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Developmental rescue of an embryonic-lethal mutation in the retinoblastoma gene in chimeric mice

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The requirement for a functional retinoblastoma gene, *Rb-1*, in murine development around days 12–15 of gestation precludes monitoring the effect of loss of *Rb-1* function on later stages of development and on tumorigenesis in adult mice. Here we describe the developmental rescue of embryonic stem cells carrying two inactive *Rb-1* alleles in chimeric mice. $Rb^{-/-}$ cells contributed substantially to most tissues in adult chimeras, including blood, liver and central nervous system, which were severely affected in pure $Rb^{-/-}$ embryos. The adult chimeric erythroid compartment appeared completely normal, but an increased number of nucleated red cells was observed during fetal liver erythropoiesis in highly chimeric embryos. No ostensive abnormalities were seen in the developing and adult CNS. However, the developing retina of chimeric $Rb^{-/-}$ embryos showed ectopic mitoses and substantial cell degeneration, while the contribution of $Rb^{-/-}$ cells to the adult retina was much reduced. Moreover, the formation of lens fibre cells was severely disturbed. No retinoblastomas developed in any of these mice. Instead, nearly all animals died of pituitary gland tumours which were exclusively derived from $Rb^{-/-}$ cells.

Key words: chimera/eye development/pituitary tumour/ $Rb^{-/-}$ ES cells/retinoblastoma

Introduction

Human retinoblastomas, which can occur as sporadic and hereditary cases, serve as a paradigm for tumorigenesis through loss-of-function mutations (Murphree and Benedict, 1984; Hansen and Cavenee, 1988; Weinberg, 1991). In either form, the loss or mutational inactivation of both alleles of the retinoblastoma gene, *RB-1*, in the developing retina is the rate-limiting step in neoplastic transformation (Knudson, 1971; Friend *et al.*, 1986; Lee,W.H. *et al.*, 1987; Bookstein *et al.*, 1988; Hong *et al.*, 1989). In hereditary cases, one inactive *RB-1* allele is carried in the germline, leading to a high incidence of retinoblastomas (90%) at an early age and osteosarcomas and soft-tissue sarcomas (10%) later in life (Abramson *et al.*, 1984; Friend *et al.*, 1987; Shew *et al.*, 1989). *RB-1* heterozygosity does not predispose to the development of other tumours, but loss of *RB-1* function is frequently found in lung, breast and bladder carcinomas (Harbour *et al.*, 1988; Lee,E.Y.-H.P. *et al.*, 1988; Cance *et al.*, 1990; Horowitz *et al.*, 1990; Xu *et al.*, 1991).

Biochemical analyses and *in vitro* studies have provided a framework to understand the role of the retinoblastoma protein (pRB) in suppressing cellular proliferation (Cobrinik *et al.*, 1992; Hamel *et al.*, 1992). pRB activity is regulated throughout the cell cycle by cyclin-dependent phosphorylation and possibly dephosphorylation (Buchkovich *et al.*, 1989; Chen *et al.*, 1989; DeCaprio *et al.*, 1989; Ludlow *et al.*, 1990). The hypophosphorylated form of pRB, found in the G_0 growth arrest state and the G_1 phase of the cell cycle, binds to a number of proteins, including the transcription factor E2F (Defeo-Jones *et al.*, 1991; Kaelin *et al.*, 1991; Helin *et al.*, 1992; Kaelin *et al.*, 1992; Shan *et al.*, 1992). Binding of E2F to pRB prevents the transcriptional activation of a number of genes required for DNA synthesis, thus blocking entry into S phase (Mudryj *et al.*, 1990). This block is relieved by cyclin-dependent phosphorylation of pRB, leading to dissociation of the pRB−E2F complex (Hinds *et al.*, 1992; Shirodkar *et al.*, 1992). One of the genes carrying an E2F-responsive element in their promoter is c-*myc* (Thalmeier *et al.*, 1989). Down-regulation of c-*myc* was hypothesized to be essential for growth arrest (Freytag, 1988; Waters *et al.*, 1991). Expression of c-*myc* is markedly reduced in cells treated with the growth inhibitor TGF-β (Laiho *et al.*, 1990; Pietenpol *et al.*, 1990). Both growth inhibition and down-regulation of c-*myc* by TGF-β were found to be linked to suppression of phosphorylation of pRB. Thus, pRB may function as the nuclear target of a signalling pathway through which extracellular growth inhibitors can interfere with the cell cycle and direct cells into quiescence. Loss of pRB function, either by genetic inactivation or by association of pRB with the transforming viral proteins E1A, E7 or large T (Whyte *et al.*, 1988; Dyson *et al.*, 1989; Ludlow *et al.*, 1989), which prevents inactivation of E2F by pRB (Bandara and La Thangue, 1991; Chellappan *et al.*, 1991), may result in the inability of cells to respond to growth-inhibiting and/or differentiation-inducing stimuli.

A clue to the normal function of the retinoblastoma gene has come from analysis of the role of the mouse homologue *Rb-1* (Bernards *et al.*, 1989) in murine development. First, *Rb-1* expression was observed in mouse embryos as early as day 9.5 of gestation, with the highest

levels found in brain, spinal cord and liver at 12.5–14.5 days (Bernards *et al.*, 1989). Secondly, the introduction of loss-of-function mutations in *Rb-1* into the mouse germline revealed the requirement for a functional Rb protein in development: homozygous *Rb* mutant embryos die around days 12–15 of gestation and show a number of developmental defects (Clarke *et al.*, 1992; Jacks *et al.*, 1992; Lee,E.Y.-H.P. *et al.*, 1992). Massive cell death, most probably by apoptosis, was seen in the post-mitotic layers of the brain and the spinal cord. In embryos surviving beyond day 14.5, complete degeneration of the spinal ganglia was frequently observed. The fetal liver showed reduced cellularity and, in contrast to wild-type or heterozygous littermates, nucleated erythrocytes were the predominant cell type in the circulation. At this stage of development, the retina appeared normal. In contrast, a highly disorganized structure of the eye lens was observed in homozygous *Rb* mutant embryos around day 14.5, probably as a result of improper fibre cell differentiation (this report). These results indicate that the lack of a functional Rb-1 protein during embryonic development affects the normal differentiation programme of a number of different cell types.

In adult mice, *Rb-1* is expressed in most if not all tissues, with highest levels found in lung, thymus and spleen (Bernards *et al.*, 1989). However, the early embryonic lethality caused by *Rb-1* nullizygosity precludes monitoring of the role of *Rb-1* at later stages of development and restricts the post-natal analysis of the effect of loss-of-function mutations in *Rb-1* on tumorigenesis. We therefore investigated the developmental and tumorigenic potential of embryonic stem cells carrying two inactive *Rb-1* alleles in chimeric mice where embryonic lethality was prevented by the wild-type fusion partner.

Results

Generation of chimeric $Rb^{-/-}$ mice

ES cells carrying a disruption in exon 19 of both copies of the retinoblastoma gene, *Rb-1*, were obtained by two rounds of homologous recombination using the previously described isogenic targeting vectors 129Rb-hyg and 129Rb-neo respectively (Te Riele *et al.*, 1992). The targeted alleles, designated $Rb\text{-}1^{t19hyg}$ and $Rb\text{-}1^{t19neo}$, can be considered as null alleles (Clarke *et al.*, 1992) and therefore the genotype of these cells will be indicated by $Rb^{-/-}$ throughout the text. Three independent $Rb^{-/-}$ ES cell clones were injected into C57BL/6 blastocysts. Notwithstanding the fact that nullizygosity of *Rb-1*, obtained by intercrossing of $Rb^{+/-}$ mice, led to early embryonic lethality, chimeric $Rb^{-/-}$ animals were readily obtained. Apparently, embryonic lethality was prevented by cells of the wild-type fusion partner. Based on coat colour, 41 chimeras were obtained from $Rb^{-/-}$ ES cell clones dRb12 ($n = 23$), dRb14 ($n = 11$) and dRb47 ($n = 7$).

In the second round of the targeting experiment, ES cell clones were obtained in which the previously disrupted *Rb-1* allele had been targeted again, leaving the wild-type allele intact. With one of these $Rb^{+/-}$ clones (sRb33) 39 chimeric $Rb^{+/-}$ animals were generated. These were used as control mice to distinguish phenotypic effects that might have resulted from the inadvertent introduction of a mutation into the ES cell clones during manipulation from those specific for the absence of a functional Rb protein.

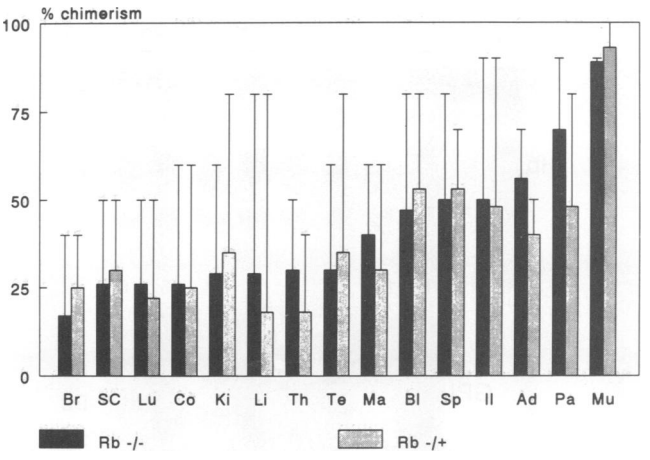

Fig. 1. Mean ES cell contribution in adult chimeric $Rb^{-/-}$ and $Rb^{+/-}$ mice. The ES cell contribution to various tissues of individual chimeras was determined by GPI analysis. The diagram represents the mean contribution to each tissue of $Rb^{-/-}$ ES cells (black columns) and $Rb^{+/-}$ ES cells (hatched columns). The horizontal bar indicates for each tissue the maximum percentage of chimerism observed in both types of chimeric mice. Br, brain; SC, spinal cord; Lu, lung; Co, colon; Ki, kidney; Li, liver; Th, thymus; Te, testis; Ma, mammary gland; Bl, blood; Sp, spleen; Il, ileum; Ad, adrenal gland; Pa, pancreas; Mu, skeletal muscle.

Tissue contribution of $Rb^{-/-}$ cells

The ES cell contribution to the different organs was estimated by the ratio of ES cell-specific versus blastocyst-specific glucose phosphate isomerase (GPI) isozymes in tissues of 23 chimeric $Rb^{-/-}$ and six chimeric $Rb^{+/-}$ mice. The mean contributions of ES cells to various tissues in chimeric $Rb^{-/-}$ mice and chimeric $Rb^{+/-}$ mice are depicted in Figure 1. Clearly, $Rb^{-/-}$ cells contributed as effectively as $Rb^{+/-}$ cells to most tissues. These included blood, liver, brain and spinal cord, tissues found to be severely affected in $Rb^{-/-}$ embryos. Although in brain the mean ES cell contribution in $Rb^{-/-}$ chimeras seemed somewhat lower than in $Rb^{+/-}$ chimeras, the maximum values that were observed did not differ. Also, in lung, thymus and spleen, tissues where *Rb-1* was found to be highly expressed in adult wild-type animals, no selection was observed against a contribution of $Rb^{-/-}$ cells. A slight difference in contribution in favour of $Rb^{-/-}$ cells was seen in the adrenal and pancreas. The extremely high contribution of both $Rb^{-/-}$ cells and $Rb^{+/-}$ cells to skeletal muscle was not related to nulli- or hemizygosity of *Rb-1*, but was intrinsic to the E14 ES cell line.

Thus, $Rb^{-/-}$ cells contributed substantially to most tissues in adult chimeras, allowing analysis of the phenotypic consequences of the mutation at later stages of development. To complete this study, 38 chimeric $Rb^{-/-}$ embryos ranging in age from days 14.5 to 18.5 of gestation were included in the analyses.

Central nervous system

Contrary to the massive degeneration of the developing central nervous system around day 14.5 in pure $Rb^{-/-}$ embryos, chimeric $Rb^{-/-}$ embryos from days 14.5 to 18.5, with ES cell contributions of up to 80% to the retinal

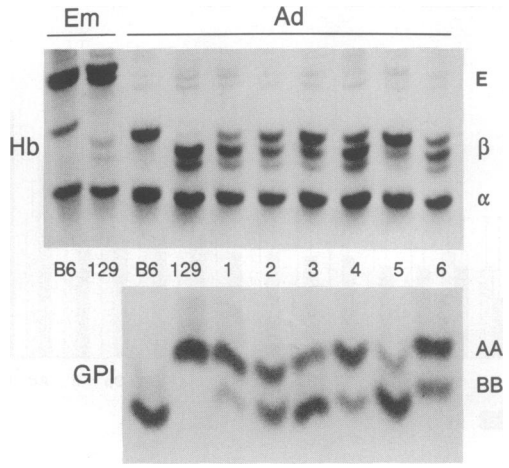

Fig. 2. $Rb^{-/-}$ ES cell-derived contribution to the blood of adult chimeric mice. (Hb) Globin chains present in the blood of six adult $Rb^{-/-}$ chimeras (lanes Ad, 1–6) were separated by Triton–acid–urea gel electrophoresis. The positions of embryonic globin chains (E) and adult β-globin chains (β) of 129 and C57BL/6 were determined by electrophoresis of blood from day 11.5 embryos (lanes Em, B6 and 129) and adult mice (lanes Ad, B6 and 129) respectively. α indicates the position of α-globin. (GPI) GPI analysis of the blood of the same six $Rb^{-/-}$ chimeras (lanes Ad, 1–6).

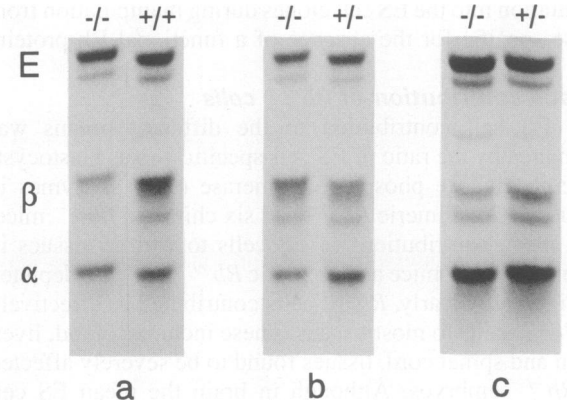

Fig. 3. Normal haemoglobin chain switching in homozygous $Rb^{-/-}$ embryos. The globin content of blood from day 13.5 embryos obtained by intercrossing $Rb^{+/-}$ heterozygotes was determined by Triton–acid–urea gel electrophoresis. Of three independent crosses a, b and c, the globin patterns of Rb-deficient (lanes −/−) and Rb-proficient (lanes +/+ or +/−) littermates are shown. E, α and β indicate the positions of embryonic, α-globin and β-globin respectively. Variations in the migration pattern of the β-globin chains in a, b and c result from variations in the genetic background of the three crosses.

pigment epithelium and the tail, showed no abnormalities in the developing brain and spinal cord. The contribution of $Rb^{-/-}$ cells to the adult central nervous system also was not accompanied by obvious histological abnormalities. In spinal ganglia, however, which completely degenerated in pure $Rb^{-/-}$ embryos, a slightly increased rate of cell death was observed in chimeric $Rb^{-/-}$ embryos (result not shown).

Erythropoiesis

$Rb^{-/-}$ cells contributed significantly to the erythroid compartment in adult chimeric $Rb^{-/-}$ mice (Figure 1). Blood smears from these animals showed normal numbers

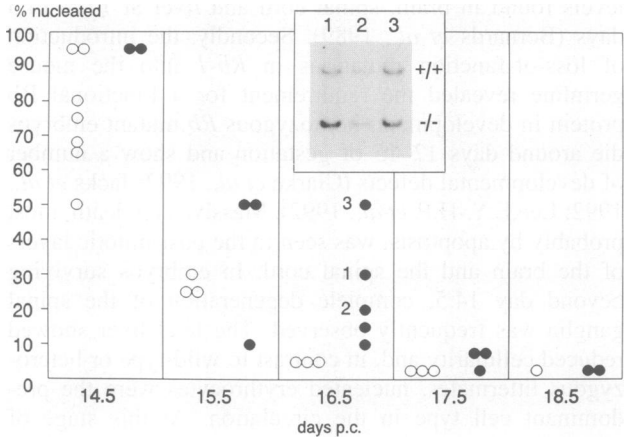

Fig. 4. Nucleated red blood cells in chimeric $Rb^{-/-}$ embryos. The percentage of nucleated red blood cells in the periphery of low-chimeric (○, <25%) and high-chimeric (●, >25%) $Rb^{-/-}$ embryos of days 14.5–18.5 was judged from histological sections. The extent of chimerism was derived from the relative amounts of the strain-specific GPIs and adult β-globin chains in blood samples of each embryo. (**Insert**) DNA extracted from the blood of day 16.5 embryos 1, 2 and 3 was digested with *Eco*RI and analysed by Southern hybridization. +/+ indicates the non-modified *Rb-1* restriction fragment present in wild-type cells; −/− indicates the disrupted *Rb-1* restriction fragment present in $Rb^{-/-}$ cells.

of mature, enucleated red cells (result not shown). To determine whether erythrocytes derived from the $Rb^{-/-}$ fusion partner synthesized normal amounts of adult haemoglobins, we took advantage of the fact that the adult β-globin chains of 129/OLA (ES cells) and C57BL/6 (blastocysts) cells migrate differently in Triton–acid–urea gels (Figure 2, Hb). Both types of β-globin chain were present in the blood of adult chimeras (Figure 2, Hb). Moreover, the extent of chimerism as measured by the relative intensity of the strain-specific β-globin bands correlated well with that of the strain-specific GPI bands (Figure 2, GPI). These results demonstrate that during adult life, when spleen and bone marrow are the major sites of haematopoiesis, $Rb^{-/-}$ cells can normally take part in erythropoiesis. Apparently, the increase in the number of nucleated red cells due to nullizygosity of *Rb-1* occurs only prenatally.

We next investigated whether the shift from yolk sac erythropoiesis (nucleated cells with embryonic globin chains) to liver erythropoiesis (enucleated cells with adult globin chains) occurred normally in pure $Rb^{-/-}$ embryos. Figure 3 shows that pure $Rb^{-/-}$ embryos synthesized similar levels of adult β-globin chains to their wild-type and hemizygous littermates. Apparently, erythropoiesis in pure $Rb^{-/-}$ embryos is not defective in switching to the synthesis of adult haemoglobin chains, suggesting that in these embryos nucleated red cells were produced in the liver, rather than resulting from continued yolk sac erythropoiesis.

We then asked whether an elevated level of nucleated red cells was also present in chimeric $Rb^{-/-}$ embryos and, if so, whether this was correlated with the extent of chimerism in these embryos and with their developmental stage. In the blood of days 14.5–18.5 chimeric $Rb^{-/-}$ embryos, the relative amounts of the strain-specific GPIs correlated well with those of the strain-specific adult β-globins (result not shown). This again indicates that

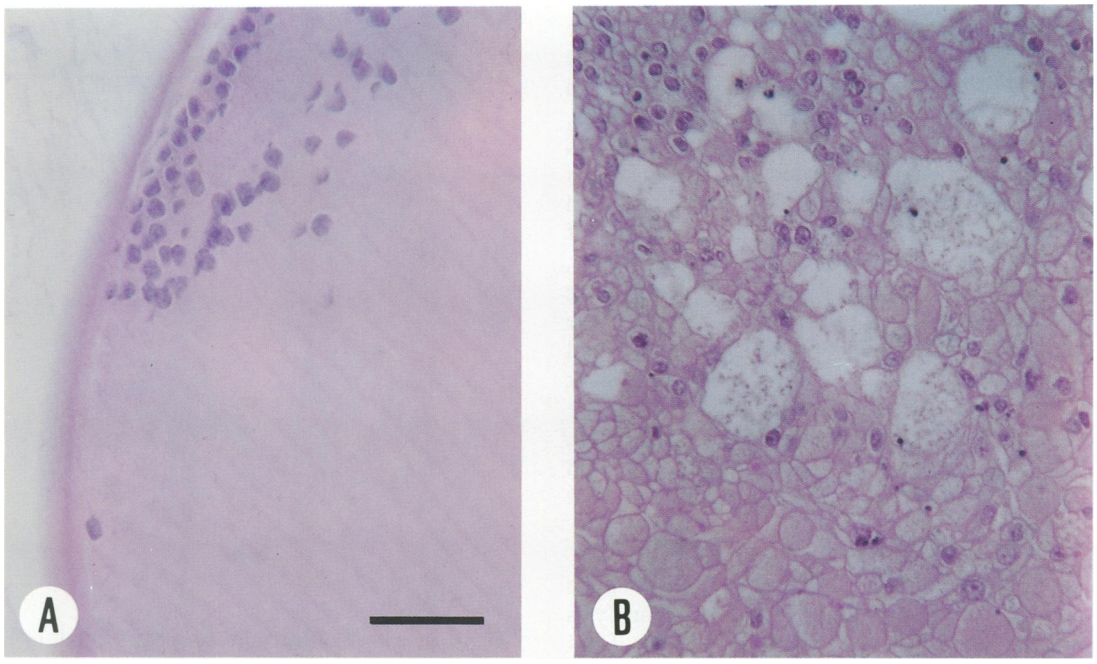

Fig. 5. Aberrant lens formation in chimeric $Rb^{-/-}$ mice. Histological sections through the eyes of chimeric $Rb^{+/-}$ (**A**) and $Rb^{-/-}$ (**B**) mice at 3 weeks of age were stained with haematoxylin–eosin. Scale bar: 50 μm.

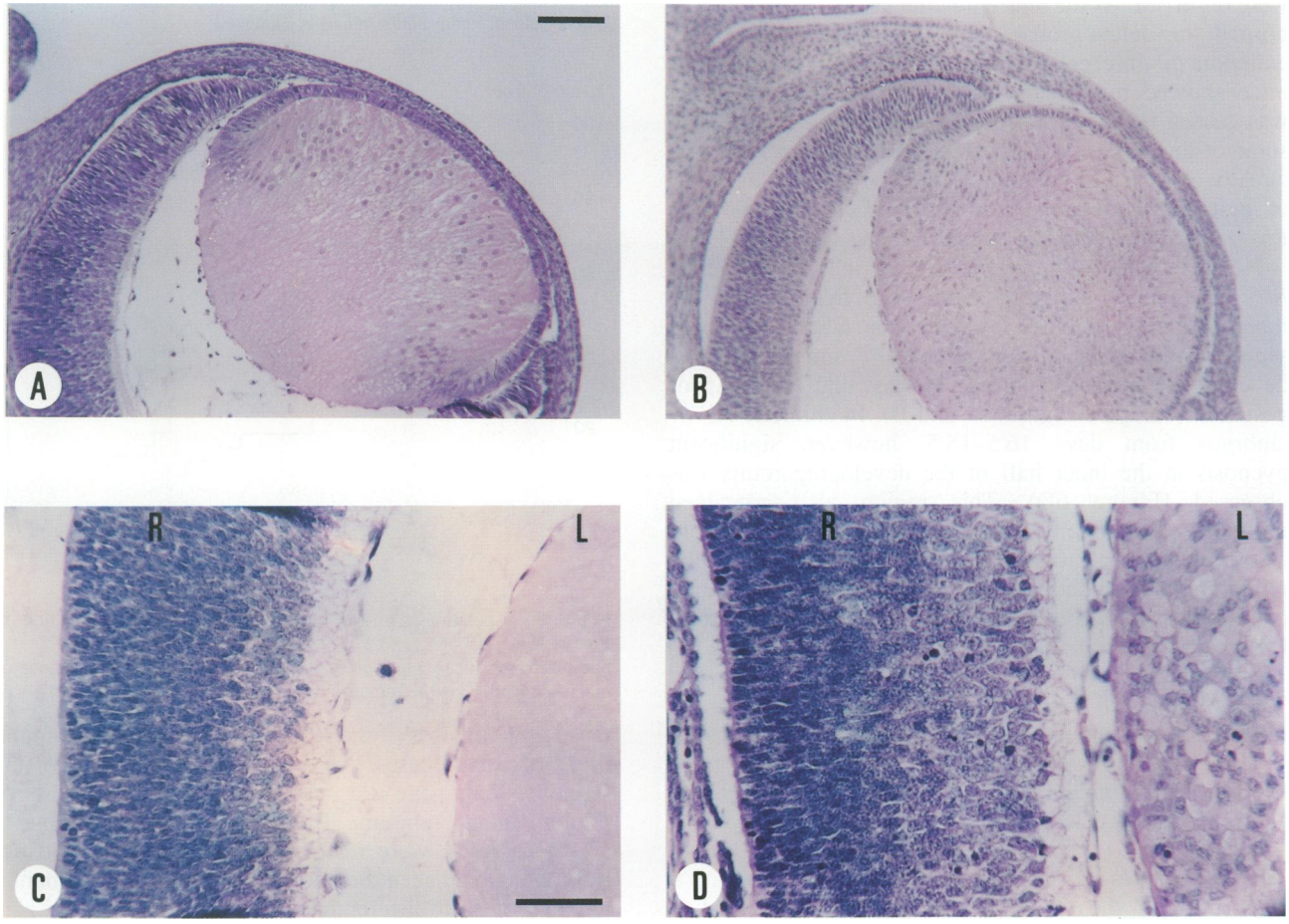

Fig. 6. Aberrant eye development in pure $Rb^{-/-}$ and chimeric $Rb^{-/-}$ embryos. (**A** and **B**) Transverse histological sections through the eyes of day 14.5 $Rb^{+/-}$ (A) and $Rb^{-/-}$ (B) embryos stained with haematoxylin–eosin. (**C** and **D**) Sagittal histological sections through retina and lens of day 16.5 chimeric $Rb^{+/-}$ (C) and $Rb^{-/-}$ (D) embryos stained with haematoxylin–eosin. R, retina; L, lens. Scale bars: (A and B) 100 μm; (C and D) 50 μm.

$Rb^{-/-}$-derived erythrocytes had normally shifted to the synthesis of adult haemoglobins. Figure 4 shows that the percentage of nucleated red cells gradually decreased from 95% in day 14.5 embryos to <5% in day 18.5 embryos. At days 15.5 and 16.5 of development, however, a relatively high percentage of nucleated red cells was seen in the highly chimeric embryos. This correlation had disappeared at days 17.5 and 18.5. Southern analysis of DNA, extracted from the blood of 16.5 day embryos, showed that both *Rb-1* wild-type and $Rb^{-/-}$ cells were present in the nucleated fraction (Figure 4, insert). These results indicate that nullizygosity of *Rb-1* leads to abnormal production of nucleated red cells only during fetal liver erythropoiesis. Moreover, as *Rb-1* wild-type cells are present in the nucleated fraction of chimeric fetal blood, this defect does not seem to reside within the erythroid compartment in a cell-autonomous fashion.

Eye

Macroscopically, 28 out of 41 adult chimeric $Rb^{-/-}$ mice showed cataracts of the eye lens. Microscopic analysis revealed a highly abnormal lens structure in most, if not all, chimeras (Figure 5B): the characteristic regular fibre pattern of the normal lens (Figure 5A) was completely absent. Instead, the lens cavity was filled with large nucleated polygonal cells and, occasionally, mitotic figures were observed. As the lens epithelial layer was completely normal, this defect probably resulted from aberrant differentiation of fibre cells. None of the 39 chimeric $Rb^{+/-}$ mice showed this phenotype, indicating that the effect was caused by the absence of a functional Rb protein in some of the lens cells. Abnormal fibre cell formation was already observed in both pure $Rb^{-/-}$ embryos at day 14.5 of gestation (compare Figure 6A and B) and chimeric $Rb^{-/-}$ embryos (compare Figure 6C and D).

No retinoblastomas were observed in any of the 41 $Rb^{-/-}$ chimeras, although some local dysplasia of the inner and outer nuclear layers of the adult retina was seen. In both pure and chimeric $Rb^{-/-}$ embryos, retina development appeared normal, at least until day 14.5 of gestation (compare Figure 6A and B). In chimeric $Rb^{-/-}$ embryos from days 16.5–18.5, however, significant pycnosis in the inner half of the developing retina was observed (Figure 6D). This region also contained mitotically active cells, which are normally only present at the outer border of the nuclear layer adjacent to the retinal pigment epithelium. In adult chimeras, the relative contribution to the retina of $Rb^{-/-}$ cells was significantly lower ($P = 0.006$) than that of $Rb^{+/-}$ cells (Figure 7). The absolute contribution of $Rb^{-/-}$ cells never exceeded 15%, whereas $Rb^{+/-}$ cells contributed up to 50% to the retina (data not shown).

These results suggest that $Rb^{-/-}$ cells were excluded from the retinal layers at a specific stage of development and replaced by wild-type cells.

Tumorigenesis

Over 90% of the chimeric $Rb^{-/-}$ mice succumbed at between 3 and 11 months of age to tumours localized in the pituitary gland (Figure 8). GPI or DNA analyses of 11 tumours showed that they were all of ES cell origin. Pituitary tumours also developed in pure $Rb^{+/-}$ animals after a 3–5 month longer latency period (Figure 8). In these tumours, loss of the *Rb-1* wild-type allele was found in 17 out of 18 cases. Apparently, functional loss of the retinoblastoma gene is a rate-limiting step in the onset of pituitary gland tumours. The tumours, although large in size (4–5 mm), were relatively benign, as they did not infiltrate into adjacent brain tissue. Most displayed characteristics of well-differentiated melanotrophs of the pars intermedia, as judged by the following. First, sections of paraffin-embedded tumours were immunostained with antibodies against the pituitary gland hormones α-MSH and β-endorphin, mainly produced by cells of the pars intermedia, and FSH/LH, exclusively produced by cells

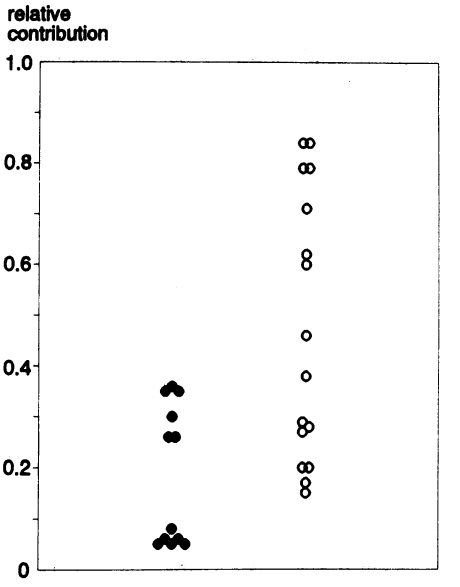

Fig. 7. Strongly reduced contribution of $Rb^{-/-}$ cells to the retina of adult chimeric mice. The ES cell contribution to both retinas and 10 other defined tissues of six $Rb^{-/-}$ (●) and eight $Rb^{+/-}$ (○) chimeras was determined by GPI analysis. The relative contribution, defined as the ratio between the contribution to each retina, precisely determined by dilution series, and the mean contribution to 10 other tissues of the corresponding chimera (brain, spinal cord, lung, kidney, liver, thymus, blood, spleen, ileum and adrenal) is indicated.

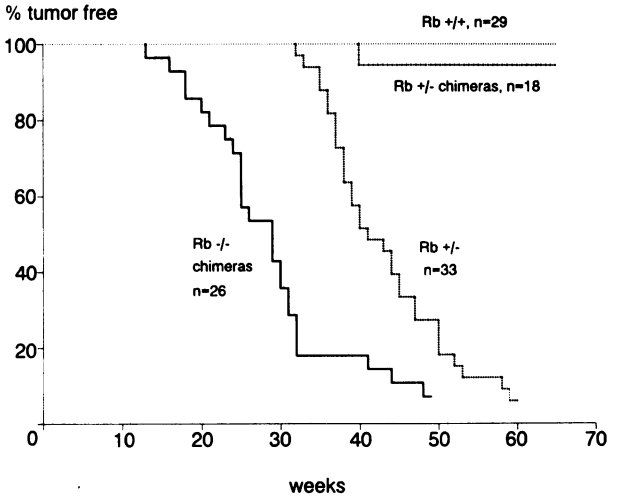

Fig. 8. Predisposition to pituitary gland tumours by loss-of-function of *Rb-1*. Chimeric $Rb^{-/-}$ and $Rb^{+/-}$ mice and pure $Rb^{+/-}$ and $Rb^{+/+}$ mice were sacrificed when moribund and examined for the presence of macroscopically visible tumours in the pituitary gland.

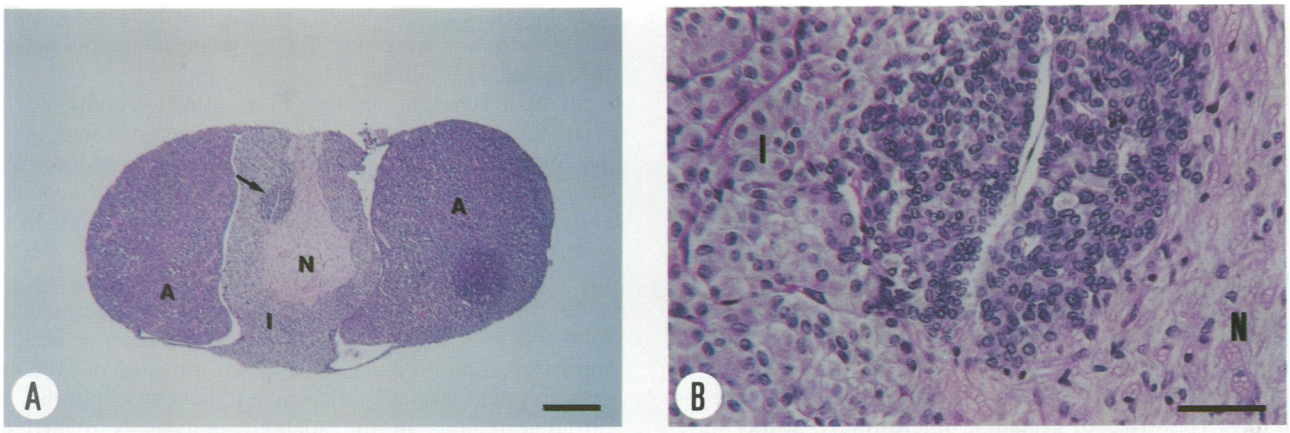

Fig. 9. Pituitary tumours originating from the intermediate lobe. *In situ* tumorigenesis in the pituitary gland of a $Rb^{+/-}$ mouse of 4 months of age at low (**A**) and high (**B**) magnification. Sections were stained with haematoxylin–eosin. A, pars anterior; I, pars intermedia; N, pars neuralis. The arrow in A indicates a neoplastic focus in the pars intermedia. Scale bars: (A) 300 μm; (B) 50 μm.

of the pars anterior. Nearly all tumours of chimeric $Rb^{-/-}$ mice (five out of six) and pure $Rb^{+/-}$ mice (four out of four) stained positively for α-MSH and β-endorphin, whereas none of the tumours were positive for FSH/LSH (results not shown). Secondly, electron microscopic analysis showed that five out of seven tumours consisted of cells indistinguishable from pars intermedia melanotrophs (Tanaka *et al.*, 1991). These cells were characterized by a well-developed Golgi apparatus with small electron-dense haloed granules and, dispersed in the cytoplasm, many larger secretory granules of variable electron density (result not shown). Thirdly, histological analysis of a macroscopically normal pituitary gland from a heterozygous $Rb^{+/-}$ mouse, sacrificed at 5 months of age, revealed a neoplastic focus of rather well-differentiated cells within the intermediate lobe (Figure 9A and B). Taken together, these results indicate that the majority of tumours originated from the intermediate lobe of the pituitary gland. Of three tumours (of the 15 that were analysed), the cell type remained unidentified, which may indicate that these cells were either poorly differentiated or of different origin.

Pre-neoplastic lesions were observed in the medulla of the adrenal gland of six out of nine chimeric $Rb^{-/-}$ mice, which had succumbed at between 4 and 7 months of age. These lesions, which were often multifocal, were not observed in the adrenal of four chimeric $Rb^{+/-}$ animals of the same age.

Discussion

Loss of function of the retinoblastoma gene, *RB-1*, was frequently found in human tumours. In view of the high expression levels of the murine homologue, *Rb-1*, in adult animals, the role attributed to *Rb-1* was to suppress neoplastic outgrowth in many different tissues. In addition, the early embryonic expression of *Rb-1* in the central nervous system and the liver pointed to a functional role for pRb in the development of these tissues. Indeed, *Rb-1* nullizygous embryos showed severe defects in neurogenesis and fetal liver erythropoiesis and died at days 12–15 of gestation.

Our results show that pRb-deficient cells can be developmentally rescued in chimeric mice. Chimeras may survive because in specific cell layers where pRb is crucial, $Rb^{-/-}$ cells were negatively selected or rescued by homo- or heterotypic *Rb-1* wild-type cells. Either possibility may explain the absence of phenotypic abnormalities in chimeric brain and spinal cord. The abrogation of embryonic lethality allowed us to study the phenotypic effects of pRb deficiency at later stages of erythropoiesis and eye development and the role of pRb deficiency in tumorigenesis.

Erythropoiesis

The high percentage of nucleated red cells in the circulation of $Rb^{-/-}$ embryos at the time when erythropoiesis had shifted from yolk sac to fetal liver was interpreted as a reflection of a cell-autonomous effect of lack of Rb function on terminal erythrocyte differentiation. Abnormal production of nucleated red cells was also observed in chimeras during fetal liver erythropoiesis, however, both *Rb-1* wild-type cells and $Rb^{-/-}$ cells were impaired in enucleation. Moreover, normal numbers of $Rb^{-/-}$ erythroid progenitors were present in the livers of $Rb^{-/-}$ homozygous embryos (day 13.5) and chimeric embryos (days 14.5 and 15.5), as judged from CFU-E assays (unpublished data). In either case, these cells gave rise to the synthesis of normal adult haemoglobin chains, indicating a normal developmental switch of erythropoiesis from the yolk sac to the liver. These observations indicate that the effect of loss of Rb function on end-stage differentiation of erythrocytes is not a cell-autonomous phenomenon.

Is the defect then related to improper liver development due to *Rb-1* nullizygosity? Two observations support this hypothesis. First, regions of reduced cellularity in the fetal liver, similar to those observed in $Rb^{-/-}$ embryos, were seen in chimeric embryos from days 14.5 to 16.5. Secondly, adult chimeric $Rb^{-/-}$ mice showed regions of polyploid hepatocytes in the liver, a phenomenon which was absent in chimeric $Rb^{+/-}$ mice of the same age (unpublished data).

Eye

Microscopical analysis of the lens of chimeric $Rb^{-/-}$ mice indicate that pRb is required to trigger the differentiation of lens epithelial cells to fibre cells in a cell-autonomous fashion. It is tempting to speculate that a differentiation block due to loss of Rb function is one of the steps in the

development of lens tumours in transgenic mice carrying the SV40 large T antigen driven by the α-crystallin promoter (Mahon *et al.*, 1987). Indeed, the severe lens aplasia in pure and chimeric $Rb^{-/-}$ embryos closely resembled the pre-malignant morphology of lenses observed in large T-expressing embryos. One has to assume that, in addition to inactivation of pRb, large T induces one or more additional phenotypic changes required for tumorigenesis in adult animals which are absent in $Rb^{-/-}$ chimeras.

Perhaps the most intriguing observation in $Rb^{-/-}$ chimeras was the abnormality in the retina. Ectopic mitoses and cellular degeneration were seen in the inner half of the developing retina of highly chimeric $Rb^{-/-}$ embryos at days 16.5–18.5 of gestation. Interestingly, at this stage of development expression of *Rb-1* in the retina was confined to this region (Szekely *et al.*, 1992). Before this stage, at least until day 14.5, $Rb^{-/-}$ cells participated normally in retina development. Therefore, this phenomenon may reflect a cell-autonomous requirement for pRb in the differentiation pathway of retinoblasts. The absence of a functional Rb protein may render these cells unresponsive to growth-arresting and/or differentiation-inducing signals, leading to additional mitoses and cell death. The cellular degeneration in the developing retina of chimeric $Rb^{-/-}$ embryos was relatively modest and confined to the inner half of the primitive nuclear layer. However, the strongly reduced contribution of $Rb^{-/-}$ cells to the retinal layers in adult chimeras is suggestive of a massive selection against $Rb^{-/-}$ cells. In this respect, it is noteworthy that the vast majority of retinoblasts differentiate as rods around or closely after birth (Carter-Dawson and Lavail, 1979; Young, 1985). Together with the observation that in transgenic mice expression of SV40 large T in rod photoreceptor cells led to their complete degeneration between days 5 and 15 post-natally (Al-Ubaidi *et al.*, 1992b), this may indicate that replacement of $Rb^{-/-}$ photoreceptor cells in chimeras occurred post-natally. Careful analysis of post-natal retina development in $Rb^{-/-}$ chimeras is required to substantiate this.

No retinoblastomas developed in any of the chimeric mice. Our results suggest that loss of *Rb-1* during end-stage differentiation of the retina leads to cell death rather than enhanced cell proliferation. Therefore, unlike the situation in man, in the mouse retina a concomitant mutation may be required to unleash the oncogenic potential of pRb deficiency. This situation may occur in retinoblastomas developing in transgenic mice expressing SV40 large T (Windle *et al.*, 1990; Al-Ubaidi *et al.*, 1992a).

Tumorigenesis

Almost all $Rb^{-/-}$ chimeras succumbed to pituitary gland tumours, which were exclusively of $Rb^{-/-}$ cell origin. Similar tumours developed in $Rb^{+/-}$ mice, and nearly all had lost the wild-type *Rb* allele. Thus, tumorigenesis in *Rb-1* heterozygotes follows Knudson's original two-hit postulate explaining the different appearance of sporadic and hereditary retinoblastoma in humans (Knudson, 1971). Apparently, loss of Rb function is an essential and rate-limiting step in the onset of these tumours, although we cannot exclude additional somatic mutations being involved. An indication in favour of the requirement for other mutations may be that the pituitary gland developed normally in chimeric embryos and that macroscopically visible tumours were not seen in young chimeras before the age of 2–3 months.

In both types of animal, most tumours consisted of well-differentiated cells, producing the intermediate lobe-specific hormones α-MSH and β-endorphin. Moreover, the presence of neoplastic foci with mitotically active cells within the intermediate lobe of pituitary glands from young heterozygous animals further identified this part of the pituitary gland as the origin of the tumour. Thus, in spite of their common embryonic origin, the intermediate lobe appears to be susceptible, while the anterior lobe is refractory, to tumorigenesis by loss of function of *Rb*. One may speculate that the variation in tumour susceptibility of different regions of the adenohypophysis is related to differences in local cell–cell interactions, through which proliferation might be regulated. In this respect, it might be significant that the intermediate lobe, but not the anterior lobe, is in direct contact with the neurohypophysis.

The tumours grew to a large size and often contained large blood-filled cavities, resembling in this respect oestrogen-induced tumours of the anterior pituitary in male Sprague–Dawley rats (Van Nesselrooij *et al.*, 1992). They did not infiltrate into adjacent brain nor spread to other sites in the body. This corresponds well with the relatively benign characteristics of pituitary tumours in man (Russell and Rubinstein, 1989). It will be interesting to see whether loss of RB function is also involved in the onset of pituitary tumours in man.

Materials and methods

Generation of chimeric mice

Cells were cultured in BRL conditioned medium as described for the parental ES cell line E14 (Hooper *et al.*, 1987). ES cell clones dRb12, dRb14, dRb47 and sRb33 were obtained by homologous recombination using the previously described $Rb^{+/-}$ ES cell clone sRb66, carrying a hygromycin resistance (*hyg*) gene in exon 19 of one allele of *Rb-1* (Clarke *et al.*, 1992), and isogenic targeting vector 129Rb-neo (Te Riele *et al.*, 1992). The electroporation conditions, G418 selection and DNA analysis of G418-resistant colonies using *Pst*I and probe A have been described (Te Riele *et al.*, 1992). After selection for G418 resistance, 10^4 colonies were obtained, of which 36% (21 of 59 tested colonies) resulted from homologous recombination. The frequency of homologous recombination at the *hyg*-containing *Rb* locus was similar to that at the wild-type *Rb* locus.

Chimeric mice were generated by injection of six to eight ES cells into C57Bl/6 blastocyst stage embryos.

GPI analysis

Frozen tissue samples were thawed and gently homogenized in PBS with a micropestle. Blood components were separated by density centrifugation of fresh blood [diluted 1:4 with 0.38% (w/v) trisodium citrate in PBS] on a lympholyte-M gradient (Cedarlane laboratories) according to standard procedures. Frozen samples of anticoagulated blood or components were thawed and diluted with PBS. After centrifugation of the homogenates, the supernatants were electrophoresed on cellulose acetate membranes in Supraheme electrophoresis buffer (Helena Laboratories) (pH 8.2–8.6) for 1.5 h at 300 V, 4°C. To 10 ml 0.9% agarose containing 15 mg fructose 6-phosphate (F6P), 2 mg nicotinamide adenine dinucleotide phosphate (NADP), 0.36 mg phenazine methosulfate (PMS), 2 mg methylthiazolium tetrazolium (MTT) at 55°C, 10 units of glucose 6-phosphate dehydrogenase (G6PDH) were added. The overlay was mixed and poured over the gel. The GPI isozyme bands appeared after a few minutes in the dark.

Histological analysis

Embryos and tissues were fixed in phosphate-buffered formalin, embedded in paraffin, sectioned at 5 μm and stained with haematoxylin and

eosin according to standard procedures. Blood smears were stained with May Grünwald–Giemsa.

Haemoglobin analysis

Fetal blood samples were obtained from the umbilical cords by aspiration into heparinized micro haematocrit capillaries and stored in heparinized tubes at −80°C. Blood from adult mice was collected in heparinized tubes after removal of the eye and stored at −80°C. Crude haemolysates were prepared by mixing 1 vol anticoagulated blood in 4 vol deionized water and subsequent incubation for 15 min at 4°C. Globin chains were separated by electrophoresis on Triton–acid–urea gels following a modification of the method of Alter *et al.* (1980), kindly provided by N.Dillon. The gels contained 12% acrylamide–6 M urea (freshly deionized by stirring for 30 min with 8% Bio-Rad AG501-X8 mixed bed resin), 2% Triton X-100, 5% acetic acid, 0.06% (w/v) ammonium persulfate and 0.5% (v/v) TEMED. Electrophoresis buffer was 0.5% acetic acid. Each well was loaded with 40 µl 1 M β-mercaptoethanol and pre-run for 30 min at 200 V and 6 h at 30 mA. Gels were run positive to negative and kept at room temperature. Up to 3 µl haemolysate was mixed with 20 µl sample buffer (6.7 M freshly deionized urea, 8.3% acetic acid, 1.2 M β-mercaptoethanol and 0.3 mg/ml pyronin Y). After centrifugation, the stroma-free samples were loaded onto the pre-run gels and electrophoresed for 20 h at 100 V. Gels were stained with Coomassie Blue, destained and dried according to standard procedures.

DNA analysis

DNA was isolated from tissues as described by Laird *et al.* (1991). The extent of chimerism and loss of heterozygosity in tumours of $Rb^{+/-}$ mice were determined by detection of the *Rb-1* wild-type and mutated *Eco*RI restriction fragments with probe A on Southern blots as described before (Te Riele *et al.*, 1992).

Electron microscopical analysis

Pituitary gland tumours were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) and cut into small blocks. Blocks, representing different parts of the tumour, were post-fixed with 1% OsO_4, stained with uranyl acetate and embedded according to routine methods. Thin sections of several blocks per tumour were stained with lead and observed in a Philips CM 10 electron microscope.

Immunostaining

The indirect immunoperoxidase assay was used as described by Ivanyi *et al.* (1989). The rabbit antisera against melanocyte stimulating hormone (MSH), β-endorphin and luteinizing hormone/follicle stimulating hormone (LH/FSH) were purchased from Seralab.

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