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Expression of the amyloid precursor protein gene in mouse oocytes and embryos

(β-amyloid/polymerase chain reaction/Alzheimer disease/Down syndrome)

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ABSTRACT **The amyloid precursor protein (APP) is thought to be processed aberrantly to yield the major constituent of the amyloid plaques observed in the brains of patients with Alzheimer disease and Down syndrome. However, the gene encoding APP is expressed widely in normal human tissues and in adult and fetal mouse tissues and is alternately spliced in a tissue-specific pattern in the adult. There is evidence that APP may function as a growth factor and as a mediator of cell adhesion and in these roles could be important in morphogenesis. As a step toward determining the role of APP in development and in determining how the adult pattern of tissue-specific splicing is established, we have used reverse transcription and the polymerase chain reaction to demonstrate APP expression in mouse oocytes, preimplantation embryos, and postimplantation embryonic stages to the late embryonic period. All three splicing forms described in mouse were present at each stage, although there were changes in the ratios of the splicing forms at different stages. Screens for APP clones in embryonic cDNA libraries from the egg cylinder stage and the early somite stage were used to confirm the results of the polymerase chain reaction, and APP clone abundance was found to increase 10-fold between these two stages.**

β-Amyloid peptide is the main component of the neuritic and cerebrovascular amyloid plaques of patients with Alzheimer disease (AD) (1) and aging individuals with Down syndrome (DS) (2). Its 42-amino acid sequence is found as part of a 695-amino acid protein, the amyloid precursor protein (APP_{695}) (3–6). APP is encoded by a gene mapped on human chromosome 21 and in the syntenic region of mouse chromosome 16 (7, 8). The β-amyloid peptide includes portions of both the extracellular domain and the single membrane-spanning domain of APP, but it is not known how the full-length protein is processed to yield the short peptide. However, it must involve a change in the normal processing of the protein, since the full-length precursor is cleaved constitutively at a site within the β-amyloid sequence (9). Two other forms of APP have been described, APP_{751} and APP_{770}, which are identical to APP_{695} except for the inclusion of either one or two additional exons, respectively, in the mRNA (10–12). Inclusion of only the second of these exons results in another form, APP_{714}, detected in several human tissues but at much lower levels than the first three forms (13). A fifth form, APP_{563}, has been cloned from a human brain cDNA library; its deduced sequence is identical to APP_{751} for the first 543 amino acids and then diverges for the remaining 20 amino acids (14). APP_{563}, a minor component of the total APP message in brain, lacks the membrane-spanning domain and presumably encodes a secreted protein. Total APP expression is highest in the brain (6); APP_{695} is expressed predominantly in neurons (15), whereas APP_{751} and APP_{770} have been detected in every adult tissue examined (6). In the mouse, only splicing forms corresponding to APP_{695}, APP_{751}, and APP_{770} have been described, and the tissue distributions of these three forms are similar to that seen in human beings (16, 17).

Both abnormal levels of APP expression and aberrant splicing regulation have been suggested as possible factors in plaque deposition in AD and DS (13, 18–20), a hypothesis that seems especially attractive in the case of DS individuals, in whom APP is known to be overexpressed (6). However, the data on the levels and splicing of APP in AD have been confusing; each group has studied a different set of brain regions and has used different techniques to measure APP levels and splicing. One group found a change in relative APP levels between two areas of hippocampus in AD (18); another found an increase in APP_{695} in neurons of the nucleus basalis and locus ceruleus but not in several other brain regions (19); and a third study found no changes in the ratio of APP_{695} to $APP_{751/770}$ in three cortical regions in AD (20). The most comprehensive study found an increase in total APP message in frontal white matter that was solely due to an increase in APP_{770} and an increase in APP_{770} at the expense of APP_{695} in cerebral meninges (13). A problem with studies on postmortem AD tissue is that only the final stage of the disease process is examined, and factors such as extensive neuronal loss in some regions certainly complicate interpretation of the results.

Overexpression of APP could be contributing to processes other than plaque formation in the case of DS individuals. Previous studies of APP in development have shown its expression in several human fetal tissues (6) and in mice at late embryonic stages (21). APP is overexpressed, more than the expected 1.5-fold, in both fetal DS tissues and trisomy 16 mice (6, 21), and its overexpression in development could contribute to the pathogenesis of defects seen in both conditions. Little is known, however, about its expression in normal development and at what points in development its overexpression could be detrimental.

Determining the role of APP expression and splicing regulation in plaque deposition and other pathologic processes will clearly require a better understanding of the normal gene regulation. The studies in cell culture systems have already proven fruitful, especially in providing information on growth factors that increase APP expression or alter its splicing pattern (22). Based on some of these studies, it has been suggested that APP is a growth factor (23), a proteoglycan core protein (24), a heparin-binding protein (25), and a mediator of cell adhesion (22). Separate experiments have provided evidence that $APP_{751/770}$ functions in the regulation of coagulation (26). The mouse embryo offers a system in which to study APP regulation, and careful description of its

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Abbreviations: APP, amyloid precursor protein; PCR, polymerase chain reaction; RT-PCR, reverse transcription followed by PCR; E, embryonic postcoital day (e.g., E3.5); AD, Alzheimer disease; DS, Down syndrome.

patterns of expression and splicing regulation will help not only to understand the gene regulation but also to decide in which developmental processes APP may be playing a role. The period between the egg cylinder stage (embryonic postcoital day 6.5, or E6.5) and the early somite stage (E8.5) is characterized by several important embryological events, such as gastrulation, formation of the body plan, and early organogenesis, in which APP could be important as a growth factor or an extracellular matrix component. As a step toward understanding the role of APP in embryogenesis, we have demonstrated its expression in mouse development from ovulated oocytes to the late embryonic stage. While the three splicing forms found in mouse are present at all stages, the relative amount of APP_{695} decreases after implantation and then increases over the period from E6.5 to the E8.5. We estimated APP message abundance at these two stages by screening cDNA libraries and showed a 10-fold increase in APP clone abundance between E6.5 and E8.5.

MATERIALS AND METHODS

Materials. *Thermus aquaticus* (*Taq*) DNA polymerase was obtained from Perkin–Elmer/Cetus, and avian myeloblastosis virus reverse transcriptase was from Life Sciences (St. Petersburg, FL). Oligo(dT) and neutralized solutions of deoxynucleotides were purchased from Pharmacia LKB. (C57BL6/J × A/J)F_1 mice were purchased from The Jackson Laboratory and ICR mice were from Harlan–Sprague–Dawley.

Collection of Embryos and RNA Isolation. Timed pregnancies were obtained by mating superovulated (C57BL6/J × A/J)F_1 females or by spontaneous matings of ICR mice. Oocytes and embryos were collected by standard methods (27). E6.5 and E8.5 embryos were dissected as described below for cDNA library construction; E7.5 embryos were dissected free of the extraembryonic membranes before RNA isolation. The care and treatment of the mice used in these experiments were in accordance with institutional guidelines. RNA was isolated by acidic phenol extractions as described (28) with 25 μg of carrier tRNA added to each sample to improve recovery.

Reverse Transcription Followed by Polymerase Chain Reaction (RT-PCR). Reactions were performed essentially as described by Rappolee *et al.* (29) with oligo(dT) primer and 5 units of avian myeloblastosis virus reverse transcriptase per 20 μl of reaction mixture. Aliquots of these cDNAs were taken for PCR having 25 cycles each of 1 min at 94°C, of 1 min at 45°C, and of 2 min at 72°C. APP primers were as shown in Fig. 1, and β-actin primers were as described by Rappolee *et al.* (29). The manufacturer's instructions were followed for PCR reagent concentrations (Perkin–Elmer/Cetus). Negative controls were done with each experiment by performing PCR on reagents with no added cDNA template. Samples were size-fractionated by gel electrophoresis on 3% Nusieve/1% SeaKem gels and were visualized by ethidium bromide staining. The DNA was transferred to nylon membranes, hybridized, and washed under standard conditions (30, 31). The probe was the isolated 5′ *Eco*RI fragment of APP as described in Fig. 1, labeled by random primer extension (32).

Screening of Embryonic cDNA Libraries. Mouse embryos were dissected at the egg cylinder stage (E6.5) with the ectoplacental cone removed and at the 8–12 somite stage (E8.5) with the extraembryonic membranes removed (33). Poly(A)$^+$ RNA was prepared and used to construct libraries in phage λgt10 (D. Weng, C. Mjaatvedt, A. Lawler, and J.D.G., unpublished data). Plaque lifts were hybridized and washed under standard conditions (34); the probe used was the 3′ *Eco*RI fragment of APP as described in Fig. 1, labeled by random primer extension (32).

RESULTS

RT-PCR (35) is an ideal method for detecting transcripts in small amounts of starting material and has been used to study gene expression in mouse embryos (29, 36). With the appropriate choice of primers, all three major splicing forms of APP can be detected in a single sample, and the relative amounts of the amplified fragments indicate the relative levels of the splicing forms. The primers used to amplify APP are shown in Fig. 1, with the expected sizes of the amplified fragments corresponding to the major splicing forms. To verify that all three splicing forms were amplified proportionally, duplicate samples containing mouse adult kidney cDNA were subjected to PCR with APP primers for various numbers of cycles, electrophoresed, and blotted as described. Over the range of 15–25 cycles, the ratios of amplified fragments remained unchanged (data not shown), showing that all forms were being amplified proportionally.

The amounts of RNA in the samples are given in embryo equivalents in Table 1 along with estimates of total RNA. Fig. 2 shows the detection of APP transcripts by RT-PCR in ovulated oocytes and in embryos from the late two-cell stage to the late embryonic stage. The β-actin PCR products, used as a control for RNA integrity and the reverse transcription, were not detectable by ethidium bromide staining in the samples from unfertilized eggs and late two-cell embryos. This is consistent with reports that maternal β-actin mRNA is deadenylylated and degraded shortly after ovulation, and β-actin transcription from the embryonic genome is only

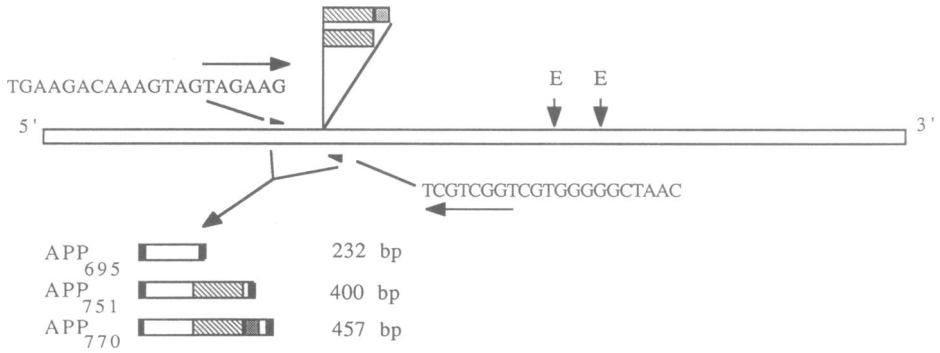

FIG. 1. Diagram and sequences of APP PCR primers. Shown are the sequences of the primers, their location relative to the alternate splice site, and the expected size of the amplified fragments corresponding to the three major splicing forms. The arrows indicate the location of the *Eco*RI sites (E) in the mouse APP cDNA used to generate the 5′ 1.7-kilobase (kb) and 3′ 1.1-kb fragments for probe templates. The full-length clone of APP_{695} was isolated from a mouse adult brain cDNA library and sequenced in this laboratory (R. Morgan, personal communication). The primers depicted were taken from the mouse sequence.

Table 1. Amounts of material from each developmental stage used in PCR, given as embryo equivalents and as estimates of total RNA

Postcoital age of ovulated oocytes, days	No. of embryos	Estimated RNA, ng
	150	53*
1.5	150	36*
2.5	45	31*
3.5	15	22*
6.5	0.6	69†
7.5	0.1	48†
8.5	0.03	100†
9.5	0.01	—
10.5	0.01	—

*Based on published values from Piko and Clegg (37) and assumption of 100% recovery durng RNA extraction.
†Based on actual amounts of RNA recovered from these stages in the construction of cDNA libraries (D. Weng, personal communication).

detectable at the four- to eight-cell stage (38, 39). The Southern blots in Fig. 2 *B* and *D* were scanned densitometrically and used to calculate the ratios of the three amplified fragments. The results, given as percentages of total optical density, are shown in Fig. 3. All experiments were repeated at least once with separately isolated RNA samples, giving consistent results both in the detection of the three splicing forms and in their relative amounts at different stages.

As additional confirmation of embryonic expression and as an estimate of the abundance of APP transcript, two cDNA libraries were screened with the APP 3′ *Eco*RI fragment. In the E6.5 library, eight positive plaques were detected of 900,000 screened; they were confirmed by plaque purification and Southern blot analysis on the inserts. The E8.5 library was screened under the same conditions, and 26 positive

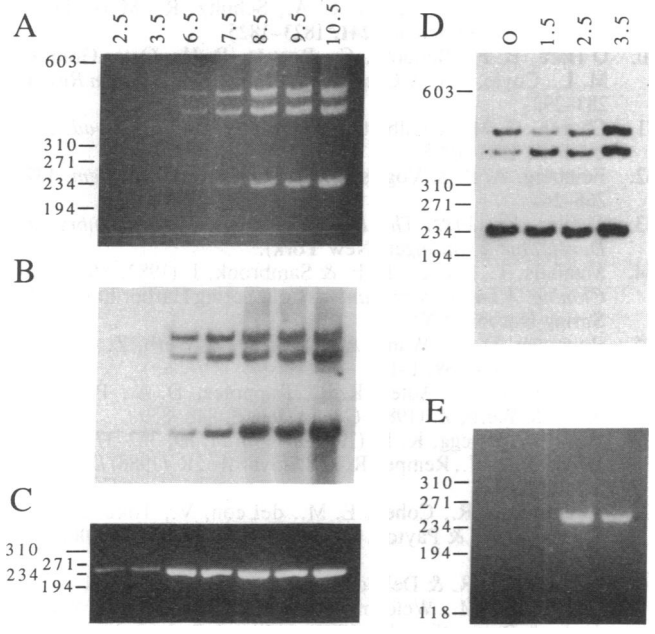

FIG. 2. (*A*) Ethidium bromide-stained gel of APP PCR products. The numbers above each lane indicate the age of the embryos in embryonic postcoital days. (*B*) Southern blot of the gel in *A*, with the same lanes. (*C*) Ethidium bromide-stained gel of β-actin PCR products from the same ages. (*D*) Southern blot of APP PCR products from preimplantation embryos. The numbers above each lane indicate the age of the embryos, with O indicating oocytes. (*E*) Ethidium bromide-stained gel of β-actin PCR products from the same ages. Autoradiography times were 1 hr for the blot in *B* and 16 hr for the blot in *D*.

FIG. 3. Ratios of APP splicing forms at different embryonic ages (in days). The ratios were calculated in arbitrary units of optical density from the intensities of the bands on the Southern blots shown in Fig. 2. Autoradiograms were scanned on an LKB Ultrascan XL densitometer. To verify that the exposures were in the linear range of the film, a PCR sample from mouse kidney RNA was serially diluted, electrophoresed, and blotted as described above. The resulting Southern blot was used to determine the linear response range of the film.

plaques were detected of 240,000 screened. Only plaques also detected by duplicate screens with the 5′ *Eco*RI fragment were purified further.

DISCUSSION

Our demonstration of APP expression in early mouse embryogenesis implies an important function for APP in normal development. We have detected all three splicing forms of APP at every developmental stage examined, including both the ovulated oocyte and late two-cell embryo. While the mouse oocyte carries a large store of maternal mRNAs, these in general are degraded by the first cleavage division, and transcription from the embryonic genome is activated in the late two-cell embryo (37, 40). Detection of APP at both stages implies either that the maternal APP mRNA is not totally degraded by the late two-cell stage or that the gene encoding APP is one of the earlier genes transcribed from the embryonic genome. The latter seems more likely because of the difference in ratios of the three splicing forms at the two stages. This is more easily explained by altered splicing regulation of the embryonic transcripts than by differential degradation of the maternal mRNAs.

While we have no direct information about the levels of APP expression in the preimplantation embryos, the amplified products were detectable only by Southern blot analysis, so the starting levels of mRNA are probably lower than in the postimplantation embryos. Since the promoter of APP has features resembling a housekeeping gene, it might be expected to be expressed at a low level in any cell (41). It has been reported that by using RT-PCR, transcripts have been detected in some cell types that clearly do not have importance to the phenotype of the cell and probably are not expressed at the protein level (42). However, those experiments were done under conditions designed to give much greater amplification than those used in this study.

One goal of this study was to identify when the alternate splicing forms of APP appear in development and to correlate the appearance of different forms with specific embryologic events. In none of our samples did we detect an amplified fragment corresponding to APP_{714}; this splicing form may not exist in the mouse. Interestingly, all three major forms, APP_{695}, APP_{751}, and APP_{770}, were present at even the earliest stages examined, so the tissue-specific splicing is clearly

acquired later in development. However, the ratios of the splicing forms varied at different stages. At preimplantation stages, we found that APP_{695} constituted 60–70% of the total, but by E6.5—the first postimplantation stage examined—the other two forms had greatly increased in relative amount. This switch may reflect an increased requirement for $APP_{751/770}$ during the early postimplantation period, which could be related to the serine protease inhibitor encoded by the first additional exon, which the two forms share. However, over the period from E6.5 to E8.5, APP_{695} increased from approximately 25% to 50% of the total APP message, and total APP clone abundance in the cDNA libraries also showed a 10-fold increase between the same stages; this translates to an increase in APP_{695} of roughly 20-fold.

Since APP_{695} is the form most abundant in the adult nervous system, its up-regulation from E6.5 to E8.5 could be due to increased and localized expression in the developing neural tube; this hypothesis can be tested by localization of the splicing forms by *in situ* hybridization. Further experiments correlating APP protein expression with detection of its message can also be done, especially to determine its importance to the phenotype of the preimplantation embryos. In addition to further descriptive studies, we can use the technique of *in vivo* mutagenesis through homologous recombination in embryonic stem cells to create a line of mice with decreased or eliminated APP expression and thereby gain more direct information about the importance of APP expression in early mouse embryogenesis.

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