Journal of Cell Science 108, 675-683 (1995)
Printed in Great Britain © The Company of Biologists Limited 1995

CDEBP, a site-specific DNA-binding protein of the 'APP-like' family, is required during the early development of the mouse

Anne Blangy, Frédérique Vidal, François Cuzin, Yin-Hua Yang, Kim Boulukos, and Minoo Rassoulzadegan*

Unité 273 de l'INSERM, Université de Nice-Sophia Antipolis, 06034 Nice, France

*Author for correspondence

SUMMARY

A murine protein, termed CDEBP, was previously shown to bind the double-stranded DNA motif GTCACATG, identical to the yeast centromeric element CDEI. The cDNA sequence showed three domains with extensive similarities to the amyloid β precursor protein (APP). The protein is homologous over its entire length to the human protein designated APPH. In situ immunofluorescence assays using antibodies raised against distinct parts of CDEBP detected discrete sites of accumulation inside the interphase nucleus, and the bulk of the protein was not associated with mitotic chromosomes. One of the complexes with double-stranded CDEI oligonucleotides detected by gel shift assay was not present when the protein had been selectively removed from nuclear extracts by immunoprecipitation. We reported previously that microinjection into one-cell mouse embryos of DNA fragments including the CDEI sequence results in an early arrest of development with abnormal nuclei containing variable amounts of DNA. The same characteristic figures were observed when embryos were treated with antisense oligonucleotides complementary to parts of the CDEBP coding region. Complexes between the CDEBP protein and CDEI sites in the mouse genome thus appear to play a critical role in the replication/segregation of the embryonic genome.

Key words: CDEI, chromatin, development, DNA binding protein, APP-like proteins

INTRODUCTION

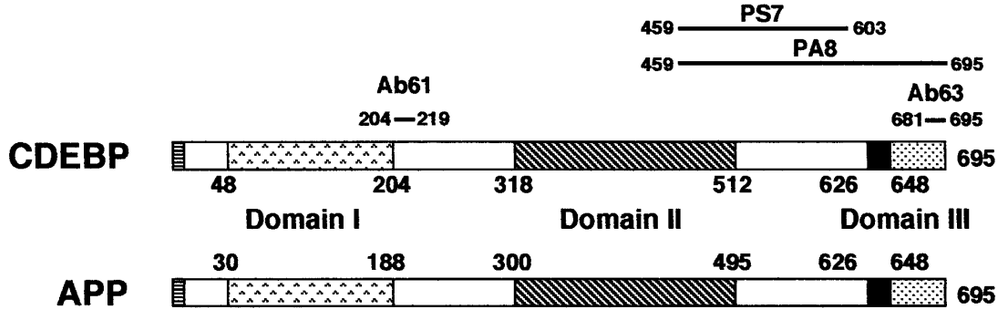
Microinjection of a unique 345 bp fragment of mouse chromosomal DNA designated p12B1 (Léopold et al., 1987) into fertilized mouse eggs results in an early arrest of their development and the accumulation of nuclei with grossly abnormal DNA contents (Blangy et al., 1991). Injection of double-stranded oligonucleotides corresponding to parts of the p12B1 sequence reproduced this effect. These experiments identified the motif GTCACATG as the minimal toxic sequence. Because of its identity with the yeast centromeric element I (Clarke and Carbon, 1985), this mouse sequence is thereafter referred to as CDEI. Gel retardation and DNase foot-printing assays have detected mouse proteins that recognize this sequence. Mutations in the CDEI sequence that interfere with protein binding also abolish its toxic effect. A cDNA for a CDEI-binding protein (CDEBP) was cloned and sequenced. A partial amino-acid sequence (Vidal et al., 1992) revealed two regions that share a high degree of similarity with the β amyloid precursor protein (APP). We and others (Hanes et al., 1993) completed the sequence and evidenced an open reading frame encoding a polypeptide of 695 amino acids with a calculated M_r value of 78,933, with three domains of similarity with APP (Fig. 1). Two proteins with the same APP-like domains have been identified in mouse and *Drosophila* (Rosen et al., 1989; Wasco et al., 1992), but they are otherwise distinct in sequence from CDEBP. The human protein designated APPH (Sprecher et al., 1993) and the partial sequence of a rat sperm protein (Yan et al., 1990) not only show the same domains of similarity with APP, but are in fact homologous with CDEBP over their entire lengths.

CDEI sites initially identified in plasmid p12B1 (Léopold et al., 1987), have subsequently been found in the genome of Bovine Papillomavirus Type 1 (BPV1) (Blangy et al., 1992). These two genetic elements are maintained as autonomous episomes in murine cells, and, in both cases, deletions and point mutations in CDEI interfere with episomal maintenance (V. Pierrefite, M.R. and F.C., unpublished data). Taken together with the toxic effect of CDEI oligonucleotides in early embryos, these observations suggested that important protein-DNA interactions occur at genomic CDEI sites. This was confirmed by the deleterious effect of CDEBP antisense oligonucleotides in preimplantation mouse embryos.

MATERIALS AND METHODS

Cell lines

Balb/c 3T3 fibroblasts (Aaronson and Todaro, 1968) and Pam 212 epithelial cells (Yuspa et al., 1980) were grown in CO_2-buffered



Domain I

```
AEPQIAMFCGKLNMHVNIQTGQWEPDPTGTKSCLGTKDEVLQYCQEIYPELQITNVMEANQPVNIDSWCRRDKRQCKS--
AEPQIAMFCGKLNMHMNVQNGKWESDPSGTKTCIGTKEGILQYCQEVYPELQITNVVEANQPVTIQNWCKRGRKQCKTHT

HIVIPFMCLVGEFVSDVLLVPDNCQFFHQEPMEVCEKHQRWHTLVKEACLTEGLTLYSYGMLLPCGVDQFHGTEYVCCP
HIVIPYRCLVGEFVSDALLVPDKCKFLHQERMDVCETHLHWHTVAKETCSEKSTNLHDYGMLLPCGIDKFRGVEFVCCP
```

Domain II

```
VDVYFETSADDNEHARFQKAKEQLEIRHRNRMDRVKKEWEEAELQAKNLPKTERQTLIQHFQAMVKALEKEAASEKQQLVETHLARVEAMLNDRRRIA
VDKYLETPGDENEHAHFQKAKERLEAKHRERMSQVMREWEEAERQAKNLPKADKKAVIQHFQEKVESLEQEAANERQQLVETHMARVEAMLNDRRRLD

LENYLAALQSDPPRPHRILQALRRYVRAENKDRLHTIRHYQHVLAVDPEKAAQMKSQVMTHLHVIEERRNQSLSLLYKVPYVAQEIQEEIDELLQ-EQ
LENYIIALQAVPPRPHHVFNMLKKYVRAEQKDRQHTLKHFEHVRMVDPKKATQIRSQVMTHLRVIYERMNQSLSLLYNVPAVAEEIQDEVDELLQKEQ
```

Domain III

```
ALIGLLVIAVAIATVIVISLVMLRKRQYGTISHGIVEVDPMLTPEERHLNKMQNHGYENPTYKYLEQMQI
AIIGLMVGGVVIATVIVITLVMLKKKQYTSIHHGVVEVDAAVTPEERHLSKMQQNGYENPTYKFFEQMQN
```

Fig. 1. Sequence similarities between the CDEBP and APP proteins. Domains of similarity termed I, II and III are drawn as identically filled blocks; open boxes correspond to regions with no detectable similarity; vertical lines indicate amino acid identity, and dots, conservative changes. Black boxes correspond to the putative transmembrane domain of APP. Dashed boxes at the amino-terminal end of each protein correspond to a putative signal peptide, similarly hydrophobic in nature in APP and CDEBP. Regions in CDEBP that were used to generate antibodies PS7 and PA8 are indicated by thick lines.

Dulbecco modified Eagle's medium supplemented with 10% fetal calf serum (Gibco).

Manipulation of mouse embryos

Harvest and culture (Hogan et al.,1986)

C57BL/6 × DBA2 F1 hybrid parents kept on light cycle from 4 am to 3 pm were mated overnight, and one-cell embryos were harvested between 2 and 4 pm the next afternoon (estimated time after fertilization: 15 hours). They were kept in M16 medium under 5% CO_2 atmosphere at 37°C.

Reverse transcription-PCR amplification

Two to five eggs were applied in 1 μl culture medium on a square fragment of Hybond Messenger Affinity (mAP) membrane (Amersham) previously wetted with 0.5 M NaCl. The eggs were lysed in situ by the stepwise addition of 1 μl drops of lysis buffer (4 M guanidinium thiocyanate, 0.1 M Tris-HCl, pH 7.5, 1% β-mercaptoethanol; total 5 μl). Binding of mRNAs to the membrane was similarly accomplished by dropwise application of 20 μl of 0.5 M NaCl to the membrane (Sheardown, 1992). The membranes were immediately washed twice by vortexing in 0.5 M NaCl (1 ml), and twice in 70% ethanol (1 ml). To the membrane were then added 20 μl of rTth-DNA Polymerase Mix (Boehringer). Reverse transcription and PCR amplification were performed according to the supplier's instructions (30 cycles of 1 minute at 94°C, 1 minute at 62°C, 1 minute at 72°C). The following primers were used: FV3 (upstream): 5′GCCTGCCTTTGTACCTCACTC3′ (nt 2994-3014), FV1 (downstream): 5′GCATAGTGGGACGAGGCACG3′ (nt 3386-3367). The expected size of the amplified product is 392 bp.

Exposure of embryos to antisense oligonucleotides

Single-stranded antisense oligonucleotides were FV1 (above) and FV2: 5′AGCATGCCATAGCTATATAAGGTCAGCC3′ (nt 646-622). The sense oligonucleotide FV4 5′GCCTGCCTTTG-TACCTCACTCACCTCCACCG3′ (nt 2994-3024) was used as a control. Embryos were cultured under 5% CO_2 atmosphere at 37°C in 200 μl of M16 medium containing 8 μg of oligonucleotide.

The IFind™ and FastDB™ programs (Intelligenetics) were used to

ascertain that the sequences of oligonucleotides FV1-4 are present neither in other APP and APP-like proteins, nor in any of the rodent sequences stored in the GenBank library.

Sequence analysis

We reported initially a partial sequence of the CDEBP coding region (Vidal et al., 1992), which was subsequently completed (Hanes et al., 1993; our unpublished results) to generate the complete sequence, available from EMBL/GenBank/DDBJ under accession number Z27070. The limits of the domains of similarity with APP are indicated in Fig. 1.

RNA analysis

For northern blot hybridization, total RNA was prepared from tissues or cell lines by the guanidinium thiocyanate method and separated from contaminating DNA by pelleting through cesium chloride (Davis et al., 1986). 20 μg RNA per lane were electrophoresed and hybridized with a ^{32}P-labeled full-length cDNA probe according to standard procedures (Sambrook et al., 1989).

Antibody production and purification

Two fusion proteins with either amino acids 459-695 or 459-603 of CDEBP (Fig. 1) attached to glutathione-S-transferase were expressed in bacteria using the pGEX system (Pharmacia). A polyclonal serum (PA8) was raised in rabbits immunized with the largest protein, using material sliced from the gel after SDS-PAGE fractionation of whole bacterial proteins. PS7 antibodies are a subclass of PA8, purified by affinity binding to the smaller fusion protein. To obtain PS7, the fusion protein was electrophoresed, transferred onto a nylon membrane and the band corresponding to the fusion protein was excised. Serum proteins (PA8) were incubated overnight at 4°C with the membrane. Purified antibodies were eluted using the Immunopure Gentle Ab/Ag Elution Buffer (Pierce).

Two other antisera were obtained against ovalbumin-conjugated peptides corresponding to parts of the CDEBP sequence (antiserum Ab61, residues 205 to 219; antiserum Ab63, residues 681 to 695, see Fig. 1). Both antipeptide antibodies were purified by affinity binding to the corresponding peptide as described above. Antibody Ab369, directed against the C-terminal part of APP was kindly provided by S. Gandy (Cornell University, NY), and anti-lamin monoclonal antibody E6 (specific to A and B2 lamins (Lehner et al., 1986)) was a gift of E. Nigg (Institut Suisse de Recherche sur le Cancer, Lausanne).

Immunochemical methods

Total cell extracts were prepared by incubating 10^7 cells per ml, in 10 mM Tris-HCl, pH 8, 1 mM EDTA, 1 mM DTT, 2% NP40, 300 mM NaCl, and protease inhibitors (1 mM PMSF, 10 μg/ml leupeptin, 0.1 μg/ml aprotinin), for 30 minutes on ice with occasional shaking. Extracts were analyzed by immunoblots as described by Harlow and Lane (1988). When non-purified, polyclonal sera were used, phosphatase conjugated anti-rabbit antibodies (Bio-Rad) were employed as second reagents and revelation was performed using Nitro Blue Tetrazolium and Bromochloroindolyl Phosphate (Sigma). When affinity purified antibodies were used, western blot analysis was performed using as second reagent horseradish peroxidase-coupled, goat anti-rabbit antibodies (Sigma), with the ECL western blot detecting kit (Amersham). Immunoprecipitation was performed by incubating cell extracts (1 ml) with polyclonal serum (10 μl) for 1 hour on ice, and pelleting the complexes with anti-rabbit IgG and Protein A-Sepharose.

For indirect immunofluorescence, cells growing exponentially on glass slides were washed with PBS and fixed in 4% paraformaldehyde in PBS for 10 minutes on ice. Cells were permeabilized with 0.2% Triton X-100 in PBS for 2 minutes. After additional washing with PBS and a 60 minute incubation in 3% low fat dry milk, the permeabilized cells were incubated with the first antibody for 45 minutes at room temperature. They were then washed extensively with PBS containing 0.2 % Tween-20. Complexes with polyclonal anti-rabbit antibodies were revealed by a 45 minute incubation at room temperature with FITC- or Texas Red-labeled donkey anti-rabbit IgG antibody (Amersham). Complexes with monoclonal antibodies were revealed with anti-mouse Ig Texas Red linked sheep antibody (Amersham). Cells were subsequently washed in PBS and incubated with 0.25 μM Hoechst 33258 dye (Sigma) for 10 minutes at room temperature. Confocal microscopy was performed using the Leica CLSM system (confocal laser scanning microscope) with an argon-krypton laser beam.

Protein binding assay

Electrophoretic retardation assays after end-labeling of the double-stranded oligonucleotide PL11 (5′AGCTTCTCAGTCACATG-GCACA3′, CDEI block underlined) were performed as previously described (Blangy et al., 1991).

Immunoprecipitation prior to the retardation assay was performed as described above, except that 15 μl of whole serum were mixed with 15 μl of 6% bovine serum albumin and incubated for 1 hour on ice before being added to 200 μl of Balb/c 3T3 cell extract for immunoprecipitation.

In vitro transcription and cell free translation

A cDNA fragment containing the complete coding region of CDEBP (nt. 37-2428) was inserted at the *Eco*RI site of plasmid pBluescript SK (Stratagene), downstream of the T7 promoter. Transcription and translation were performed using the TNT T7 Coupled Reticulocyte Lysate System and T7 RNA polymerase (Promega), essentially according to the manufacturer's instructions, with 1 μg of CDEBP DNA. The reaction was performed at 30°C for 60 minutes. [^{35}S]Methionine-labeled protein products were revealed by SDS-PAGE using Laemmli buffer and 10% polyacrylamide gel.

RESULTS

Expression of CDEBP RNA in mouse cells

Northern blot hybridization with the CDEBP cDNA probe detected an apparently unique RNA species of the expected size (3.6 kb). It was present with variable abundance in all the tissues tested (lung, brain, liver, kidney, spleen, heart, testis, skeletal muscle; Fig. 2a), as well as in established cell lines (not shown, see Figs 4 and 5). Since the existence of a CDEI-binding protein had been initially inferred from experiments performed in early mouse embryos (Blangy et al., 1991), we analyzed RNA of unfertilized oocytes, and of one-cell and two-cell embryos. After reverse transcription and PCR amplification, a DNA fragment in the expected size range hybridized with a CDEBP cDNA probe (Fig. 2b).

The CDEBP protein is localized in the interphase nucleus

In vitro synthesis by a reticulocyte lysate primed by a full-length CDEBP cDNA clone under the T7 RNA-polymerase promoter generated several polypeptide bands with apparent molecular masses between 90 and 110 kDa (Fig. 3a,b). These values higher than that calculated from the sequence (78,933) are likely be due to *O*-glycosylation of the polypeptide, a reaction which is known to occur in reticulocyte lysates (Starr et al., 1990). Presence of *N*-acetylglucosamine in the in vitro product was confirmed by its quantitative retention on a wheat

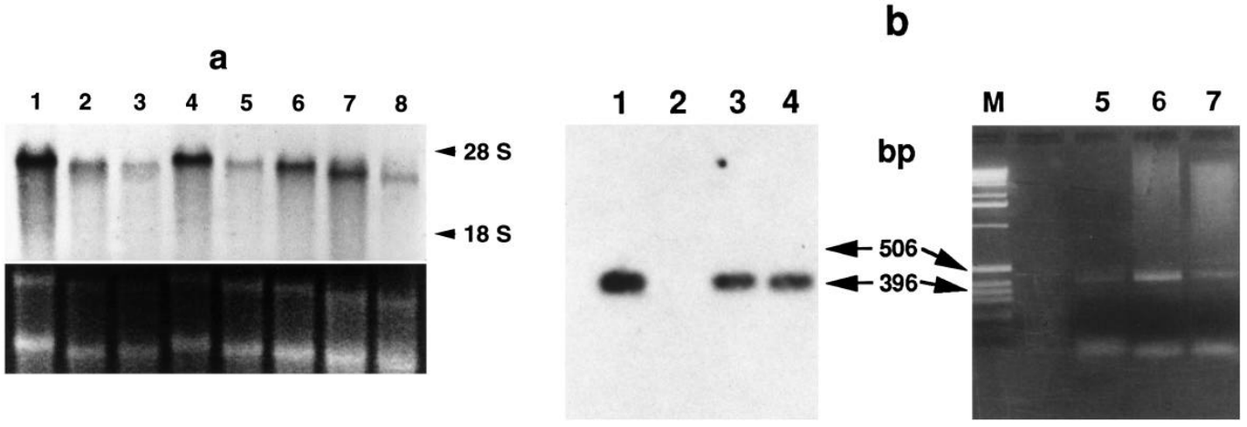


Fig. 2. CDEBP mRNA in mouse tissues and cell lines. (a) Top: northern blot hybridization of mRNA from representative mouse tissues with a probe corresponding to the full-length CDEBP cDNA. Lane 1, lung; 2, brain; 3, liver; 4, kidney; 5, spleen; 6, heart; 7, testis; 8, skeletal muscle. Bottom: ethidium bromide staining (loading control). (b) CDEBP expression in oocytes and early embryos. Southern blot analysis of PCR amplification products obtained after reverse transcription of polyadenylated RNAs from unfertilized oocytes (lane 1), one-cell (3) and two-cell (4) mouse embryos. Lane 2 is the same as 1, but the reverse transcription step was omitted. After amplification, 1/10th of the reaction product was loaded on a 2% agarose gel, electrophorezed, transferred onto nitrocellulose and hybridized with the CDEBP probe. Lanes 5-7, aliquots (1/20) of the reaction mixtures corresponding to lanes 1, 3 and 4 were further amplified for 20 cycles; marker, 1 kb DNA scale (Gibco BRL), only positions of the relevant markers (396 and 506 bp) indicated.

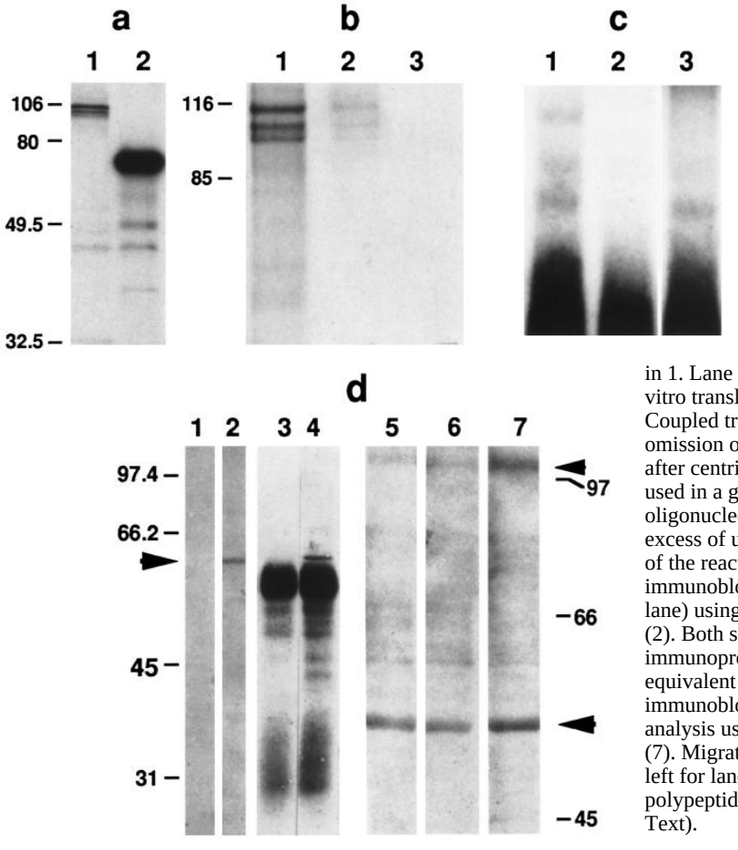


Fig. 3. In vitro translation and immunochemical detection of CDEBP. (a) In vitro translation: CDEBP cDNA was transcribed in vitro using T7 polymerase, and translated in a rabbit reticulocyte lysate (see Materials and Methods; total volume: 50 µl). Lane 1, [^{35}S]methionine-labeled reaction products fractionated by SDS-PAGE were revealed by autoradiography. Lane 2, product of the reaction directed by the luciferase control DNA (supplied by the manufacturer). (b) Lane 1, same experiment as in a, lane 1, except that electrophoresis was run for a longer time to obtain a better resolution of the 90-110 kDa products. Lane 2, the reaction product (5 µl) was immunoprecipitated using PA8 antibodies; complexes with Protein A-Sepharose were centrifuged, redissolved in electrophoresis buffer and analysed by SDS-PAGE as in 1. Lane 3, same as in 2, but preimmune antibodies were used. (c) In vitro translated protein binds an oligonucleotide with the CDEI motif. Coupled transcription translation was performed as in a, except for the omission of radiolabeled methionine. 2 µl (lane 2) of the supernatant after centrifugation of the reaction mixture (2,000 ***g***, 5 minutes) were used in a gel retardation assay with ^{32}P-labeled double-stranded PL11 oligonucleotide; lane 2, same experiment in the presence of a 100-fold excess of unlabeled PL11; lane 3, same experiment with the product of the reaction directed by the control luciferase DNA. (d) Lanes 1-2, immunoblot analysis of Balb/c 3T3 extract (10^5 cell equivalent per lane) using preimmune serum (1) and anti-CDEBP antiserum Ab63 (2). Both sera were used at a 1:200 dilution. Lanes 3-4, protein immunoprecipitated from the same Balb/c 3T3 extract (10^6 cell equivalent per lane) using preimmune (3) and immune PA8 serum (4); immunoblots were revealed with Ab63. Lanes 5-7, immunoblot analysis using affinity-purified antibodies Ab61 (5), Ab63 (6) and PS7 (7). Migration of molecular mass markers (kDa) is indicated on the left for lanes 1-4 and on the right for lanes 5-7. Arrows indicate the polypeptide species which are specifically immunoprecipitated (see Text).

germ agglutinin affinity column chromatography (not shown). The in vitro-synthesized polypeptide was immunoprecipitated by the anti-CDEBP polyclonal antibody PA8 (Fig. 3b), and it was capable of binding the CDEI sequence (Fig. 3c). Western blot analysis performed on nuclear extracts detected mostly polypeptides of a smaller size, with a prominent 58 kDa band

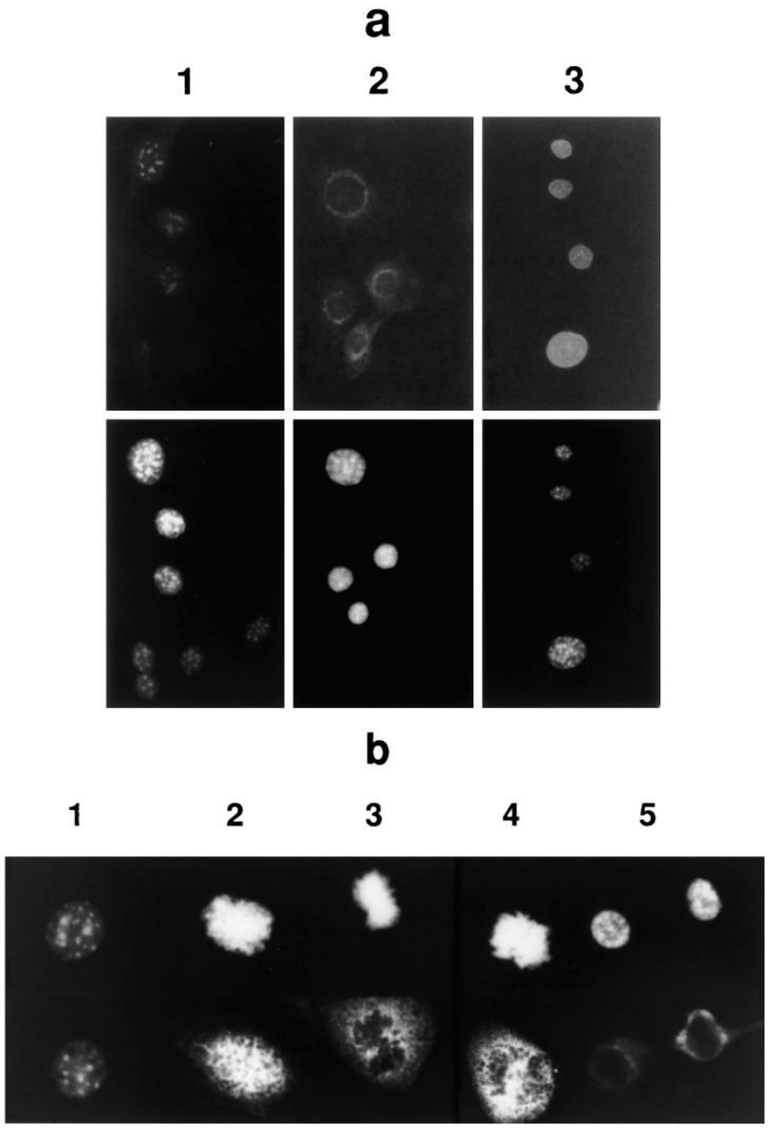


Fig. 4. Cellular localization of CDEBP. (a) Nuclear localization in interphase cells. Top: indirect immunofluorescence staining of Balb/c 3T3 cells using anti-CDEBP PS7 antibodies (1), anti-APP antibody 369 (Buxbaum et al., 1990) (2), anti-lamin antibodies (3). Bottom: staining of DNA with Hoechst 33258. ×500. (b) CDEBP is not associated with mitotic chromosomes. Pam 212 cells were stained with the anti-CDEBP antibody Ab61 (lower panel) and with Hoechst 33258 (upper panel); 1, interphase; 2, prophase; 3, metaphase; 4, anaphase; 5, telophase. ×1,000. The same immunofluorescence patterns (not shown) were generated using PS7 on Pam 212 and Ab61 on Balb/c 3T3 cells.

(Fig. 3d) suggestive of proteolytic cleavage, and variable amounts of material remaining at the same electrophoretic position as the in vitro product (lanes 5-7).

The intracellular distribution of CDEBP was determined by indirect immunofluorescence assays on mouse cells. Antibodies PS7 reacted with a nuclear component in Balb/c 3T3 fibroblasts (Fig. 4a). The antipeptide antibodies Ab61 and Ab63 generated the same picture in Balb/c 3T3 as well as Ab61 (Fig. 4b), PS7 and Ab63 (not shown) in the epithelial cell line Pam 212. The label was concentrated in localized spots in the nucleus. They did not correspond to a cytological entity recognizable at the resolution level of optical microscopy. They appeared to coincide with a fraction of the bright spots stained by Hoechst 33258 dye in mouse nuclei. In the case of Ab61 and Ab63, the specificity of the labeling reaction was confirmed by its extinction by an excess of the corresponding peptide (not shown).

During cellular division, immunofluorescence staining was not associated with the mitotic apparatus (Fig. 4b). At the beginning of prophase, it was progressively excluded from the condensing DNA, and, after nuclear envelope breakdown, it was distributed throughout the cell. During telophase, a preferential localization of staining in the region of nuclear membrane reformation was observed, and, immediately after the end of telophase, all the fluorescence was found in reconstituted nuclei.

By confocal microscopy, the subnuclear structures containing the protein appeared to be distributed within the whole volume of the nucleus (Fig. 5), a localization clearly distinct from the two controls: the lamins, localized under the nuclear membrane, and APP, which was mostly perinuclear, as expected from its known localization in the ER and Golgi apparatus (Zimmermann et al., 1988).

Indirect immunofluorescence staining performed on fertilized mouse eggs did not generate a detectable signal (not shown). This result may suggest that early embryos contain a

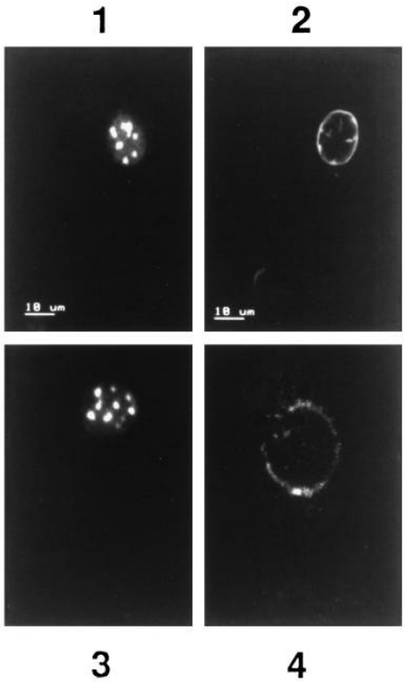


Fig. 5. Intranuclear localization of CDEBP. Immunofluorescence staining of Balb/c 3T3 cells using anti-CDEBP antibodies PS7 (1), anti-lamin antibodies (2), anti-CDEBP antibodies Ab61 (3) and anti-APP monoclonal antibodies (4). Preparations were examined by confocal microscopy, the images correspond to a slice from the middle of the nucleus. ×500.

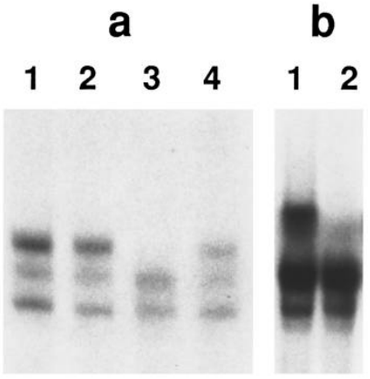


Fig. 6. Effect of anti-CDEBP antibodies on CDEI-binding activities. Gel shift assays with ^{32}P end-labeled double-stranded oligonucleotide PL11 were performed as previously described (Blangy et al., 1991), using 5 μl of extract from Balb/c 3T3 cells (10^7 cells per ml). (a) Lane 1-2, controls using untreated nuclear extract (1) and supernatant after incubation with Protein A-Sepharose alone (2). Lane 3-4, incubation of extract with serum and Protein A-Sepharose followed by centrifugation prior to the addition of the radiolabeled oligonucleotide: PA8 antiserum (3); PA8 preimmune serum (4). (b) Lane 1, cell extract, no antibody added; lane 2, same with 0.1 μg purified PS7 antibodies.

limited pool of the protein. On the other hand, we cannot exclude that this is not due to technical problems (e.g. fixation procedure) specific to fertilized eggs.

One of the three protein-CDEI complexes revealed by gel shift assays contains CDEBP

The ability of the CDEBP protein to bind to the CDEI motif is independently indicated by several lines of evidence. The initial cDNA clone was selected by expression screening and a GST-CDEBP fusion protein was shown to bind a CDEI oligonucleotide in gel retardation assays (Vidal et al., 1992). This is also the case of the polypeptide synthesized in vitro from a complete CDEBP cDNA sequence (Fig. 3c), but these observations do not exclude that other cellular proteins could bind the same motif. To answer this question, we analyzed in more detail the effects of anti-CDEBP antibodies on the binding of CDEI by mouse nuclear proteins. Without antibody treatment, three DNA-protein complexes with different electrophoretic mobilities are detected (Fig. 6). The same assay was then performed after removal of CDEBP by immunoprecipitation with PA8 antibodies. Under these conditions, no immunoreactive protein could be detected in the supernatants by western blot analysis (not shown) and the slow migrating DNA-protein complex was not present. It was also not present when the binding reaction was performed with the complete extract, but in the presence of antibody PS7. The combined data show that, while CDEBP is clearly a member of one of the complexes with the CDEI block, other proteins, still to be identified, bind the same sequence.

Effect of antisense oligonucleotides suggests that CDEBP is required for the correct segmentation of preimplantation mouse embryos

The toxic effect of microinjected DNA molecules that contain an intact CDEI box previously led us to assume that protein-DNA interactions at genomic CDEI site(s) were important during the early development of the mouse embryo (Blangy et al., 1991). It seems clear, however, that proteins other than CDEBP bind CDEI (Fig. 6). To determine whether the latter plays a role during early development, we took advantage of the efficient uptake of oligonucleotides by preimplantation embryos, antisense oligonucleotides thus providing a proven method for at least a partial inhibition of the synthesis of defined proteins (Bevilacqua et al., 1988; Rappollee et al., 1992). After 36 hours of culture in the presence of the antisense oligonucleotides FV1 and FV2, about 90% of the embryos showed a variable number of cells of unequal sizes with a more or less extended cytopathic effect (Fig. 7 and Table 1). These abnormalities were similar to those previously observed after microinjection of oligonucleotides and DNA fragments containing a CDEI site (Blangy et al., 1991). A majority (ca 70%) of the embryos which had been cultivated for the same time in the presence of the sense oligonucleotide FV4 developed into normal looking stage 4-6 morulas. The minority that did not develop properly remained arrested at the one-cell stage and did not show the characteristic unequal divisions observed in the presence of the antisense oligonucleotides. Although it was not possible to directly demonstrate a decrease in content of CDEBP RNA, because of the purely qualitative nature of PCR assays performed on isolated one-cell embryos (data not shown), these results suggest that CDEBP interaction with the CDEI motif plays indeed a role in the early development of the mouse.

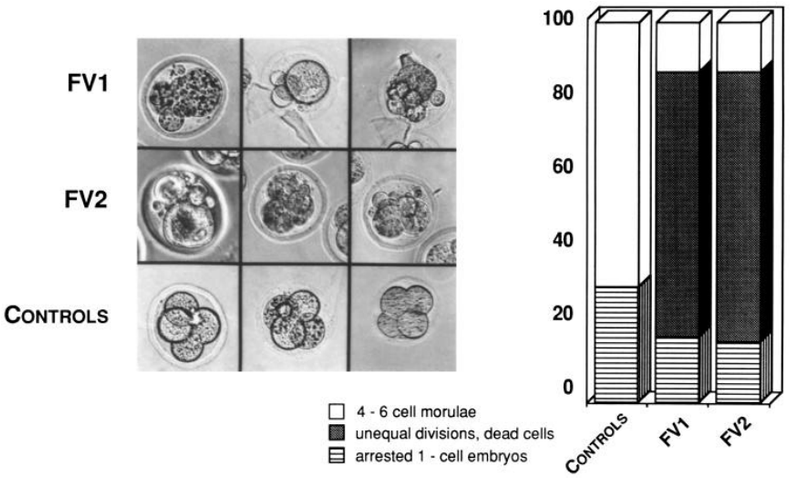


Fig. 7. CDEBP antisense oligonucleotides induce abnormal cleavage and cell death in preimplantation embryos. Fertilized mouse eggs were treated with two antisense oligonucleotides, one of them (FV1) complementary to a sequence in the 3′ part of the mRNA, the other one (FV2), to a sequence at the 5′ end of the coding region (see Materials and Methods). 36 hours later normally developed embryos (4-6 cell stage), embryos arrested at the 1-cell stage, and embryos with gross structural abnormalities and unequal cell division were numbered. Representative pictures are shown in the left panel. The values expressed for each group as percentage of the total number of embryos are shown on the right. The complete results of eight independent experiments are shown in Table 1.

Table 1. CDEBP antisense oligonucleotides inhibit the development of early mouse embryos

		Stage of development after 36 hours in oligonucleotide-containing medium		
Exp.	Oligo-nucleotide	1-Cell	4-Cell normal embryos	Abnormal
1	FV1	1	2	5
	FV2	1	2	5
	FV4	2	6	0
2	FV1	2	0	8
	FV2	1	0	8
	FV4	4	8	0
3	FV2	2	0	8
	FV4	1	5	0
4	FV1	2	0	13
	FV2	3	1	11
	FV4	6	7	0
5	FV2	2	2	6
	FV4	4	11	0
6	FV2	3	3	14
	FV4	3	7	0
7	FV1	4	4	12
	FV4	4	11	0
8	FV1	3	3	9
	FV2	3	3	9
	FV4	4	11	0

Mouse embryos were harvested 15 hours after fertilization and kept for 36 hours in culture medium containing the indicated oligonucleotide as indicated in Materials and Methods. FV1 and FV2 oligonucleotides are complementary to sequences in the most 3′ region and in the 5′ part, respectively, of the CDEBP coding region; FV4 is identical in sequence to the RNA (see Materials and Methods for nucleotide sequences). After 36 hours, embryos with cells of unequal sizes and dead cells were counted (see Fig. 7).

DISCUSSION

We identified a murine protein, termed CDEBP, that binds the CDEI motif GTCACATG. The complete nucleotide sequence of the corresponding cDNA contains an open reading frame with a coding capacity for a protein of 695 amino acids. The predicted sequence shows the same domain similarities with the amyloid β precursor protein (APP) as other recently described members of the 'APP-like' family, namely the mouse APLP (Wasco et al., 1992), human APPH (Sprecher et al., 1993) and *Drosophila* APPL (Rosen et al., 1989) proteins. The published APPH sequence differs essentially from CDEBP by two additional sequences, of 56 and 12 amino acids, inserted at positions that would be in CDEBP between residues 289 and 290, and 538 and 539, respectively. However, variants lacking either one or both of these sequences are also found in human cells (D. Goldgaber, personal communication). The genomic sequence of CDEBP is currently being completed in our laboratory. It shows two candidate alternative exons, of the expected length (168 and 36 nt, respectively) and coding capacity. Preliminary results of RT-PCR analysis indicate that, at least in some cell types, the two alternative exons are present in CDEBP RNAs. Establishing whether the four possible RNA species are produced, and whether different cell types synthesize different variants will, however, require more detailed studies.

All APP-like proteins have in common three conserved domains corresponding, respectively, to the cysteine rich extracellular domain of APP, to a second extracellular portion of the protein, and to its transmembrane and cytoplasmic carboxyterminal region. The high degree of conservation of these sequences in different proteins and species clearly suggests common biochemical function(s). Further speculation is, however, difficult, since there is no indication yet for a function of any of the APP-like proteins. It is of special interest in this respect, however, to note that the cytoplasmic region of APP, to which the C-terminal part of CDEBP shows extended similarity, has been shown to interact in brain with a major GTP-binding protein, G_o (Nishimoto et al., 1993).

The APLP, APPL and APP proteins are found in the endoplasmic reticulum and the Golgi apparatus (Zimmermann et al., 1988; Wasco et al., 1992). In contrast, we observed a nuclear localization of CDEBP. Its most amino-terminal sequence has the potential to function as a signal peptide, so that the polypeptide might initially translocate into the ER. Final localization to the nucleus, rather than to cytoplasmic membranes via the Golgi apparatus, implicates the presence of other signals. Candidate nuclear localization signals can be found in a region rich in basic residues between residues 411 and 480. As previously reported in other instances (Starr and Hanover, 1990), reticulocyte extracts appear to catalyze *O*-glycosylation of the newly synthesized CDEBP polypeptide. It is therefore

likely that the protein is glycosylated in vivo, as it is the case of a number of nuclear proteins (Jackson and Tjian, 1988; Haltiwanger et al., 1992). In cellular extracts, the bulk of immunoreactive material accumulates upon storage as shorter forms, with a prominent 58 kDa band. Whether this proteolytic process corresponds to a site-specific cleavage and might correspond to a possible processing of the protein in vivo remains to be established.

By indirect immunofluorescence assays, the protein appears localized to the interphase nucleus, in dense spots which could not be related to a known nuclear structure. They coincide with part of the spots that are stained in murine cells by Hoechst 33258. The nature of the latter has not been completely elucidated. A fraction of them correspond to the centromeric regions, which, in the mouse, contain AT-rich repeated sequences, but the larger structures that coincide with the immunofluorescence stain, have not, as far as we know, been identified. During cell division, CDEBP is neither associated with the mitotic apparatus, nor with the compacted chromosomes. It is also not detected in the compacted nuclei of post-meiotic male germ cells (P. Abbe and M.R., unpublished results). Association of CDEBP with DNA would thus appear to be characteristic of the expanded structure of the interphase chromatin.

Preimplantation mouse embryos that had received high concentrations of CDEI oligonucleotide arrested their development after a few unequal cellular and nuclear divisions. Although mouse cells contain more than one CDEI-binding protein, the direct involvement of CDEBP was indicated by the occurrence of the same characteristic figures of unequal division after treatment of the embryos with antisense oligonucleotides. Whether or not a requirement for CDEBP is specific of the early embryo, rather than being a more general feature of mouse cells, remains to be established. In transfected cells of established lines, we did not observe any significant toxicity of DNA fragments that contain CDEI sites (F.V., J. Vailly, M. G. Mattei,, P. Léopold, F.C. and M.R., unpublished data). Unlike fertilized eggs which contain a limited stock of protein and RNA, these cells could continuously synthesize CDEBP and thus overcome its inhibition by an excess of binding sites. On the other hand, nuclear abnormalities and unequal divisions occur spontaneously in cultures of established mouse lines at a rate that prevents a significant observation of an additional effect of the transfected sequences.

One hypothesis consistent with all our present data is that the CDEI motif is involved in the stabilization of genomic structures. In addition to the observed effects on the embryonic genome, this is consistent with the fact that CDEI boxes are present in two genetic elements stably maintained as autonomous episomes in mouse cells, the p12B1 plasmid and the genome of BPV1. In both instances, deletion of the CDEI box prevented their autonomous establishment (F.V. et al. and V. Pierrefite et al., unpublished data). However, the CDEBP-CDEI interaction is likely to be only one part of a more complex system. We have seen that proteins other than CDEBP recognize the CDEI sequence (Fig. 6). In addition, both in p12B1 and in BPV1 DNA, the CDEI boxes are included in a complex set of binding sites for distinct proteins, all of them required for episomal maintenance (our unpublished results).

We thank P. Stanley for helpful discussion and advice. We are indebted to D. Goldgaber for the communication of unpublished results, to S. Gandy for the gift of anti-APP antibody, to E. Nigg, for that of anti-lamin antibody. We thank C. Sardet for access to the confocal microscopy equipment. The expert technical assistance of P. Abbe, M. Cutajar and C. Minghelli is gratefully acknowledged. A.B. was an ASC fellow of the Institut National de la Recherche Agronomique (France). This work was made possible by grants from Association pour la Recherche sur le Cancer and Ligue Nationale Française Contre le Cancer.

REFERENCES

Aaronson, S. A. and Todaro, G. J. (1968). Basis for the acquisition of malignant potential by mouse cells cultivated in vitro. *Science* **162,** 1024-1026.

Ashall, F. and Goate, A. M. (1994). Role of the β-amyloid precursor protein in Alzheimer's disease. *Trends Biochem. Sci.* **19,** 42-46.

Bevilacqua, A., Erickson, R. P. and Hieber, V. (1988). Antisense RNA inhibits endogenous gene expression in mouse preimplantation embryos: lack of double-stranded RNA "melting" activity. *Proc. Nat. Acad. Sci. USA* **85,** 831-835.

Blangy, A., Léopold, P., Vidal, F., Rassoulzadegan, M. and Cuzin, F. (1991). Recognition of the CDEI motif GTCACATG by mouse nuclear proteins and interference with the early development of the mouse embryo. *Nucl. Acids Res.* **19,** 7243-7250.

Blangy, A., Carle, G. F., Pierrefite, V., Rassoulzadegan, M. and Cuzin, F. (1992). Mammalian and viral DNA sequences which interfere with the maintenance of a centromeric vector in yeast. *Biochem. Biophys. Res. Commun.* **187,** 737-743.

Buxbaum, J. D., Gandy, S. E., Cicchetti, P., Ehrlich, M., Czernick, A. J., Fracasso, R. P., Ramabhadran, T. V., Unterbeck, A. and Greenberg, P. (1990). Processing of Alzheimer ss/A4 amyloid precursor protein: modulation by agents that modulate protein phosphorylation. *Proc. Nat. Acad. Sci. USA* **87,** 6003-6006.

Clarke, L. and Carbon, J. (1985). The structure and function of yeast centromeres. *Annu. Rev. Genet.* **19,** 29-56.

Davis, G. L., Dibner, M. D. and Battey, J. F. (1986). *Basic Methods in Molecular Biology.* New York: Elsevier.

Haltiwanger, R. S., Blomberg, M. A. and Hart, G. W. (1992). Glycosylation of nuclear and cytoplasmic proteins. *J. Biol. Chem.* **267,** 9005-9013.

Hanes, J., von der Kammer, H., Kristjansson, G. I. and Scheit, K. H. (1993). The complete cDNA coding sequence for the mouse CDEI binding protein. *Biochim. Biophys. Acta.* **1216**, 154-156.

Harlow, E. and Lane, D. (1988). *Antibodies: A Laboratory Manual.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Hogan, B., Costantini, F. and Lacy, L. (1986). *Manipulating the Mouse Embryo: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Jackson, S. P. and Tjian, R. (1988). *O*-glycosylation of eukaryotic transcription factors: implications for mechanisms of transcriptional regulation. *Cell* **55,** 125-133.

Lehner, C. F., Kurer, V., Eppenberger, H. M. and Nigg, E. A. (1986). The nuclear lamin protein family in higher vertebrates. Identification of quantitatively minor lamin proteins by monoclonal antibodies. *J. Biol. Chem.* **261,** 13293-13301.

Léopold, P., Vailly, J., Cuzin, F. and Rassoulzadegan, M. (1987). Germ line maintenance of plasmids in transgenic mice. *Cell* **51,** 885-886.

Nishimoto, I., Okamoto, T., Matsuura, Y., Takahashi, S., Okamoto, T., Murayama, Y. and Ogata, E. (1993). Alzheimer amyloid protein precursor complexes with brain GTP-binding protein G_o. *Nature* **362,** 75-79.

Rappollee, D. A., Sturm, K. S., Behrendtsen, O., Schultz, G. A., Pedersen, R. A. and Werb, Z. (1992). Insulin-like growth factor II acts through an endogenous growth pathway regulated by imprinting in early mouse embryos. *Genes Dev.* **6,** 939-952.

Rosen, D. R., Martin-Morris, L., Luo, L. and White, K. (1989). A *Drosophila* gene encoding a protein resembling the human β-amyloid protein precursor. *Proc. Nat. Acad. Sci. USA* **86,** 2478-2482.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular Cloning: A*

Laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Sheardown, S. A. (1992). A simple method for affinity purification and PCR amplification of poly(A)$^+$ mRNA. *Trends Genet.* **8,** 121.

Sprecher, C. A., Grant, F., Grimm, G., O'Hara, P. J., Norris, F., Norris, K. and Foster, D. C. (1993). Molecular cloning of the cDNA for a human amyloid precursor protein homolog: evidence for a multigene family. *Biochemistry* **32,** 4481-4486.

Starr, C. M. and Hanover, J. A. (1990). Glycosylation of nuclear pore protein p62. Reticulocyte lysate catalyzes *O*-linked *N*-acetylglucosamine addition *in vitro*. *J. Biol. Chem.* **265,** 6868-6873.

Vidal, F., Blangy, A., Rassoulzadegan, M. and Cuzin, F. (1992). A murine sequence-specific DNA binding protein shows extensive local similarities to the amyloid precursor protein. *Biochem. Biophys. Res. Commun.* **189,** 1336-1341.

Wasco, W., Bupp, K., Magendantz, M., Gusella, J. F., Tanzi, R. E. and Solomon, F. (1992). Identification of a mouse brain cDNA that encodes a protein related to the Alzheimer disease-associated amyloid β protein precursor. *Proc. Nat. Acad. Sci. USA* **89,** 10758-10762.

Yan, Y. C., Bai, Y., Wang, L., Miao, S. and Koide, S. S. (1990). Characterization of cDNA encoding a human sperm membrane protein related to A4 amyloid protein. *Proc. Nat. Acad. Sci. USA* **87,** 2405-2408.

Yuspa, S. H., Hamley-Nelson, P., Koehler, B. and Stanley, J. R. (1980). A survey of transformation markers in differentiating epidermal cell lines in culture. *Cancer Res.* **40,** 4694-4703.

Zimmermann, K., Herget, T., Salbaum, J. M., Schubert, W., Hilbich, C., Cramer, M., Masters, C. L., Multhaup, G., Kang, J., Lemaire, H.-G., Beyreuther, K. and Starzinski-Powitz, A. (1988). Localization of the putative precursor of Alzheimer's disease-specific amyloid at nuclear envelopes of adult human muscle. *EMBO J.* **7**, 367-372.

(Received 18 April 1994 - Accepted 12 October 1994)