Volume 17 Number 2 1989 Nucleic Acids Research

The PreA4$_{695}$ precursor protein of Alzheimer's disease A4 amyloid is encoded by 16 exons

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Received November 17, 1988; Revised and Accepted December 22, 1988 Accession no. X13428

ABSTRACT
Alzheimer's disease (AD) is characterized by the cerebral deposition of fibrillar aggregates of the amyloid A4 protein. Complementary DNA's coding for the precursor of the amyloid A4 protein have been described. In order to identify the structure of the precursor gene relevant clones from several human genomic libraries were isolated. Sequence analysis of the various clones revealed 16 exons to encode the 695 residue precursor protein (PreA4$_{695}$) of Alzheimer's disease amyloid A4 protein. The DNA sequence coding for the amyloid A4 protein is interrupted by an intron. This finding supports the idea that amyloid A4 protein arises by incomplete proteolysis of a larger precursor, and not by aberrant splicing.

INTRODUCTION
Alzheimer's disease (1) is the most common cause of dementia, afflicting about two million people in the USA (2). It is characterized by the formation of intraneuronal neurofibrillary tangles (3,4,5), extracellular amyloid plaques (3,4,5) and cerebrovascular amyloid deposits (5,6) in the brain. The major constituent of these depositions is the amyloid A4 protein or β-protein (3,4,6).

Recently, we isolated and sequenced a full-length cDNA clone encoding the fetal brain precursor of the amyloid A4 protein and localized the gene (PAD gene (7)) on chromosome 21 (8). The structure of the deduced amino acid sequence suggests that the fetal brain PreA4$_{695}$ protein is a glycosylated cell-surface receptor consisting of an N-terminal signal sequence, three extracellular domains, a transmembrane region and a cytoplasmic domain. The membrane spanning domain corresponds to residues 625–648 of the PreA4$_{695}$ protein and overlaps with the amyloid A4 peptide sequence (597–639). Three other groups reported the finding of longer transcripts of the PAD gene which all contain an extra exon encoding a peptide that is very similar to the Kunitz family of protease inhibitors (9,10,11). Schubert et al. (12) showed residues 18–44 of the amyloid A4 precursor protein to be very similar to a heparan sulfate proteoglycan core protein found in the nerve cell line PC12. Here we report exon–intron boundaries of the PAD gene. Our work excludes the possibility that the amyloid A4 peptide could be the product of alternative splicing.

MATERIALS AND METHODS
Genomic libraries and screening conditions
Four different libraries were used: a) a chromosome 21 library (*Hind*III fragments in *lambda* charon 21A, courtesy of Dr M. Van Dilla) which was constructed at the Lawrence Livermore National Laboratory, Livermore, CA, under the auspices of the National Laboratory Gene Library Project, sponsored by the US Department of Energy, b) a

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1                                                          57
|                                                          |
ATGCTGCCCGGTTTGGCACTGCTCCTGCTGGCCGCCTGGACGGCTCGGGCGCTGGAGgtg
MetLeuProGlyLeuAlaLeuLeuLeuLeuAlaAlaTrpThrAlaArgAlaLeuGlu
1                                                       19

ggtgccgcgcctcggaaggggggggaggctgcacggtggggacgcgatacccccaatac

cttaacccaagtctttaatgcagagaagccgggggtccgtcaatgggacccctctcct..

........tggcacaaatattattttcagtcttggtcatcaggcagggcctggcaggcat

catgctctgtgtgagtcagtggagtttattataagaatgcggtagcctccacagtgatta

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tctcatatgggaaaacgtgtaaatttttcatgaataaattctttcggtattggtaatttc

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atacataacacttaatctaaaggagtgttgaagaccgggctgattcctaattgagaatga

gcaagaatagaactcttttgatagttatccctgttcttcctccaagcctctgccttgga

                                          58
                                          |
gctatggatactataactaactgaagcttcttctttcagGTACCCACTGATGGTAATGCT
                                          ValProThrAspGlyAsnAla
                                          20

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ValGlnAsnGlyLysTrpAspSerAspProSerGlyThrLysThrCysIleAspThrLys

                             225
                             |
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GluGlyIleLeuGlnTyrCysGlnGlu
                          75

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gagtgcagtggcaagattatggcttactgtagccttgacctactgggctcaagcaatcct

cccacctcagcctcccaagttcctggactataggcacatgccaccaggcctgactgattt

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                                              226
                                              |
ggctcaagcgatcctc..................tgctctcccagGTCTACCCTGAACTG
                                              ValTyrProGluLeu
                                              76

CAGATCACCAATGTGGTAGAAGCCAACCAACCAGTGACCATCCAGAACTGGTGCAAGCGG
GlnIleThrAsnValValGluAlaAsnGlnProValThrIleGlnAsnTrpCysLysArg

                                                          355
                                                          |
GGCCGCAAGCAGTGCAAGACCCATCCCCACTTTGTGATTCCCTACCGCTGCTTAGgtgag
GlyArgLysGlnCysLysThrHisProHisPheValIleProTyrArgCysLeuV
                                                          119

ccg.......................cttgaagtctatctttccttgatgtcttctgcg

                                                         356
                                                         |
gtaagaacactgtgatacagatggaatgacgggaagtggttttcctttctttcagTTGGT
                                                         alGly
                                                           120

GAGTTTGTAAGTGATGCCCTTCTCGTTCCTGACAAGTGCAAATTCTTACACCAGGAGAGG
GluPheValSerAspAlaLeuLeuValProAspLysCysLysPheLeuHisGlnGluArg

                                                   468
                                                   |
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MetAspValCysGluThrHisLeuHisTrpHisThrValAlaLysGlu
                                                 156

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.....ttgtgaaattggttcctaatatattgggtctgcatgttgattattttatgtggag

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469
|
ACATGCAGTGAGAAGAGTACCAACTTGCATGACTACGGCATGTTGCTGCCCTGCGGAATT
ThrCysSerGluLysSerThrAsnLeuHisAspTyrGlyMetLeuLeuProCysGlyIle
157

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GATTCTGCTGATGCGGAGGAGGATGACTCGGATGTCTGGTGGGGCGGAGCAGACACAGAC
AspSerAlaAspAlaGluGluAspAspSerAspValTrpTrpGlyGlyAlaAspThrAsp

            662
            |
TATGCAGATGGGAGgtaaggtggcctttgtgttcagcctcagagatgctgaaacatcttg
TyrAlaAspGlySe
            221

                                                   663
                                                   |
tatggagtatttgtatcctgtaaattaatc...................TGAAGACAAA
                                                 rGluAspLys
                                                   222

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GACGAGGACGATGAGGATGGTGATGAGGTAGAGGAAGAGGCTGAGGAACCCTACGAAGAA
AspGluAspAspGluAspGlyAspGluValGluGluGluAlaGluGluProTyrGluGlu

GCCACAGAGAGAACCACCAGCATTGCCACCACCACCACCACCACCACAGAGTCTGTGGAA
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            865 *
            |
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            289

cgagtggattattctgttgttgttggctttttttctcaaacctccttctcttctacttt

 * 866
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    290

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                 999
                 |
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               333

............aaaggatcgaaactgatgaactttaaattcaaatgtttccttaattta

  1000
   |
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   ValMetArgGluTrpGluGluAlaGluArgGlnAlaLysAsnLeuProLysAlaAsp
   334

                1074
                |
AAGAAGGCAGTTATCCAGgtaaaacctgaacccatttccta................aaa
LysLysAlaValIleGln
               358

acgtagaattagtcaatgttggaatgactatgcagtctttaggatactttttagcactag

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                                          1075
                                          |
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                                    HisPheGlnGluLysValGluSer
                                    359

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GAAGCCATGCTCAATGACCGCCGCCGCCTGGCCCTGGAGAACTACATCACCGCTCTGCAG
GluAlaMetLeuAsnAspArgArgArgLeuAlaLeuGluAsnTyrIleThrAlaLeuGln

              1233
              |
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AlaValProProArg
             411

acagtaagggtggggcactgggaactggaagccatacaaaaagaatgaggagaaatgcct

tgagcactgttattcagaggttcaacccctgtccattccatcttgaaggtcaaagggtca

                                                  1234
                                                  |
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                                                   ProArgHis
                                                   412

GTGTTCAATATGCTAAAGAAGTATGTCCGCGCAGAACAGAAGGACAGACAGCACACCCTA
ValPheAsnMetLeuLysLysTyrValArgAlaGluGlnLysAspArgGlnHisThrLeu

                                                         1362
                                                            |
AAGCATTTCGAGCATGTGCGCATGGTGGATCCCAAGAAAGCCGCTCAGATCCGGTCCCAG
LysHisPheGluHisValArgMetValAspProLysLysAlaAlaGlnIleArgSerGln
                                                         454

                    1363
                    |
...................tgatgcagGTTATGACACACCTCCGTGTGATTTATGAGCGC
                           ValMetThrHisLeuArgValIleTyrGluArg
                           455
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ATGAATCAGTCTCTCTCCCTGCTCTACAACGTGCCTGCAGTGGCCGAGGAGATTCAGGAT
MetAsnGlnSerLeuSerLeuLeuTyrAsnValProAlaValAlaGluGluIleGlnAsp

    1462
    |
GAAGTTGgtaagtaagcttttcttttatct...........................tt
GluValA
    488

gaatgccatgtgcctcagttttcacatctgtaaaagggagatgataatggtacctatgtc

atggctctaaacgcgatcatgcacgtgaaagcagttgaagtcttgcctggcagaagtaaa

tggtggctgctgctgctgctgctgttgtgattgttgttactcaccaaagagatggttttg

          1463
          |
tttggtttagATGAGCTGCTTCAGAAAGAGCAAAACTATTCAGATGACGTCTTGGCCAAC
          spGluLeuLeuGlnLysGluGlnAsnTyrSerAspAspValLeuAlaAsn
            489

ATGATTAGTGAACCAAGGATCAGTTACGGAAACGATGCTCTCATGCCATCTTTGACCGAA
MetIleSerGluProArgIleSerTyrGlyAsnAspAlaLeuMetProSerLeuThrGlu

ACGAAAAACCACCGTGGAGCTCCTTCCCGTGAATGGAGAGTTCAGCCTGGACGATCTCCAG
ThrLysThrThrValGluLeuLeuProValAsnGlyGluPheSerLeuAspAspLeuGln

                                                        1684
                                                        |
CCGTGGCATTCTTTTGGGGCTGACTCTGTGCCAGCCAACACAGAAAACGAAGgtaagagt
ProTrpHisSerPheGlyAlaAspSerValProAlaAsnThrGluAsnGluV
                                                        562

                                            1685
                                            |
cccctgagccagcaagg...........................TTGAGCCTGTTGATGCCCGC
                                            alGluProValAspAlaArg
                                              563

                                   1738
                                   |
CCTGCTGCCGACCGAGGACTGACCACTCGACCAG..........................
ProAlaAlaAspArgGlyLeuThrThrArgProG
                                   580

tttaagagtcatttatcaattttcttctaacttcaggcctagaaagaagttttgggtagg

ctttgtcttacagtgttattatttatgagtaaaactaattggttgtcctgcatactttaa

                 1739
                 |
ttatgatgtaatacagGTTCTGGGTTGACAAATATCAAGACGGAGGAGATCTCTGAAGTG
                 lySerGlyLeuThrAsnIleLysThrGluGluIleSerGluVal
                   581

                                                          1839
                                                          |
AAGATGGATGCAGAATTCCGACATGACTCAGGATATGAAGTTCATCATCAAAAATTGgta
LysMetAspAlaGluPheArgHisAspSerGlyTyrGluValHisHisGlnLysLeu
                                                        613

cgtaaaataatttacctctttccactactgtttgt...................aaag

taagcatcaaatatttgaccaaccagttgggcagagaatatactgaaactttttatataa

cctcatccaaatgtccccgtcatttaagaaatgaaattcttctaattgcgtttataaatt

                                                       1840
                                                       |
gtaaattatattgcatttagaaattaaaattctttttcttaatttgttttcaagGTGTTC
                                                       ValPhe
                                                       614

TTTGCAGAAGATGTGGGTTCAAACAAAGGTGCAATCATTGGACTCATGGTGGGCGGTGTT
PheAlaGluAspValGlySerAsnLysGlyAlaIleIleGlyLeuMetValGlyGlyVal

GTCATAGCGACAGTGATCGTCATCACCTTGGTGATGCTGAAGAAGAAACAGTACACATCC
ValIleAlaThrValIleValIleThrLeuValMetLeuLysLysLysGlnTyrThrSer

                    1986
                    |
ATTCATCATGGTGTGGTGGAGgtaggtaaacttgactgcatgtttccaagtgggaattaa
IleHisHisGlyValValGlu
                    662

gactatgagagaattaggcttagcttttgctaagaactagctaagtatctcttttaaaa

aacgaatcagtgtgcttccatgatgcttgggttacagttgttctttctt...........

.tgggcaaaggcgttctgctccaagatgtcaaagtgggggagaaaattatgggtgttctg

caatcttggcaaaagaagagtactgtactccttatctttttactgcttctccatgttcac

                                                      1987
                                                      |
ccttaaaagattgatttttatttttactcagctctcctcttgtttttcagGTTGACGCC
                                                      ValAspAla
                                                      663

GCTGTCACCCCAGAGGAGCGCCACCTGTCCAAGATGCAGCAGAACGGCTACGAAAATCCA
AlaValThrProGluGluArgHisLeuSerLysMetGlnGlnAsnGlyTyrGluAsnPro

                               2088
                               |
ACCTACAAGTTCTTTGAGCAGATGCAGAACTAG
ThrTyrLysPhePheGluGlnMetGlnAsnEnd
                           695
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Figure 1: Exon–intron boundaries of the human PAD-gene.
The DNA sequence of the 16 $PreA4_{695}$ exons are written in capital letters and numbered as the corresponding $PreA4_{695}$-cDNA (8). The DNA sequences of the partially sequenced $PreA4_{695}$ introns are written in small letters and the points interrupting the intronal sequences indicate regions within the introns which have not been sequenced. The varying number of points within the different introns do not correspond to the size of these gaps. The asterisk's at the bases 865 and 866 indicate the exon–intron boundary where the further exons mentioned in the text may be inserted. The amino acid sequence written under the exons corresponds to the deduced amino acid sequence of the $PreA4_{695}$-cDNA.

chromosome 21 library (*Hind*III and *Eco*RI fragments in *lambda* charon 21A) from the American Type Culture Collection (ATCC) in Rockville, Maryland, USA, c) a human genomic library (*lambda* vector L47.1, courtesy of Dr B. Horsthemke, University of Essen), and d) a human leukocyte genomic library (*lambda*-EMBL 3) purchased from Genofit, Heidelberg. Different DNA fragments were isolated from the $PreA4_{695}$cDNA (8) and used as screening probes. Hybridization conditions for the first three libraries mentioned above have been described (7). Hybridizations with the human genomic library from Genofit were performed in 6×SSC, 5×Denhardt's solution, 0.5% SDS, 0.05% Na-pyrophosphate, 100μg/ml salmon sperm DNA at 65° with 5 × 10^5c.p.m/ml of randomly primed probe (13). Putative positive *lambda*-clones were rescreened under the same conditions and the *lambda*-DNA was isolated as described (14).

Treatment of positive lambda clones

In each case the DNA inserts of positive *lambda* clones from the chromosome 21 libraries were cloned completely into the plasmid vector pUC19 (15). The DNA inserts of positive *lambda* clones from the human genomic libraries were digested with different restriction endonucleases and subcloned into pUC19. Exon-containing fragments were detected by dideoxy sequencing (16,17) using exon-specific primers.

Polymerase chain reaction (PCR)

Amplifications with *Taq*I-polymerase (18) were performed in 100μl reaction mixtures containing 1μg genomic DNA (human embryonic liver) in 50mM KCl, 10mM Tris-HCl, pH 8.5, 2.5mM $MgCl_2$, each primer at 200nM, each dNTP (dATP, dCTP, dGTP, dTTP) at 200μM, gelatine at 200μg/ml and 2.5 units of polymerase (Cetus, Perkin-Elmer). The samples were overlaid with several drops of mineral oil and subjected to 40 cycles of amplification as follows. The samples were heated from 70° to 95° over a 2-minute period (denaturation), cooled to 55° over 2 minutes (annealing), heated to 70° for 1 minute (extension reaction). Thermal cycling was performed in a programmable heatblock (Perkin-Elmer, Cetus Instruments). After the final extension step, the samples were precipitated with ethanol and resuspended in 100μl TE buffer (19). 80μl of each sample was resolved on a 1.2% seaplaque-agarose gel and the fragment of interest was isolated as described (19). The amounts of synthesized fragments were approximately 200–500ng in each case. 50ng of each sample was used for cloning into the plasmid vector pUC19. The cloning of the exon-representing products was confirmed by sequence analysis.

DNA-sequencing

The sequences presented in figure 1 were derived by the chain termination method (16) using Klenow polymerase on single-stranded denatured plasmid DNA templates (17). Exon-specific synthetic primer-oligomers were synthesized on a Model 380 A DNA synthesizer (Applied Biosystems).

RESULTS AND DISCUSSION

Exon-specific clones isolated from the genomic libraries

The following fragments were isolated from the chromosome 21 libraries and cloned completely into the plasmid vector pUC19 (15): H1.30(2.8kb,exon1), E6.BA(1.8kb,exon2), H3.31(4.5kb,exon3), H2.31(7.5kb,exons4,5), 4A(7.0kb,exon7), H1.41(3.8kb,exons 8,9), APC1(1.2kb, exon 14), E4.5(2.8kb, exon 15), H1.23(7.0kb, exon 16). The lengths of the different fragments and the numbers of the corresponding exons are shown in brackets. Clone P1.21(1.2kb,exon12) was isolated from the human genomic library from B.Horsthemke and represents only a part of a larger insert from the corresponding *lambda* clone. Clone HG440(appr.15kb) was isolated from the human leukocyte genomic library (Genofit) and a 4.5kb fragment containing exon 11 was subcloned in pUC19.

Exon-specific clones synthesized by PCR (18)

Lambda clones specific for the exons 6,10 and 13 could not be detected in the genomic libraries mentioned above. Three *lambda* clones specific for exon 6 were isolated from the human leukocyte genomic library (Genofit) but up to now they have not been subcloned into pUC19. In order to find out whether the putative exons 6,10 and 13 were interrupted by further introns we probed the DNA by the PCR amplification method (18) as follows. The exon–intron boundaries of exons 5 and 7,9 and 11, 12 and 14, respectively had been determined by the analysis of neighbouring genomic clones. So we used two synthetic oligonucleotides with their 5′ ends corresponding to the first or last position of the putative exon. The products of amplification procedures were shown to have the same size as the corresponding cDNA fragments on a 2% agarose gel (data not shown), and sequence analysis of the cloned fragments revealed no further introns.

Exon−intron boundaries determined by DNA sequence analysis
Partial sequence analysis of the various genomic clones revealed that the $PreA4_{695}$ transcript is a splicing product containing 16 exons (figure 1). In each case the exon−intron boundary as well as the whole exon was sequenced. As expected (20), each intron starts with a 'GT' and ends with a 'AG'.

Only two of the genomic clones isolated from the genomic libraries contain two exons (clones H2.31, 7.5Kb, exons 4 and 5, and H1.41, 3,8kb,exons 8 and 9). All the other clones contain one exon each, even HG440 (appr. 15 Kb) and P1.21 (appr. 14 Kb). Hybridization experiments (data not shown) showed that the neighbouring genomic clones did not overlap, thus the size of the gene cannot yet be determined. From the known insert sizes a minimal length of 50 Kb can be calculated.

Recently, three groups reported the cloning of preA4-cDNAs containing an extra exon encoding a protease-inhibitor like sequence (9,10,11). Kitaguchi et al. (10) isolated a cDNA containing the exon coding for the protease-inhibitor like sequence together with a 3′-adjacent small exon coding for a peptide similar in sequence to the MRC OX2 antigen (21). They proposed (10) that the three different preA4-mRNA's are due to alternative splicing of a single PAD gene. For this form the PAD gene provides 18 exons. The use of the $PreA4_{695}$ cDNA in the identification of the genomic structure of the PAD gene would have failed to reveal the trypsine inhibitor coding exon or any further exons. In figure 1 the positions of the exon−intron boundaries where the trypsin-inhibitor-like exon and the other additional exon (10) have been found are marked by asterisk's (bases 865, 866).

Exon−intron boundaries and the structure of the $PreA4_{695}$ protein.
Comparison of the exon−intron-structure of the PAD-gene to the deduced protein sequence shows that exon 1 contains the coding region for the signal peptide (22). Mita et al. (23) recently reported a cDNA coding for a further 73 amino acids at the N-terminus of the precursor. The significance of this finding is still unclear. Exons 2,3,4 and 5 span the cysteine-rich region, and exons 5 and 6 provide the highly negatively charged domain (8). The amino acid sequence encoded by exon 6 shows some similarity (31 %) to human prothymosin alpha-1 (24) but this similarity is mainly based on the acidic amino acids in these two peptides. The protein sequence encoded by exons 7 to 13 contains the two putative N-glycosylation sites (8,25) of the $PreA4_{695}$-protein. The amyloid A4 protein extends across the border between exons 14 and 15, a fact that supports the idea that accummulation of this peptide in the brain tissue is due to degradation of the precursor and not to aberrant splicing. Finally the putative transmembrane region is completely contained in exon 15 and most of the putative cytoplasmic domain is coded for by exon 16.

ACKNOWLEDGEMENTS

We thank A.Starzinski-Powitz for the human embryonic DNA, B.Horsthemke for the human genomic library, J.Hesselbach and A.Lee for excellent technical assistance, and B.Kisters for help with the computers. This work was supported by a grant of the Fritz Thyssen Stiftung to B.M.H. and grants of the Deutsche Forschungsgemeinschaft through SFB 317, the Bundesministerium für Forschung und Technologie and the Fonds der chemischen Industrie to K.B.

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