 cells that was mirrored by a strong increase of the relative MG-APPex15 level to 74 ± 2% (n = 5) and to 81 ± 2% (n = 5) in comparison to 52 ± 4% (n = 6) in NIH3T3 cells expressing the wild type APP-minegene (Fig. 5A, B7 and B8 versus MG2). None of the truncated APP minigenes influenced the inclusion of exon 15 in the minegene-derived mRNA transcripts of P19 cells (Fig. 5A). One possible explanation of this result is that the enhancement of exon 15 recognition observed in NIH3T3 cells transfected with the constructs B7 and B8 is due to the removal of a splice repressor sequence. Alternatively, the activating effect could solely depend on the size of the deleted intronic area. To discriminate between these two possibilities, the potential regulatory region in intron 15 (position +21 to +158) was deleted (Fig. 5B, ΔDCR), or the removed intronic fragment was reintroduced in the opposite direction (Fig. 5B, RCD). These mutations significantly improved the recognition of exon 15 in NIH3T3 cells (Fig. 5B, lanes 1 and 2 versus 3) resulting in an increase of the relative MG-APPex15 levels to 78 ± 8% (n = 13, ΔDCR) and to 73 ± 3% (n = 6, RCD) compared with 50 ± 8% MG-APPex15 (n = 13) in control cells transfected with the wild type minegene MG2. In P19 cells only the deletion present in the ΔDCR construct slightly increased the relative MG-APPex15 amount from 93 ± 4% (n = 11) to 98 ± 2% (n = 11), whereas the usage of exon 15 was not enhanced in cells transfected with the RCD vector (Fig. 5B, lanes 4 and 5 versus 6). In summary, these data suggested the presence of regulatory sequence elements located in intron 15 (position +21 to +158) that block the inclusion of exon 15 in NIH3T3 cells. This region was therefore designated DCR.

Both cis-Elements Act in a Distance-dependent Manner Regulating the Recognition of Exon 15—From the deletion analysis of the intronic regions proximal to exon 15, we concluded that these sequences contain cis-acting elements (UCR and DCR, respectively), both having an inhibitory influence on the exon 15 inclusion in non-neuronal cells. To test the functional relationship between these cis-elements and exon 15, we increased the distance between exon 15 and UCR from 68 to 418 nt and between exon 15 and DCR from 20 to 711 nt (Fig. 6A, UCR1 and DCR1). In both cases an increase of the distance between exon 15 and the corresponding cis-element resulted in an enhanced inclusion of exon 15 in the minegene mRNA of NIH3T3 cells. The observed effect was associated by an increase of the relative MG-APPex15 amount up to 72 ± 3 (n = 4, UCR1) and 82 ± 2% (n = 8, DCR1) in comparison to 52 ± 5% MG-APPex15 (n = 26, MG2) in wild type-transfected control cells (Fig. 6, A and B, lanes 1 and 3 versus 6). However, the spacing between UCR and exon 15 did not affect the usage of the differential exon in P19 cells (Fig. 6B, lane 7). Enlarging the distance between DCR and exon 15 slightly improved the usage of exon 15 in P19 cells (Fig. 6B, DCR1, lane 9) similar to that observed in cells expressing a DCR-deficient minegene (Fig. 5B, ΔDCR, lane 5). Thus, these results suggested that the proxim-
The inhibitory effect of UCR duplication on the recognition of exon 15 described in the previous section implicated a redundant architecture of this sequence element. UCR from both the human and the mouse APP gene displays a high pyrimidine content of ~75% (Fig. 7A). The majority of the pyrimidine residues are organized in three stretches, each consisting of between 8 and 14 successive pyrimidines. We therefore referred to these sequence elements as polypyrimidine-rich sequences PPSI, PPSII, and PPSIII (Fig. 7A). Furthermore, PPSII and PPSIII are flanked at one or both ends, respectively, by a tetranucleotide motif UGCU. To examine the role of PPSI, PPSII, and PPSIII in the inclusion of exon 15 in non-neuronal cells, point mutations were introduced into each element as depicted in Fig. 7A, and the expression pattern of the corresponding constructs was examined in NIH3T3 and P19 cells. All point mutations in PPSI, PPSII, and PPSIII (Fig. 7A, PM25, PM26, and PM23, respectively) weakened the inhibitory function of UCR in non-neuronal cells that was reflected by an increase of the relative MG-APPex15 amount in NIH3T3 cells between 67 ± 2 and 74 ± 4% in comparison to control cells expressing the parental APP construct MG2 (49 ± 4%). In contrast, these mutations did not significantly affect the usage of exon 15 in P19 cells (Fig. 7A). The strength of this effect was in the range of that observed for the constructs ∆UCR (79 ± 3%) and RCU (80 ± 3%), both completely destroying this regulatory element (Fig. 4B). This indicates that PPSI, PPSII, and PPSIII are involved in the repression of exon 15 recognition in non-neuronal cells.

Similar analysis was performed for the other cis-regulatory region DCR (Fig. 7B). In contrast to UCR, the pyrimidine content of human and mouse DCR is more balanced making up ~60% of all residues. Approximately 50% of the pyrimidines are clustered in four (murine DCR) or five (human DCR) short stretches between 5 and 16 nt in length, located at both ends of the DCR. Two of these pyrimidine-rich sequences contain a short conserved repeat GCCUCUC(U)(U) and a truncated version GCUCUC (Fig. 7B). The involvement of these elements in the inhibitory function of DCR was tested by mutating them to the complementary sequences shown in Fig. 7B. Point mutations present in the constructs PM13, PM14, and PM15 had no significant effect on exon 15 usage neither in NIH3T3 nor in P19 cells (Fig. 7B). Since the function of an intronic cis-element is often mediated by a combination of different sequence motifs (27), in minigenes PM34.1 and PM35 parts of the mutated sequences in PM13 or PM15 were combined with the mutation of construct PM14, respectively (Fig. 7B). These double mutations drastically impaired the inhibitory function of DCR in NIH3T3 cells increasing the relative amount of APPex15 to 78 ±

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**Fig. 6. Properties of UCR and DCR.** A, left panel, maps of constructs for analyzing distance, enhancing and cooperative effects of UCR and DCR. On top the genomic map of MG2 is shown. Below the genomic PacI/AII fragments of MG2 and the mutated minigenes are enlarged, indicating exon 15 (gray box), introns (bold solid lines), cis-elements UCR, DCR (white boxes with cross-hatching), and deletions (open triangle). Size of sequence elements is marked by numbers but not drawn in scale. Right panel, quantitative analysis of exon 15 inclusion in the minigene-derived mRNA molecules from NIH3T3 and P19 cells. At least two independent transfection experiments were performed in duplicate and analyzed by using a Phosphorimager. The mean of MG-APP for minigene MG2 was calculated by using all values that were determined together with the mutated minigenes (NIH3T3, n = 26; P19, n = 26). The bars symbolize the mean ± S.E. of percent exon 15 inclusion (gray P19, dark gray NIH3T3). The two vertical dashed lines indicate the percent of exon 15 usage for MG2 in both cell lines. The p values of mutant constructs versus MG2 were determined by Student’s t test; *, p < 0.05; **, p < 0.01; and ***, p < 0.001. B, autoradiograms showing the splicing pattern of the minigenes described above.
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**Fig. 7.** Point mutation analysis of UCR and DCR. **A**, left panel, on top, an alignment between the mouse (positions -138 to -69) and human UCR RNA sequence is depicted, and identical nucleotides are marked by **boldface** letters. Polypyrimidine-rich sequences (PPSI–III, typed in **boldface** nucleotides) are framed by **black lines**. Below the point mutations PM23, PM25, and PM26 are shown, indicating the mutated sequences. Right panel, the mean ± S.E. of percent exon 15 inclusion measured in two independent experiments with double samples is shown. **B**, left panel, on top an alignment between the first 47 nucleotides of the mouse and human DCR RNA sequence is shown, using the same symbols as in **A**. The conserved RNA motifs GCCUCUCU(U) and GCCUCUCU are typed in **boldface** letters and framed by **black lines**. Right panel illustrates the mean ± S.E. of percent exon 15 inclusion, determined in two independent experiments performed in duplicate.

4% (n = 4) for PM34.1 and 74 ± 1% (n = 4) for PM35 compared with 53 ± 10% (n = 12) for the MG2-transfected control cells. In P19 cells, these mutations caused no significant alteration of the splicing pattern (Fig. 7B). Therefore, we concluded that at least two of these RNA motifs in the DCR sequence are necessary to inhibit the usage of exon 15 in NIH3T3 cells.

**UCR and DCR Regulate the Inclusion of a Hybrid Exon in a Constitutive Splicing Background**—The results reported in the preceding sections showed that the identified cis-elements block the recognition of exon 15 in NIH3T3 cells. To examine the ability of UCR and DCR to regulate the cell type-specific inclusion of a chimeric exon, both elements were grafted into the constitutive splicing background of the human APLP1 gene, a member of the APP gene family (28–30). For this purpose, a 553-bp genomic segment of the APLP1 gene, encompassing the constitutively spliced exons 15–17, was amplified by PCR from genomic DNA and cloned into the pZeoSV vector, generating APLP1 minigene MG4. To minimize possible effects of potential cis-acting elements in exon 16 (144 bp length) of APLP1 and to reduce the size of the exon to that of the APP exon 15 (54 bp), the minigene construct E16Δ90 was created by deleting 90 bp of the central part of APLP1 exon 16 (Fig. 8A). Since P19 cells did not accept the minigene-derived pre-mRNA molecules as splicing substrates (data not shown), for this particular study the neuroblastoma cell line N2a was used as a neuronal splicing system. After transient transfection of MG4 and E16Δ90 into NIH3T3 and N2a cells, the splicing pattern of the minigene-derived mRNA molecules was monitored by qualitative RT-PCR using minigene-specific primers spZEO1 and apZEO2 (Fig. 2A). The identity of each PCR product was confirmed by sequencing (data not shown). As expected, APLP1 exon 16 was constitutively included in chimeric mRNA molecules from both cell lines (Fig. 8B, lanes 1 and 2). However, removing 90 bp of the internal sequence in exon 16 (exon Δ90) yielded a very different result, reflected by the generation of two splice products of 341- and 287-bp length in both cell lines (Fig. 8B, lanes 3 and 4). The faster migrating band corresponds to mRNA species lacking the exon Δ90. To test whether UCR and DCR could block the inclusion of exon Δ90 in NIH3T3 cells, the first 10 nt of exon Δ90 with the flanking intronic sequences were exchanged against the corresponding regions of exon 15 with UCR and DCR (Fig. 8A, C1). Expression of this chimeric construct in NIH3T3 showed that the recognition of the chimeric exon was impaired, producing mainly splicing variants without this exon (Fig. 8B, lane 5). In contrast, the genomic APP portions of minigene C1 did not block the usage of this exon in N2a cells (Fig. 8B, lane 6). To confirm whether UCR and DCR were responsible for blockage of the chimeric exon in NIH3T3 cells, both cis-elements were deleted (Fig. 8A, minigene C2). The removal of both regulatory regions neutralized the observed blockage of chimeric exon in NIH3T3 cells (Fig. 8B, lane 7). Furthermore, this deletion led to a strong activation of the chimeric exon in NIH3T3 cells, resulting in its constitutive usage (Fig. 8B, lanes 7 and 8). These results demonstrated first that UCR and DCR regulated the recognition of the chimeric exon in a cell type-specific manner, indicated by its inhibition in NIH3T3 cells, whereas in N2a cells the repression of the chimeric exon was not enhanced; and second that these effects could be reversed in NIH3T3 cells by deleting UCR and DCR.

**DISCUSSION**

Alternative splicing plays a fundamental role in the regulation of eukaryotic gene expression, generating different mRNA isoforms from a single pre-mRNA precursor. In most cases, the usage of an alternatively spliced exon is controlled in a tissue-, developmental stage-, or sex-specific manner (reviewed in Refs. 31 and 32). The usage of an alternatively spliced exon is controlled in a tissue-, developmental stage-, or sex-specific manner (reviewed in Refs. 31 and 32).

**Cell Type-specific Recognition of Exon 15 Is Conserved between Mouse and Rat**—Consistent with previously published data on the distribution of L-APP transcripts in rat (11), our expression analysis revealed the ubiquitous occurrence of L-APP in mouse tissues. On average, L-APP constituted 55% of the total APP message in non-neuronal organs of mouse, and the measured relative L-APP levels were between 2 and 20% higher than those determined in rat. In both rodent species
only low levels of L-APP were detected in brain (5–6%). The major source of brain L-APP appear to be cells of non-neuronal origin, since L-APP was undetectable by our sensitive RT-PCR assay in RNA preparations from primary cultured mouse neurons (Fig. 1C). In contrast, L-APP consisted of 60% total APP transcripts in primary cultured microglial cells, in agreement with the previously reported relative abundance of L-APP in cultures of rat microglia and astrocytes (11). Taken together these data strongly indicate that the cell type-specific recognition of APP exon 15 is conserved between both rodent species. Therefore, the mouse model is a suitable system to analyze the molecular mechanism controlling the cell type-specific usage of APP exon 15 in non-neuronal cells and neurons.

**Fig. 8.** Cell-specific regulation of a chimeric exon by UCR and DCR. A, left panel, maps of APLP1 and chimeric APLP1/APP minigenes. Exons and introns are marked by boxes and bold solid lines. Genomic portions of human APLP1 and mouse APP are drawn in gray and black, and small numbers indicate the size of the genomic fragments (numbering of the APLP1 exons corresponding to Ref. 50). On top parental APLP1 minigene MG4 containing a full-length exon 16 (144 nt). Below E16Δ90 derived from MG4 by a 90-bp deletion of the central part in exon 16, marked by an open triangle. C1, 10 nt of exon Δ90 + 50 nt of the upstream intron and 10 nt of exon Δ90 + 63 nt of the intronic downstream region were exchanged by the corresponding one of APP exon 15 with UCR and DCR. C2, both cis-elements were deleted, symbolized by open triangles. B, qualitative analysis of the minigene splicing pattern in NIH3T3 and N2a cells by performing an RT-PCR with 30 cycles using 1/20 of a cDNA preparation and the primer pair spZEO1/apZEO2. Ethidium bromide staining of two 1.5% agarose gels is shown. The minigene-derived splicing products are marked by their exon structures symbolized by gray boxes. CE, chimeric exon; T, NIH3T3; N, N2a.

Sequence conservation within intronic regions proximal to exon 15 of the human and mouse APP gene suggested the presence of cis-acting elements that might be involved in splicing regulation (Fig. 2B). Deletion scan analysis of the intronic region upstream and downstream of exon 15, using our APP expression system, led to the identification of two regulatory elements (Figs. 4 and 5) as follows. (i) The UCR is located 69 nt...
upstream of the intron 14/exon 15 border and spans at least 67 nt. (ii) the DCR starts 21 nt downstream of exon 15/intron 15 region and encompasses at least 138 nt.

Both cis-elements mediated a splice repressor function, blocking the recognition of exon 15 in non-neuronal cells by acting in a sequence- and distance-dependent manner (Figs. 4B, 5B, and 6). Furthermore, UCR and DCR are the key players for regulating the exclusion of exon 15 in the non-neuronal splicing system, since their complete removal impaired the cell-specific recognition of exon 15 in non-neuronal cells (Fig. 6, M2). The cell type-specific splice repressor function of UCR and DCR is underlined by the fact that the recognition of a chimeric exon in a constitutive splicing background is cell-specifically blocked in non-neuronal cells by the presence of both cis-elements, whereas in the neuronal splicing system these regulatory elements do not negatively influence the usage of this exon (Fig. 8, C1 and C2). In summary these experimental data clearly showed that these regulatory elements represent cell type-specific acting sequences controlling exon 15 exclusion in the non-neuronal splicing system we used.

One common feature of many intronic regulatory sequences, e.g. the neuron-specific N1 enhancer of c-src gene (25, 27), is their redundant and complex architecture. This is reflected by the presence of multiple binding sites for certain factors, the latter being part of a protein complex that regulates splice site selection (26, 37). Hence, the enhanced repression of exon 15 observed in non-neuronal cells after duplicating UCR can be interpreted as a reflection to its redundant architecture. Moreover, duplication of UCR interferes with its cell type-specific function, since the recognition of exon 15 was slightly impaired in neuronal cells, too (Fig. 6, dUCR). This could be caused by cooperative interactions between different binding sites of the two duplicated cis-elements in neuronal cells causing a basal level of inhibition, similar to the activation of a heterologous exon by the duplicated neuron-specific N1 enhancer in non-neuronal cells (27). Alternatively, the increased number of potential binding sites could titrate for limited trans-acting factor(s) that neutralize(s) the negative function of UCR in neuronal cells. The latter case is very unlikely, since a titration experiment with different dUCR plasmid DNA amounts (transfection of 50 ng to 5 μg by liposome-mediated transfection) showed no enhanced function of UCR in P19 cells. In contrast, duplication of DCR did not strengthen its negative effect neither in P19 nor in NIH3T3 cells (Fig. 6, dDCR). This result appears surprising, but in the context of the complex architecture of splicing regulatory sequences it is possible that DCR, spanning position +21 to +158, contains only a part of the target sequences for mediating its repressor function. Thus, a duplicated DCR did not amplify its inhibitory influence on exon 15 recognition, since cooperative interactions between certain components were still lacking. A similar effect has been reported for the neuron-specific intronic enhancer of the N1 exon; duplication of the enhancer core sequence (position +38 to +70) moderately activated the differential exon in neuronal cells, whereas a multimerization of a larger area (position +38 to +142) strongly increased the amount of mRNA transcripts including N1 exon (25, 27).

**Models for the Regulation of the Alternatively Spliced Exon 15**—The recognition of an alternatively spliced exon in a three-exon system, like the APP minigene, is determined by the balance of splice site strength between the flanking exons and the internal exon, leading to the inclusion and/or exclusion of the internal exon. Common features of the pre-mRNA molecule like exon length (34, 38), intron size (39), identity with splice site consensus (40, 41), and RNA secondary structures (42, 43) influence the splice site strength. A shift between both splicing pathways can be mediated by changing the strength of involved splice sites, mainly that of the alternatively spliced exon. In this context splicing enhancer and repressor elements located in intronic and exonic sequences play an important role in modulating the splice site strength of an alternatively spliced exon in a tissue-, sex-, or development-specific manner. In the case of the APP minigene our presented data suggest that UCR and DCR are key players controlling the cell-specific recognition of exon 15 in the non-neuronal splicing system. In principal, the inhibitory function of both cis-elements can be mediated by two different mechanisms as follows. (i) UCR and DCR are involved in the formation of an RNA secondary structure, masking the splice sites of exon 15 for its recognition by the spliceosomal components, as it was observed for exon 6B of the chicken β-tropomyosin pre-mRNA (42). (ii) UCR and DCR contain multiple binding sequences for assembling a proteinaceous repressor complex in non-neuronal cells.

We propose that both regulatory regions serve as target sequences for the formation of a protein repressor complex, since RNA secondary structure predictions identified no potential stem loop structures that can be involved in splice site selection.

The genomic localization, presence of pyrimidine-rich sequence elements, and inhibitory function of UCR and DCR resembled the situation of the intronic elements URE and DRE flanking exon 3 of α-tropomyosin. These regulatory elements repress the recognition of α-tropomyosin exon 3 in smooth muscle cells (44). Key mediator for the inhibition of exon 3 in smooth muscle cells is the “polypyrimidine tract binding protein” (PTB) that binds to pyrimidine-rich elements within DRE (45–47). Regarding its high pyrimidine content of almost 80%, the UCR sequence could serve as a PTB-binding target, too. Comparison of the UCR sequence with the PTB binding consensus (|U/G/C(A/N)|GCCUG(V/G)AGUCCYYCCYYGG(V/G)-CCC) (46) identified three potential PTB-binding sites sharing 17–18 nucleotides with the PTB consensus, whereas the pyrimidine-rich sequences (PSII to III) of UCR form the internal pyrimidine core of the potential PTB-binding sites: PSPII (UCACAAAGGUCCUUUUUUUUCGACG), PSPIII (CCAG-CUGU-CCUUUUUGCAGCU), and PSPIII (UAUUCAAAGGU-UCUUCCUUUGCU) (where Y is pyrimidine; identical nucleotides with PTB consensus are in boldface letters; pyrimidine core is underlined; dash indicates missing nucleotide). A possible involvement of PTB in the regulation of APP exon 15 splicing is strengthened by the fact that the destruction of PSII, -II, or -III weakened the inhibitory function of UCR (Fig. 7A). The second regulatory region, DCR, contained the conserved RNA motifs GCCUCU(C)(U) two times and a truncated version GCCUCUC which shows sequence homology to the intronic CUCUCUC element located upstream of c-src gene N1 exon. This short element blocks the inclusion of the neuron-specific N1 exon in non-neuronal cells (48) by binding PTB (49). The mutation analysis of these elements in DCR indicated that this could also be the case in exon 15 splicing, since the destruction of two of these RNA motifs impaired the cell-specific exclusion of exon 15 in non-neuronal cells (Fig. 7B). Therefore, PTB is an attractive candidate that mediates the repressor function of UCR and DCR in the APP exon 15 splicing system.

Since the presence or absence of the region encoded by exon 15 influences the proteolytic processing of APP to Aβ peptide (18) and neurons do not produce L-APP, one could speculate that APPex15 and L-APP might play a different role in the progression of Alzheimer’s disease. If this is the case the identified regulatory sequences could serve as a target to modulate

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2 C. Bergsdorf and K. Beyreuther, unpublished data.
neuronal APP splicing and may be beneficial in preventing or treating Alzheimer’s disease.

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