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APLP2, a member of the Alzheimer precursor protein family, is required for correct genomic segregation in dividing mouse cells

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The mouse amyloid precursor-like protein 2 (APLP2) belongs to the Alzheimer peptide precursor family. A possible role in pre-implantation development had been suggested previously, and was investigated further by creating a large deletion in the genomic locus. While heterozygous mice developed normally, homozygous embryos were arrested before reaching the blastocyst stage. One-cell embryos which contained protein of maternal origin underwent a limited number of cleavages. The progressive disappearance of the protein at stages 4 and beyond correlated with the appearance of extensive cytopathological effects. Nuclear DNA contents of the arrested embryos departed widely from the normal 2–4C value, thus suggesting a role for the protein in replication and/or segregation of the embryonic genome. Embryonic mortality was not due to the untimely initiation of programmed cell death, and it occurred before the stage at which apoptotic cells normally appear. The same abnormal distribution of DNA contents was seen in primary cultures of *Aplp2* +/– embryonic fibroblasts following transfection of an expression vector for *Aplp2* antisense RNA with green fluorescent protein (GFP) expressed from a co-transfected construct. Daughter cells derived from a GFP-positive cell showed abnormal DNA contents both >4C and <2C, thus indicating a role for the protein in the mitotic segregation of the genome and establishment of the proper nuclear structure.

Keywords: ALPLP2 protein/Alzheimer peptide precursor/mitotic segregation/nuclear structure

Introduction

The amyloid precursor-like protein 2 (APLP2) belongs to the Alzheimer peptide precursor (APP) family (Vidal *et al*., 1992; von der Kammer *et al*., 1994a,b; von Koch *et al*., 1995; Yang *et al*., 1996). After the discovery of APP, the precursor of the βA4 peptide accumulated in the brain of Alzheimer patients (reviewed by Selkoe, 1989), a group of related genes was identified, all evolutionarily well-conserved and, therefore, potentially important. The list includes the mouse *Aplp1* (Wasco *et al*., 1992), the rat *Aplp2* (Sandbrink *et al*., 1994a), the human *Aplp2* (also termed *Apph*) (Sprecher *et al*., 1993; Wasco *et al*., 1993), *apl-1* of *Caenorhabditis elegans* (Daigle and Li, 1993) and *appl* in *Drosophila* (Rosen *et al*., 1989), as well as the murine *Aplp2* gene. Their products all share with APP three domains of similarity interspersed with completely divergent regions (Figure 1), and their respective functions have remained a matter of speculation. A functional role in the central nervous system was indicated for APP by the phenotype of homozygous negative mutant mice (Müller *et al*., 1994; Zheng *et al*., 1995). Development was only slightly impaired, but the mutants exhibited behavioural abnormalities. No such evidence has been obtained for the other genes of the family. The situation is made even more complex by the occurrence for each protein of isoforms generated by alternative splices. Four such forms have been described for APLP2, corresponding to the different combinations generated by two alternatively spliced exons (Sandbrink *et al*., 1994b). Some of these isoforms undergo post-translational modifications, in the case of APLP2 by chondroitin sulfate glycosaminoglycan addition (Thinakaran and Sisodia, 1994). A possible function in axogenesis was proposed on the basis of the preferential accumulation of one of the APLP2 isoforms in the olfactory tract of the mouse (Thinakaran *et al*., 1995).

Our previous results led us to suggest a different function for an APP family protein. The same gene which was designated *Aplp2* on the basis of the sequence similarities between APP and the encoded protein (von der Kammer *et al*., 1994b; von Koch *et al*., 1995) initially had been described under the name *Cdebp* as that of a DNA-binding protein which recognizes the sequence [A/G]TCAC[G/A]TG, identical to the CDEI element of the yeast centromere (Vidal *et al*., 1992; Hanes *et al*., 1993). Immunocytochemical analysis localized the protein at discrete spots in the interphase nucleus (Blangy *et al*., 1995), and a series of convergent observations then suggested a role in the replication and/or segregation of genomic DNA. Protein binding to a CDEI motif in the genome of bovine papillomavirus type 1 was found to be required for maximal efficiency of replication of the viral DNA in transfected cells and for its subsequent episomal maintenance in stable transformants (Pierrefite and Cuzin, 1995; Pierrefite *et al*., 1996). A strong inhibitory effect on early development had been observed upon microinjection into fertilized mouse eggs of double-stranded oligonucleotides containing the CDEI sequence, and after treatment of one-cell embryos with antisense oligonucleotides. In both instances, the development was arrested before the blastocyst stage, with the characteristic accumulation of abnormal nuclear structures and DNA contents (Blangy *et al*., 1991, 1995), suggestive of a possible role for the protein in DNA replication and/or segregation.

To investigate further the biological function(s) of the APLP2 protein, we first isolated the entire genomic structure, mapped it to mouse chromosome 9 and elucidated its exon–intron organization (Yang *et al*., 1996). We

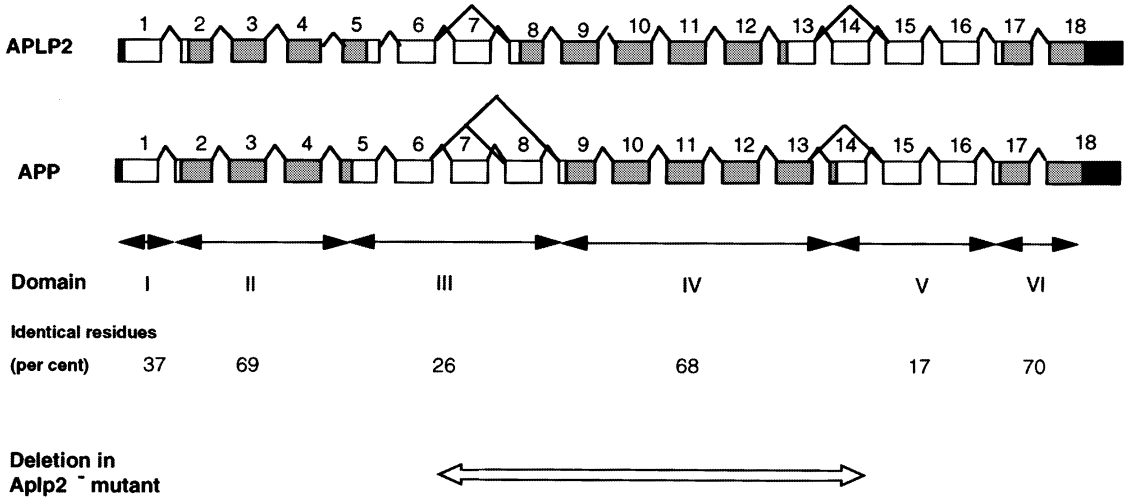

Fig. 1. The three domains of similarity between the APP and APLP2 proteins. Diagram (not to scale) of the respective exon–intron structures of the genomic loci encoding the APP and APLP2 proteins (from Yang *et al.*, 1996). The exons indicated by grey boxes encode protein domains with 68–70% identical residues. No significant similarity is found in the other exons, shown as open boxes. Closed boxes correspond to the non-translated 5′ and 3′ mRNA sequences. The open double-headed arrow shows the position of the deletion in the *Aplp2*$^{-}$ mutant.

then generated an intragenic 11.35 kb deletion, which abolishes the expression of all the known isoforms. No obvious developmental defect was noted in the heterozygotes, which consistently were produced with normal Mendelian ratios. In sharp contrast, homozygotes failed to reach the blastocyst stage.

Results

Generation of the Aplp2-deleted mice

The replacement targeting vector pYY-V8 (Figure 2) was designed to create a null mutation in the *Aplp2* (*Cdebp*) gene. It contains a 3 kb fragment covering exons 5 and 6, with the adjacent and intervening intron sequences linked to a *neo* cassette and to 2.9 kb of sequences from intron 14. The size and location of the expected deletion (11.35 kb corresponding to eight exons and seven introns) were chosen in such a way that none of the isoform mRNAs could possibly be generated from the mutated allele. WW6 embryonic stem (ES) cells were electroporated with linearized pYY-V8 DNA. Positive/negative selection was applied in medium containing both G418 and ganciclovir. A first screen by Southern blot hybridization after *Bam*HI cleavage (see below) detected 18 homologous recombinants among 78 survivors analysed. They exhibited identical genomic structures, with the *neo* cassette inserted between exon 4 on the 5′ side and exon 15 on the 3′ side. Long-distance PCR amplification (primers p44 and p45; Figure 2B) yielded the expected 8.5 kb fragment hybridizing with the *neo* probe. Results of PCR analysis were confirmed by subsequent Southern blot analysis of tail DNA after germline transmission of the mutant allele (see below).

Two clones, designated 8-W54 and 8-W76, were used to generate chimeric mice by blastocyst microinjection. For each clone, mouse colonies were established from the agouti progeny of two different chimeras, by back-crossing heterozygous animals twice onto either BALB/c or B6/D2 genetic backgrounds. The properties of these four families of mice were identical and will not be described separately.

Although a detailed analysis remains to be performed, no obvious developmental or behavioural defect was noted in heterozygous animals. Litters in crosses with wild-type partners were of normal sizes and frequencies. Half of the offspring were heterozygous for the targeted allele, with an equal representation of males and females. Transmission of the targeted allele was checked by Southern blot analysis of tail DNA using two probes, A and B, on both sides of the deletion, and one, C, corresponding to the *neo* sequences (Figure 2). Probe A detected the expected 2.7 kb recombined *Bam*HI fragment, which also hybridized with the *neo* probe (not shown), *Bgl*II generated a fragment of 7.3 kb reacting with probes A and B, *Bcl*I, a 12 kb fragment hybridizing with probes A and B, and *Eco*RI, a fragment of 7.3 kb detected by probe B.

Absence of homozygous mutants in the progeny of heterozygous mating

To find evidence for a possible role for *Aplp2* in development, crosses between heterozygotes were performed to generate homozygous animals. Litters were significantly smaller, yielding an average of seven pups as compared with nine in crosses with wild-type mice, with one-third of the mice homozygous for the wild-type allele, and two-thirds, heterozygous (Table I). The proportion of heterozygous and wild-type and the lack of the homozygous genotypes are consistent with the notion that homozygous embryos die before birth. If this is the case, death must occur at an early embryonic stage, since the same distorted allelic distribution was found among embryos dissected at about mid-gestation (see below).

Development of the Aplp2 –/– homozygous embryos is arrested before the blastocyst stage

Examination of the earliest developmental stages of the *Aplp2*-mutated embryos showed a fraction of morphologically abnormal embryos increasing in number between fertilization and the blastocyst stage, up to values close to the expected Mendelian proportion of homozygotes (Table II). These embryos showed a grossly distorted morphology, with a small number of cells of unequal sizes

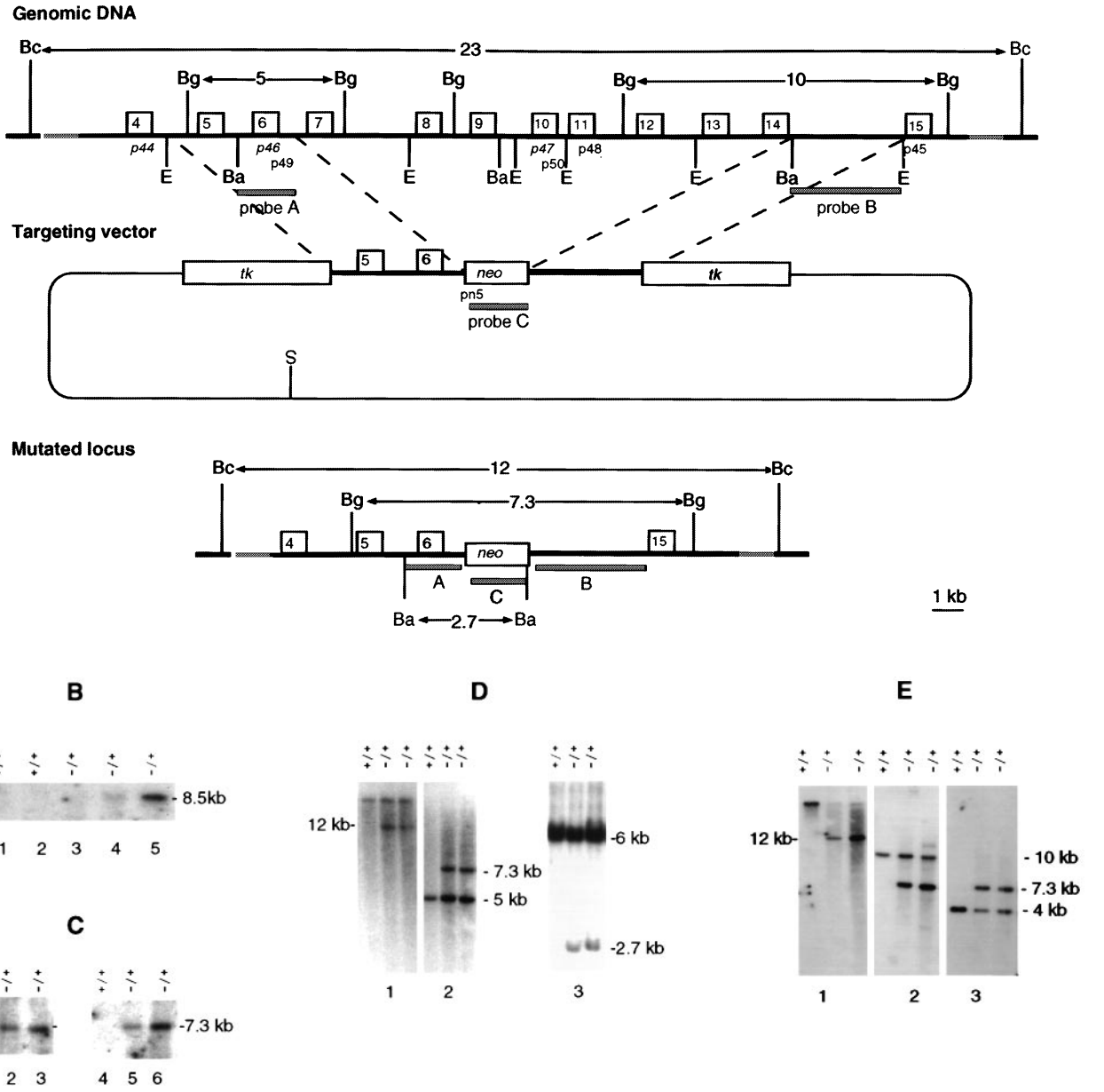

Fig. 2. Homologous recombination at the *Aplp2* (*Cdebp*) locus of the mouse. (**A**) Map of the genomic locus (Yang *et al*., 1996), of the targeting vector pYY-V8 and predicted structure of the targeted locus. Restriction sites: Ba, *Bam*HI; Bc, *Bcl*I; Bg, *Bgl*II; S, *Ssp*I. The targeting vector includes a total of 5.9 kb of sequences homologous to the genomic locus, with 3 kb covering exons 5 and 6 linked to 2.9 kb from the intron between exons 14 and 15. The expected recombinant allele has therefore deleted eight exons and seven introns (11.35 kb). The *neo* and *tk* genes allow positive and negative selection, respectively, of the homologous recombinants. p45–p50: oligonucleotide primers, those with the same 5′–3′ orientation as the mRNA are indicated by italics. The vector was linearized by *Ssp*I cleavage prior to electroporation. (B–E) PCR and Southern blot analysis of wild-type mice (+/+) and of putative heterozygous mutant mice (+/–), initially identified by positive hybridization of tail DNA with *neo* sequences (probe C) in the F1 progeny obtained from chimeric animals. (**B**) Long PCR analysis: hybridization with probe C (*neo* sequences) of the product amplified from primers p44 (exon 4) and p45 (exon 15) in the presence of increasing concentrations of dimethylsulfoxide: 1% (lane 3), 5% (lanes 1 and 4) and 10% (lane 2 and 5). Lanes 1 and 2, wild-type DNA; lanes 3–5, heterozygous mutant DNA. The product expected from the recombined locus is a 8.5 kb fragment hybridizing with probe C. (**C**) Southern blot hybridization with the *neo* probe after cleavage with *Bgl*II (lanes 1–3) and *Eco*RI (lanes 4–6) of a wild-type (lanes 1 and 4) and two putative recombinants (lanes 2, 5, 3 and 6). Both enzymes are expected to generate from the recombined locus *neo*-containing fragments of nearly identical sizes (7.3 kb). (**D**) Southern blot analysis of one of the wild-type and the two putative recombinants identified in (C) using the 5′ probe A, after cleavage with *Bcl*I (1), *Bgl*II (2) and *Bam*HI (3). (**E**) Same as in (D), but hybridization is with the 3′ probe B; cleavage with *Bcl*I (1), *Bgl*II (2) and *Eco*RI (3).

surrounded by the zona pellucida (Figure 3). For 39 arrested embryos analysed at day 3.5, the average number of cells per embryo was 11.4 (± 0.03), as compared with 44.8 (± 2.4) for their normal littermates (nine blastocysts analysed). At that time, where normal embryos had undergone compaction, cells of the abnormal embryos were dissociated readily by incubation in Ca^{2+}-free medium. They did not attach when cultivated in ES cell medium with or without feeders, and after 1 week in culture, most of them had become Trypan Blue-positive.

We then set up a PCR procedure for the genotypic analysis of individual embryos at these early stages. A set

Table I. Litter size and hereditary transmission

Parent genotypes	No. of litters	Total progeny	Litter size	Genotypes[a]		
				+/+	+/−	−/−
+/−×+/+	59	516	8.7 ± 0.12[b]	279	237	0
+/−×+/−	42	292	6.9 ± 0.18	99	193	0

[a]Tail DNA was analysed by Southern blot hybridization after *Bam*HI cleavage (see Figure 2).
[b]Average ± SEM; $P < 0.01$.

Table II. Development of morphological abnormalities among the early embryos from mating of two heterozygous parents

d.p.c.	Developmental stage	No. of litters	No. of embryos	Litter size	Abnormal embryos	Frequency (%)
0.5	1-cell	17	146	8.6	7	4.8
2.5	4- to 8-cell	28	196	7.0	18	9.2
3.5	blastocyst	19	152	8.0	28	18.3

of oligonucleotide primers was chosen in exons 10 and 11 to allow amplification of a fragment of 0.5 kb from the wild-type allele (p47 and p48; Figure 2), and another set (p46 and pn5) in exon 6 and in the *neo* cassette, to generate a 1 kb fragment from the recombined allele. We genotyped a total of 82 embryos (10 litters) from the two-cell to the blastocyst stage (Table III; Figure 3). Homozygous *Aplp2* –/– embryos, characterized by amplification of the 1 kb fragment, but lacking the 0.5 kb fragment from the wild-type allele, were detected with the expected 25% frequency. Embryos had been sorted individually under the microscope before PCR amplification, allowing us to establish that only the morulae with abnormal structures were homozygous for the deletion. Altogether, these results therefore demonstrate that the *Aplp2* gene is required for normal development of the mouse as early as the 4- to 8-cell morula stages.

We next asked whether the limited and irregular number of cleavages accomplished by homozygous mutant embryos reflected the possibility that division may occur in the absence of APLP2 or, alternatively, whether it resulted from the presence in the fertilized egg of protein of maternal origin.

The apparently normal development of the null mutant embryos up to stages 2–4 may be accounted for by the presence of protein of maternal origin

The distribution of the protein throughout the cell volume in pre-implantation blastomeres, both in wild-type and heterozygous embryos (Figure 4A), appears to be similar to that previously observed in mitotic somatic cells (Blangy *et al.*, 1995). Since it is generally admitted that zygotic transcription starts in the mouse embryo at the two-cell stage, the protein present at early times after fertilization is likely to be synthesized from maternal RNA. Consistent with this conjecture is the observation that all the one- and two-cell embryos of litters generated by two heterozygous parents appeared equally labelled by immunofluorescence assay. When they progressed further to the advanced morula stages, a fraction of them showed progressive

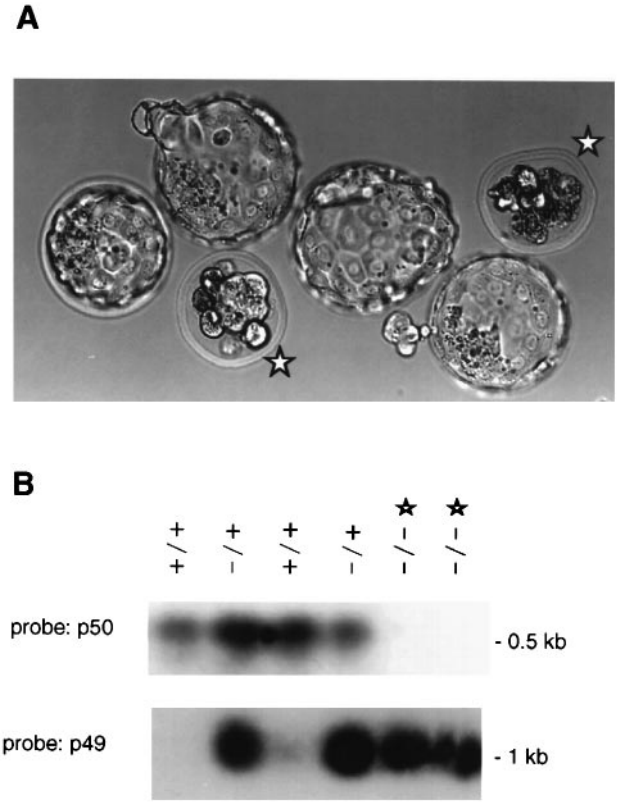

Fig. 3. Death of homozygous *Aplp2* –/– embryos prior to the implantation stage. (**A**) Non-hatched blastocysts from a mating between two heterozygotes recovered by flushing the uterus at day 3.5 p.c. (**B**) PCR amplification was performed on individual embryos for 35 cycles as described in Materials and methods. From primers p47 (exon 10) and p48 (exon 11), a fragment of 0.5 kb hybridizing with oligonucleotide p50 is amplified from the wild-type allele. Primers p46 (exon 6) and pn5 (*neo* cassette) allow amplification from the recombinant allele of a 1 kb fragment hybridizing with oligonucleotide p49. The stars in (A) indicate two embryos with abnormal morphological appearance and in (B), the corresponding amplification products.

Table III. Genotype distribution in the progeny of two heterozygous parents

Genotype[a]	Embryonic day and developmental stage	
	E2.5 (4–8-cell morula)	E9.5 (somite formation)
+/+	10	32
+/−	21	66
−/−	8	0

[a]Embryos recovered at the indicated time (five litters at day 2.5, 14 litters at day 9.5) were genotyped by PCR amplification as described in Figure 3B; results for E9.5 embryos were confirmed by Southern blot hybridization after *Bam*HI cleavage as shown in Figure 2.

weakening of the label and, concomitantly, the first unequal cellular divisions (Figure 4B).

Is the arrest of homozygous embryos due to untimely programmed cell death?

We considered the possibility that the arrest in the development of homozygous embryos could be due to the triggering of programmed cell death (PCD). PCD occurs, mostly by apoptosis, at some defined periods of the development of the vertebrate embryo. Programming of cell death is

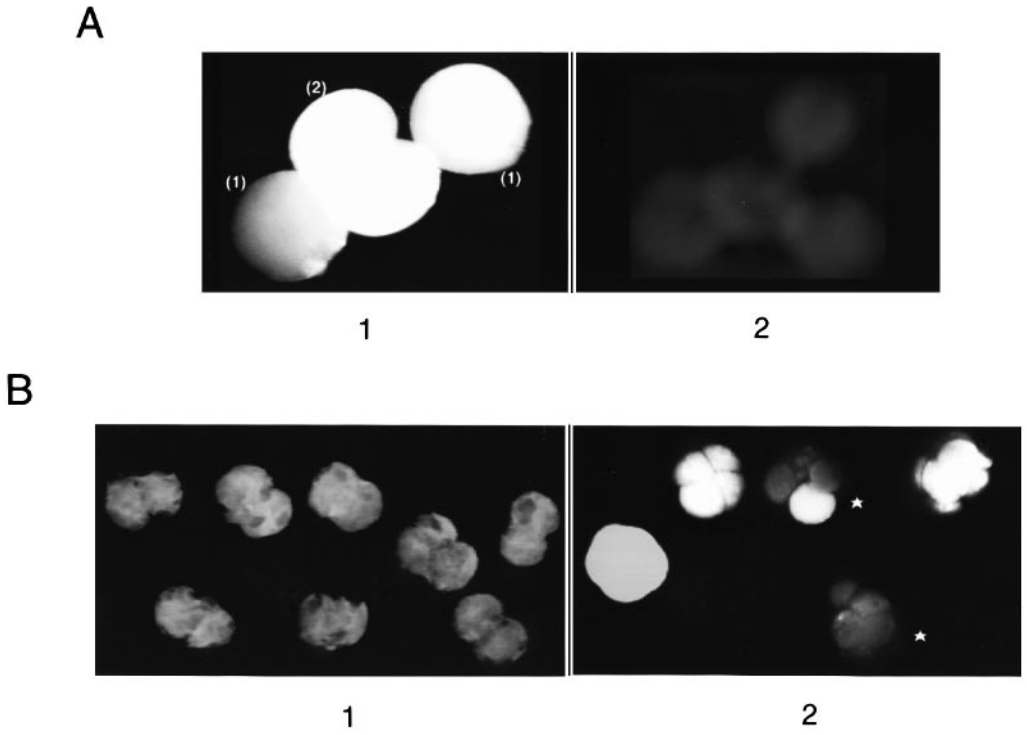

Fig. 4. Distribution of the APLP2 in wild-type and mutant early embryos. Embryos were recovered at 3.5 d.p.c., fixed in 4% paraformaldehyde and treated for immunofluorescence analysis (see Materials and methods) using antibody Ab61 (Blangy *et al*., 1995), which recognizes APLP2 and not APP. (**A**) 1, immunofluorescence localization of the protein in wild-type mouse embryos at stages 1 and 2; 2, control without addition of Ab61. (**B**) Representative litters of embryos from two heterozygous parents. 1, stage 2; 2, morula. The two embryos indicated by stars show both unequal cell divisions and decreased immunofluorescence staining (overexposed photograph to show the residual label in the mutant embryos).

part of the formation of the proamniotic cavity in the implanted embryo (Coucouvanis and Martin, 1995), and the occurrence of cell death has been documented at earlier stages of development (El-Shershaby and Hinchliffe, 1974). Death of the *Aplp2* –/– mice could be interpreted as the untimely initiation of the PCD programme, as was reported in cases of growth factor deprivation during development (reviewed by Coucouvanis and Martin, 1995). We therefore examined homozygous and heterozygous *Aplp2* mutants and wild-type embryos at successive pre-implantation stages (Figure 5) using the TUNEL (TdT-mediated dUTP nick end-labelling) assay for the detection of the endonucleolytic cleavage of nuclear DNA characteristic of apoptosis. During normal development, positive cells were not found before the blastocyst stage. At that stage, some of the trophectoderm cells were found engaged in apoptosis in all blastocysts. Among the embryos generated by two *Aplp2* +/– parents, however, about one-quarter did not show TUNEL-labelled cells. They were the same embryos that showed cytopathic effects. We therefore conclude that the arrest of development of the embryos lacking a functional *Aplp2* gene is not due to an abnormal PCD signal and, moreover, that it is actually effective before the stage of normal development at which apoptosis takes place in the trophectoderm.

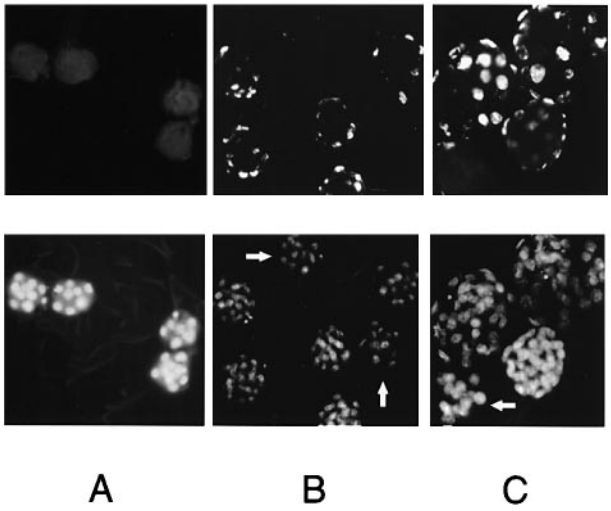

Fig. 5. *Aplp2* –/– embryos do not initiate programmed cell death. Embryos from two heterozygous parents were recovered at 3.5 d.p.c. and fixed in 4% paraformaldehyde. The top row shows TUNEL staining patterns of apoptotic nuclei, the bottom row, Hoechst 33258 staining of the same fields. (**A**) Eight-cell stage; (**B**) early blastocysts; (**C**) fully expanded blastocysts at higher magnification. Arrows point to morphologically abnormal embryos.

Abnormal nuclear DNA contents in the arrested embryos suggest a defect in DNA replication and/or mitotic segregation

Embryos collected at 2.5–3.5 d.p.c. were fixed and stained with Hoechst 33258, and the DNA content of individual nuclei was estimated by *in situ* determination of fluorescence intensity (Figure 6). Normal blastocysts showed the expected complement of DNA, with most values comprised between 2C and 4C (S phase), and a small proportion of G_1 (2C) or G_2 (4C) nuclei. The nuclei of arrested embryos exhibited a much wider distribution, most of them scoring either below the 2C or above the

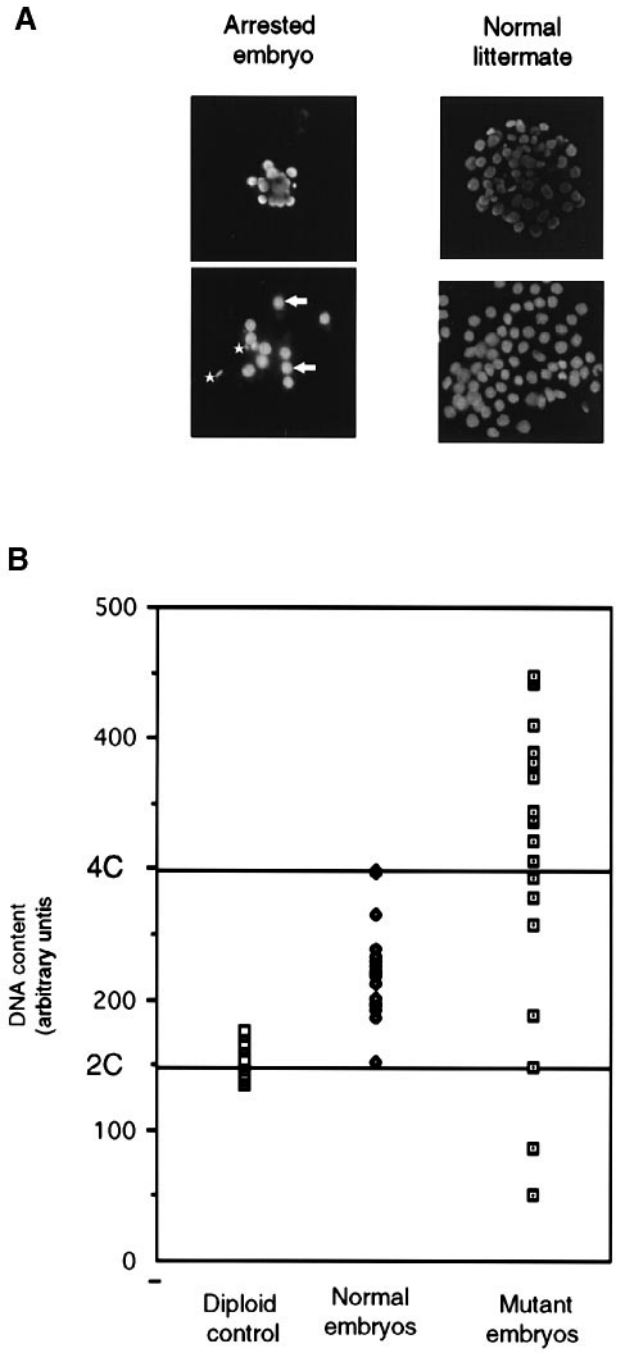

Fig. 6. Unequal distribution of nuclear DNA in arrested *Aplp2* –/– embryos. Blastocysts collected at embryonic day 3.5 in crosses between two heterozygous parents were fixed and stained with Hoechst 33258. (**A**) Arrested embryos (left) and normal littermates (right); upper row, whole embryos; lower row, isolated blastomeres. All pictures were taken with identical exposure times: note the presence in the abnormal embryos of nuclei with either higher (arrows) or lower than normal (stars) fluorescence intensity. (**B**) Nuclear DNA contents were determined by fluorescence intensity reading performed on digitized images of individual nuclei. Nuclei of morphologically altered (homozygous) and normal (heterozygous and wild-type) embryos were measured separately. Data are presented for one of the normal and the two abnormal embryos of the same litter. The same results were obtained on a total of 10 litters from heterozygous parents. Control measurements were performed on G_0-arrested circulating lymphocytes.

4C values. Such an effect of the *Aplp2* deletion is suggestive of a role for one of the gene products in the replication and/or segregation of nuclear DNA.

Segregation analysis in cell cultures

Experiments were designed to determine whether the role of the APLP2 protein was restricted to the pre-implantation embryo. Previous experiments performed on established cell lines had been inconclusive because of the frequent occurrence of abnormal nuclear structures and DNA contents in most immortalized mouse lines (our unpublished results). Further experiments were performed on two cell types which maintain diploid karyotypes in culture, ES cells and primary embryonic fibroblasts. Starting from the initial *Aplp2* +/– targeted ES clones, we first attempted to select homozygous mutants by growth in medium containing elevated concentrations of G418 (400–3000 μg/ml), toxic for heterozygotes with only one *neo*^{r} allele (Mortensen *et al*., 1992). Drug-resistant clones appeared with low frequencies, but out of 85 clones tested, none of them exhibited the Southern blot profile corresponding to the homozygous deletion (data not shown). This result is compatible with a lethal effect of the homozygous deletion in ES cells. However, to obtain a positive confirmation, further experiments were performed by antisense inhibition in diploid primary cultures of *Aplp2* +/– heterozygous fibroblasts from mid-gestation embryos. Starting from the complete litters of two heterozygous parents, pure *Aplp2* +/– cultures were selected in G418 medium. Morphology and growth patterns were indistinguishable from those of control wild-type fibroblasts, as expected from the fully normal phenotype of the heterozygous mice, but we hypothesized that these cells should be sensitive to antisense RNA inhibition. Transfection was performed with a 1:1 mixture of two plasmid constructs. pSVGFP expresses the green fluorescent protein (GFP) under the control of the early simian virus 40 (SV40) promoter, and in pCdebp-pbedc, the *Aplp2* promoter (Yang *et al*., 1996) drives the transcription of the *lac*Z reporter fused to a region complementary to the 5′ 1.1 kb of *Aplp2* mRNA. Controls were performed by replacing this antisense expression vector by the same vector with *lac*Z sequences only. In cultures fixed at 1 day intervals, *in situ* (X-Gal) staining for β-galactosidase activity identified a Lac-positive fraction in cultures transfected with either the antisense or the control constructs amounting to 0.5–1%, the usual range after transfection of primary cultures. GFP fluorescence was seen in a comparable fraction of the cell population. Cultures were then stained with Hoechst 33258 and examined by fluorescence microscopy. As X-Gal staining interferes with the fluorescence of Hoechst 33258–DNA complexes, the GFP label was used to identify transfected cells, and their DNA contents were assessed by cytofluorometry.

Control cultures showed the 2C–4C distribution of DNA contents characteristic of cycling cells, both in the total population and in the GFP-positive fraction after co-transfection of the control plasmid pCdebp-lacZ (Figure 7). In contrast, DNA determination performed on GFP-positive cells in cultures that also received the antisense expression vector pCdebp-pbedc showed a number of cells with abnormal DNA contents. These abnormal nuclei were not seen before 48 h after transfection, the time at which

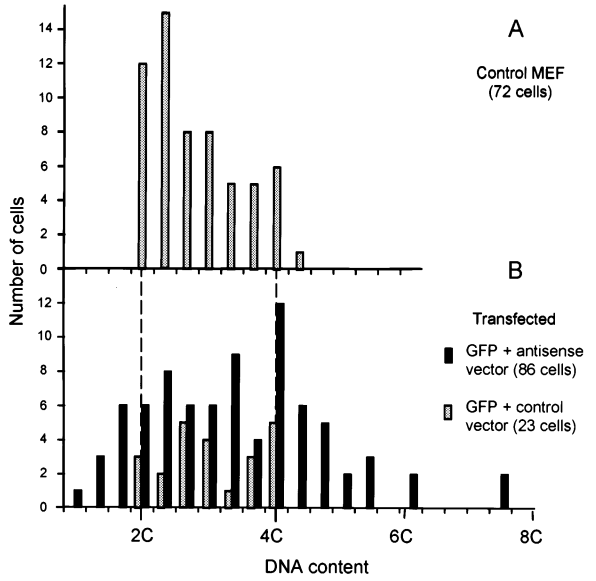

Fig. 7. Abnormal mitotic segregation in *Aplp2* +/– fibroblasts transfected with an *Aplp2* antisense expression vector. Primary cultures of heterozygous embryo fibroblasts were co-transfected with expression vectors for GFP and for antisense *Aplp2* RNA. Controls received the GFP-expressing construct together with a plasmid lacking the antisense transcription unit (see Materials and methods). DNA contents of individual nuclei were estimated 72 h later by fluorescence assay after Hoechst 33258 staining. (**A**) Distribution of DNA contents in the non-transfected (GFP-negative) growing culture shows the expected G_1–S–G_2 distribution with the fluorescence intensities corresponding to the 2C and 4C amounts (G_1 and G_2 phases, respectively) indicated. (**B**) The GFP-positive cells after co-transfection of the control construct (hatched bars) show the same 2C–4C distribution. Cells that received the *Aplp2* antisense expression vector (closed bars) show a much wider distribution of DNA contents. Abscissa values corresponding to 2C–8C were deduced from the histogram in (A).

the transfected cultures had resumed their growth, thus suggesting that their appearance is in some way correlated with cell-cycle event(s). The possibility of an elementary pedigree analysis over the first few cellular generations after transfection was offered by the analysis of GFP staining patterns as a function of time. Whereas until 48 h after transfection, only isolated fluorescent cells were detected, examination of either the antisense or the control cultures at later times (72–96 h) showed small clusters of 2–4 positive cells, mitotic products from one transfected parent cell (Figure 8). One nucleus in each GFP-positive cluster had a >4C DNA content, while one or several nuclei exhibited DNA contents <2C. The segregation of DNA copies and regular partition of the nuclear structure during mitosis thus appear profoundly altered upon interference with APLP2 expression.

Discussion

To elucidate the role of the APLP2, we engineered mutant mice in which the gene is disrupted by a large deletion (11.35 kb). Heterozygous mice were apparently normal, but embryos homozygous for the disrupted locus underwent only a limited number of cleavages, corresponding to the period where they contain protein of maternal origin. From previous experiments performed on early mouse embryos (Blangy *et al*., 1991) as well as on a model viral replicon (Pierrefite and Cuzin, 1995; Pierrefite *et al*., 1996), we had proposed that the protein plays an essential role in the replication and/or maintenance of the genomes. The phenotype of the *Aplp2*-null mutants confirmed an important role for at least one of the protein isoforms in the dividing blastomeres. Experiments performed in primary cell cultures extended this role to one other cell type, the fibroblasts from mid-gestation embryos.

Aplp2 is expressed as a series of four differentially spliced isoforms (Sandbrink *et al*., 1994b): isoform 763 includes exons 7 and 14; 751 contains exon 7 but not 14; 706 contains exon 14 but not 7; and 694 contains neither exon (Thinakaran *et al*., 1995; Yang *et al*., 1996). In the rat, all isoforms were found to be expressed ubiquitously in peripheral tissues as well as in the central nervous system, with, however, variable levels of expression in different tissues. Two of them, isoforms 694 and 751, encode the chondroitin sulfate glycosaminoglycan-modified forms preferentially accumulated in defined regions of the central nervous system (Thinakaran *et al*., 1995). These different proteins with distinct localizations may exert distinct functions in the organism. The mutation that we generated eliminates all the known alternative splicing sites. None of the four isoforms can be produced, necessitating further studies to determine which protein(s) is required for genomic segregation and nuclear partition.

Death of the *Aplp2* –/– embryos does not appear to be due to apoptosis. In the normal embryo, a significant proportion of cells undergo PCD (El-Shershaby and Hinchliffe, 1974; Coucouvanis and Martin, 1995). Under the conditions we used, PCD was only observed after the beginning of trophectoderm and inner cell mass differentiation, thus constituting a convenient marker for an early stage of blastocyst formation. This stage was not reached by the mutants lacking *Aplp2* expression. They also did not undergo compaction, and were not able to attach in culture, thus reinforcing the conclusion that trophectoderm differentiation did not take place. Only the presence of protein of maternal origin could apparently ensure the progression of the homozygous embryos through the first cleavages, and its expression from the zygotic genome thereafter was absolutely required.

Taken together with the site-specific DNA-binding property of the protein (Blangy *et al*., 1991; Vidal *et al*., 1992), these results are indicative of a role either in chromosome replication or segregation (or both). A direct analysis of segregation was undertaken, by fluorescence *in situ* hybridization with chromosome-specific probes. However, it was made difficult by the small number and abnormal structure of nuclei in either arrested embryos or antisense-transfected fibroblasts. Further investigation on the function of the gene in embryonic and adult cells will in fact require its targeted inactivation at specified differentiation stages, by conditional systems such as Cre-dependent deletions (reviewed by Porter, 1998). At the present time, a segregation defect appears as a most likely possibility and this conclusion is reinforced by one additional observation. It was found consistently that the total DNA content of arrested homozygous morulae was close to that expected after four rounds of DNA replication, even though most of the cells showed DNA contents either above or below the normal 2C–4C range (Figure

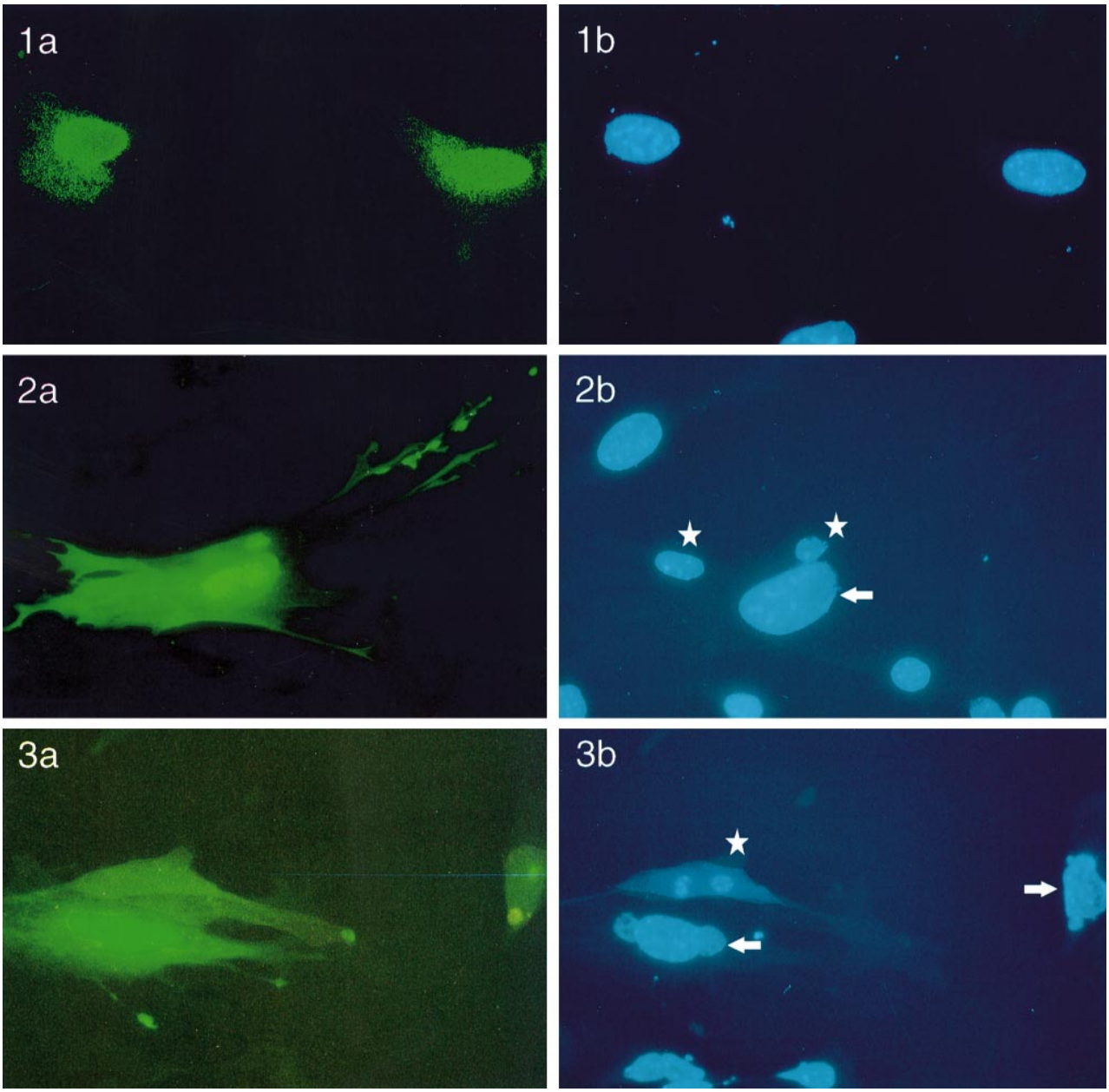

Fig. 8. Abnormal nuclear structure and DNA contents in cells transfected with *Aplp2* antisense expression vector. Same experiment as in Figure 7. 1a,b: control cells transfected with a mixture of plasmid pSVGFP and pCdebp-lacZ DNA. 2a,b and 3a,b: GFP-positive cells after co-transfection of the antisense expression vector pCdebp-pbedc. Daughter cell nuclei show >4C (arrows) and <2C (stars) DNA contents. 1a-3a: GFP protein fluorescence. 1b-3b: Hoechst 33258 fluorescence of the same cells.

6). The same conclusion is also suggested by studies conducted on *Aplp2* +/– heterozygous embryo fibroblasts in which expression of the remaining allele has been inhibited by an antisense transcript. Since the GFP marker allowed us to analyse in these experiments the progeny of individual transfectants, the regular finding of pairs of daughter cells with nuclear DNA contents lower and higher, respectively, than the expected 4C value, was direct evidence for an incorrect mitotic segregation. This conclusion is also consistent with our previous observations that a binding site for the protein in the episomal genome of bovine papillomavirus type 1 is necessary for its transient replication in transfected cells and its establishment in stable transformants (Pierrefite and Cuzin, 1995; Pierrefite *et al*., 1996). Finally, it is also relevant to note that the presenilin proteins recently were suggested to play a role in chromosome segregation (Li *et al*., 1997). These proteins are functionally related to APP, with a possible topological interaction in the cell membrane. One might therefore speculate that they are also functionally connected with APLP2.

Materials and methods

Selection of homologous recombinants

From *Aplp2* (*Cdebp*) C57BL/6 genomic clones (Yang *et al*., 1996), a 3 kb fragment including exons 5 and 6 and a 2.9 kb fragment containing intron 14 were prepared and inserted between *Xba*I, and the *Bam*HI and *Hin*dIII sites, respectively, of vector pSSC-9 (Chauhan and Gottesman, 1992). The resulting construct (Figure 2) has two *tk* genes at both ends of the homologous sequence, and a *neo*^{r} gene replacing a 11.35 kb

region of the *Aplp2* gene that contains eight exons and seven introns. The WW6 ES cell line (Ioffe *et al*., 1995) was cultured on mitomycin C-treated STO feeder cells in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL) containing 10% fetal bovine serum (FBS; HyClone) and 1000 U/ml leukaemia inhibitory factor (Gibco-BRL).

pYY-V8 DNA was linearized by digestion with *Ssp*I prior to electroporation, which was performed on 1×10^8 ES cells with 10–20 μg of linear DNA using the Bio-Rad Gene Pulser apparatus at 400 V and 250 μF. The cells were then plated onto 100 mm plates, and G418 (Gibco-BRL) (200 μg/ml) and 1 μM ganciclovir (Syntex) were applied 48 h later. The double-resistant colonies were picked after 8–10 days.

Screening of recombinants

Genomic DNA prepared from the drug-resistant clones and from mouse tails was tested by Southern blot analysis by standard methods (Sambrook *et al*., 1989) with probes A and B subcloned from genomic DNA (see Figure 2) and a vector probe corresponding to the *neo* coding sequences.

Amplification with the *Taq* polymerase was performed according to the specifications of the manufacturer (Boehringer Mannheim). Long-distance PCR amplification was performed using the Expand™ Long Template PCR System (Boehringer Mannheim) on 1 μg of ES cell DNA. PCR conditions for 4–5 kb DNA fragments: 3 min 94°C, (10 s 94°C, 10 s 52°C, 4 min 68°C) for 10 cycles; (10 s 94°C, 10 s 52°C, 4 min 30 s 68°C) for 25 cycles; and 7 min 68°C. For 7–10 kb DNA fragments: 3 min 94°C, (10 s 94°C, 10 s 52°C, 8 min 68°C) for 10 cycles; (10 s 94°C, 10 s 52°C, 8 min 30 s 68°C) for 25 cycles; and 7 min 68°C.

The position and orientation of the oligonucleotide primers are indicated in Figure 2. Nucleotide sequences are: pn5, 5′-CAAAACCAC-ACTGCTCGAC-3′; p44, 5′-GCACCAGCGCTGGCACACG-3′; p45, 5′-GCACCAATCAGTCCCCCGTC-3′; p46, 5′-CGAGGGCCATTT-CAG-3′; p47, 5′-GAAGCCATGCTGAATGAC-3′; p48, 5′-CTTTGTT-CTCAGCACGGAC-3′; p49, 5′-CATCGTGAACAATCTCCTTGTC-3′; p50, 5′-CGAGGTGGGTCAGACTGCAG-3′.

Generation of mutant mice

ES cells 8-W54 and 8-W76 were injected into blastocysts from MF1 mice and transferred to pseudopregnant females as described (Robertson, 1987). Chimeric males obtained from each ES line were bred with BALB/c and with B6D2 partners, yielding in each case agouti offspring indicative of germline transmission. A total of four mutant families were thus generated, which subsequently were maintained by crossing heterozygous mice.

PCR analysis of isolated embryos

Embryos were recovered at E0.5–E3.5 and individual embryos were placed in PCR tubes. Amplification of genomic DNA was performed by heating at 94°C for 5 min in water, digestion with proteinase K for 3 h at 37°C, heating again at 94°C for 5 min, followed by PCR amplification for 35 cycles (1 min 94°C, 1 min 54°C, 1 min 72°C).

Culture and transfection of embryonic fibroblasts

Cultures were prepared from E12.5 embryos according to published procedures (Seif and Cuzin, 1977). DNA was transfected by the calcium phosphate precipitation procedure. In plasmid pCdebp-lacZ, expression of the *lac*Z gene is driven by a 2.4 kb *Hin*dIII fragment of the upstream region of *Aplp2* with promoter activity (Yang *et al*., 1996). The *Aplp2* fragment was treated with Klenow polymerase to create blunt ends, ligated with *Xho*I linkers and inserted in the *Xho*I site of plasmid pnassβ (Clontech). In plasmid pCdebp-pbedc, a 1.1 kb *Hin*dIII fragment of the *Aplp2* cDNA (nucleotides 125–1233) was inserted in the inverse orientation into the *Bpu*1102I site (nucleotide 3347) of pCdebp-lacZ. Plasmid pSVGFP was constructed by inserting a *Hin*dIII–*Eco*RI fragment from plasmid pEGFP (Clontech), encoding the GFPmut1 variant of the GFP, at the same sites in pECE DNA, downstream of the early SV409 promoter (Ellis *et al*., 1986).

Immunofluorescence staining of eggs and embryos

Embryos were fixed overnight in 4% paraformaldehyde in M2 medium (Hogan *et al*., 1986) at 4°C. They were permeabilized with 0.2% Triton X-100 in M2 for 2 min. After additional washing in phosphate-buffered saline (PBS) and 1 h incubation in 0.2% trypsin at room temperature, they were maintained in M2 + 1% bovine serum albumin (BSA) and 10% FBS. They were incubated with the first antibody for 1 h at room temperature and washed in PBS containing 0.2% Tween-20. Complexes were revealed by a 45 min incubation at room temperature with fluorescein isothiocyanate (FITC)-labelled anti-rabbit IgG antibody (Amersham). Embryos subsequently were washed in PBS and mounted in mounting medium with Hoechst 33258 (Vectashield/H-1200).

In situ detection of apoptotic cells

Apoptotic cells in pre-implantation embryos were identified by the TUNEL technique using the *in situ* Cell Death Detection Kit, Fluorescein (Boehringer Mannheim) following the manufacturer's instructions, on embryos fixed and permeabilized as described above for immunofluorescence assays.

DNA content of individual cells

Embryos were collected at 3.5 d.p.c. The zona pellucida was removed by treatment with acidic tyrode solution (Hogan *et al*., 1986), and the embryos were fixed overnight in 4% formaldehyde in M2 medium. They were incubated individually for 15 min in 20 μl drops of 50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% NP-40, dried, washed with PBS, then stained and mounted in mounting medium with Hoechst 33258 (Vectashield/H-1200). An inverted microscope (Zeiss Axiophot) equipped with a CCD colour camera (Hamamatsu) was used for fluorescence reading. Image analysis was performed using the 'MacBas v2.2' program (Kohshin Graphic System, Inc.).

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