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Deletion of *RB* Exons 24 and 25 Causes Low-Penetrance Retinoblastoma

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Summary

A deletion in the tumor-suppressor gene, *RB,* discovered by quantitative multiplex PCR, shows low penetrance (LP), since only 39% of eyes at risk in this family develop retinoblastoma. The 4-kb deletion spanning exons 24 and 25 (Δ24–25) is the largest ever observed in an LP retinoblastoma family. Unlike the usual *RB* mutations, which cause retinoblastoma in 95% of at-risk eyes and yield no detectable protein, the Δ24–25 allele transcribed a message splicing exon 23 to exon 26, resulting in a detectable protein ($pRB^{\Delta 24-25}$) that lacks 58 amino acids from the C-terminal domain, proving that this domain is essential for suppression of retinoblastoma. Two functions were partially impaired by Δ24–25—nuclear localization and repression of E2F—consistent with the idea that LP mutations generate "weak alleles" by reducing but not eliminating essential activities. However, Δ24–25 ablated interaction of pRB with MDM2. Since a homozygous LP allele is considered nontumorigenic, the pRB/MDM2 interaction may be semi- or nonessential for suppressing retinoblastoma. Alternatively, some homozygous LP alleles may not cause tumorigenesis because an additional event is required (the "three-hit hypothesis"), or the resulting imbalance in pRB function may cause apoptosis (the "death allele hypothesis"). $pRB^{\Delta 24-25}$ was also completely defective in suppressing growth of Saos-2 osteosarcoma cells. Targeting $pRB^{\Delta 24-25}$ to the nucleus did not improve Saos-2 growth suppression, suggesting that C-terminal domain functions other than nuclear localization are essential for blocking proliferation in these cells. Since Δ24–25 behaves like a null allele in these cells but like an LP allele in the retina, pRB may use different mechanisms to control growth in different cell types.

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Introduction

Retinoblastoma results from homozygous inactivation of both alleles of the *RB* tumor-supressor gene in developing human retina. In ~50% of patients, both alleles are mutated in only the single retinal cell that forms the tumor. In the other patients, the first allele is mutated in the germ line, as either a new or an inherited mutation. The second allele is mutated in several developing retinal cells as a stochastic event following a Poisson distribution (Knudson 1971), leading to multiple tumors, usually bilateral, in family members with the germ-line mutation. In 70% of retinoblastoma tumors, the second hit is loss of heterozygosity (LOH), with duplication of the mutant allele by nondisjunction or mitotic recombination (Cavenee et al. 1983; Zhu et al. 1989), but 30% of the time the second hit is a different mutation. The common type of *RB* mutations lead to premature STOP codons and presumed truncated proteins (pRB), which are not detectable in retinoblastoma tumors (Dunn et al. 1989; Horowitz et al. 1990). Such mutations manifest >95% penetrance in families (Gallie et al. 1995).

In uncommon, low-penetrance (LP) families, a significant proportion of obligate carriers develop either no tumors or only unilateral tumors (Macklin 1960; Strong et al. 1981; Connolly et al. 1983; Onadim et al. 1990, 1991, 1992; Sakai et al. 1991; Munier et al. 1992; Lohmann et al. 1994). In some families, tumors in distantly related individuals are caused by distinct, sporadic *RB* mutations: these are examples of pseudo-LP pedigrees (Dryja et al. 1993; Munier et al. 1993; Bia and Cowell 1995). Genuine LP has been attributed to a delayed second hit (Knudson 1971; Herrman 1976), host resistance factors (Matsunaga 1978), immunological effects (Gallie et al. 1979), and "weak alleles" (Sakai et al. 1991; Gallie et al. 1995).

The weak-allele hypothesis is that the inherited LP *RB* allele is partially but not completely impaired. LOH would result in two copies of the weak allele, which would still be sufficiently active to prevent tumorigenesis (Gallie et al. 1995); tumors would arise only when the second mutation is a null, completely inactivated allele.

Therefore, individuals with a germ-line LP *RB* mutation would have a reduced chance of developing retinoblastoma tumors, since LOH would be insufficient for tumorigenesis.

The *RB* mutations have been identified in several LP retinoblastoma families and support the weak-allele hypothesis, since each is in-frame and would result in detectable pRB. Three families have point mutations in the promoter region, which markedly reduce but do not extinguish promoter activity (Sakai et al. 1991; Cowell et al. 1996). Several mutations within the *RB* coding region are associated with LP: Arg661Trp (exon 20) in three separate families (Lohmann et al. 1992; Onadim et al. 1992), Cys712Arg (exon 21) (authors' unpublished data), and deletion of Asn480 (exon 16). These mutations lie within the important pocket domain (exons 12–22) (Hu et al. 1990), which characterizes the *RB* gene family (Ewen et al. 1991; Hannon et al. 1993; Li et al. 1993; Mayol et al. 1993). The pocket domain is critical for transcriptional repression (Hamel et al. 1992; Hiebert et al. 1992; Flemington et al. 1993; Helin et al. 1993; Adnane et al. 1995; Bremner et al. 1995), growth suppression by pRB (Templeton et al. 1991; Qian et al. 1992; Qin et al. 1992), and interaction of pRB with a variety of viral and cellular proteins (DeCaprio et al. 1988; Whyte et al. 1988; Dyson et al. 1989; Hu et al. 1990; Defeo-Jones et al. 1991; Huang et al. 1991; Qian et al. 1992; Qin et al. 1992; Dowdy et al. 1993; Ewen et al. 1993; Hagemeier et al. 1993*a;* Wang et al. 1993). An exon 4 deletion causing LP retinoblastoma results in loss of 40 amino acids from the N-terminal portion (N-domain) of pRB (Dryja et al. 1993); in vitro–generated mutations overlapping this region render pRB defective in growth suppression and phosphorylation (Hamel et al. 1990; Qian et al. 1992).

Using analysis of size and copy number of each exon and the promoter, we have identified a large *RB* deletion spanning exons 24 and 25 (Δ24–25) as the causative mutation in a large LP family. We define several functions and interactions for which $pRB^{\Delta 24-25}$ is deficient because of the protein domains that are missing. Nuclear localization and repression of E2F-mediated transcription are partially impaired, indicating that this *RB* mutation is indeed "weak." Interaction with the MDM2 protein was ablated, an observation that has led us to suggest new models for LP retinoblastoma. Although Δ24–25 is associated with an LP phenotype in the retina, this allele behaves like a null allele in the assay for growth suppression of an osteosarcoma cell line, Saos-2. Thus, pRB may not use the same mechanism to suppress growth in different cell types.

Material and Methods

Cell Culture and Nucleic-Acid Preparation

Both culture of Epstein-Barr virus (EBV)–transformed lymphocytes and preparation of genomic DNA and RNA from either these cells or peripheral blood lymphocytes were as described elsewhere (Goddard et al. 1988; Dunn et al. 1989).

Fragment Analysis

Fragment analysis involves amplification of the promoter region and all 27 exons of *RB* with fluorescein-labeled primers in multiplex sets. Multiplex PCR was performed by use of an approach similar to that described elsewhere (Group 1992; Ioannou et al. 1992; Schwartz et al. 1992). A 50-μl reaction mixture contained 0.5 μg of genomic DNA, 150 ng of each primer, 3.6 μM each dNTP, 10% dimethyl sulfoxide, 16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl (pH 8.8), 6.7 mM $MgCl_2$, 42.5 μg of BSA, 1 mM β-mercaptoethanol, 6.8 μM EDTA (pH 8.0), and 5 units of *Taq* DNA polymerase. One primer of each pair was fluorescein-labeled at the 5′ end. Samples were denatured at 94°C for 3 min and were amplified by 18 PCR cycles (94°C for 50 s, 52°C for 50 s, and 72°C for 2 min) and a final elongation cycle of 70°C for 10 min. Seven microliters of each PCR product was added to 7 μl of loading buffer, denatured for 2 min at 100°C, rapidly cooled on ice, and electrophoresed on a 6% polyacrylamide gel run on a Pharmacia A.L.F. sequencer to detect subexonic deletions and insertions. To detect whole-exon deletion or duplication, each multiplex set of three to seven pairs of *RB* exon primers also contained control primers for C4, which amplify exon 4 of the retinaldehyde-binding protein gene on chromosome 15. Four external controls were also tested, by use of DNA known to be nullisomic (WERI-*RB*1 cells) (McFall et al. 1977), monosomic (EL) (Benedict et al. 1983), diploid (normal), and trisomic (ALI) (authors' unpublished data) for *RB*. The number of copies of a fragment in a test sample was calculated by comparing fluorescence intensity of the fragment with these standards. Fragment detection and subsequent calculations were performed automatically by Fragment Manager 2.1 (Pharmacia).

Long PCR

Long PCR (Barnes 1994; Cheng et al. 1994) was performed by use of a Perkin-Elmer kit with intron 23 primer GGG AAG TAG TAA AGA ATG AGA GGG GGA TTA and intron 26 primer ATG CAT AAA CAA ACC TGC CAA CTG AAG AAA. Target fragments were amplified from 500 ng of genomic DNA in a 100-μl reaction. Other components were as specified by the kit manufacturer. Two-step PCR was performed (20 cycles of 94°C for 1 min and 64°C for 10 min, followed by 17 cycles of 93°C for 1 min and 62°C for 10 min, with a final extension at 72°C for 9 min).

Reverse Transcriptase–PCR, Subcloning, and Sequencing

Ten micrograms of RNA from normal and carrier EBV-transformed lymphocytes was reverse-transcribed

by use of 1.5 μg of oligo(dT) as primer and 60 units of avian myeloblastosis virus reverse transcriptase for 90 min at 42°C. One-tenth of the resulting cDNA was amplified in the presence of primers JD2 (GGA ATT CAC CCC TGA AGA GTC C) and O*RB5* (CGG GGA TCC AGA GGT GTA CAC AG), which hybridize to sequences in exons 23 and 27, respectively. The sense primer (JD2) contains an *Eco*RI site whereas the antisense primer (O*RB5*) contains a *Bam*HI site. Amplification of wild-type and Δ24–25 RNA with these primers generates 420-bp and 246-bp fragments, respectively. These fragments were gel-purified, digested with *Eco*RI+*Bam*HI, subcloned into pGEM7 (Promega), and sequenced with T7 and SP6 primers.

Western Analysis

Detection of pRB from EBV-transformed lysates was performed as described elsewhere (Bremner et al. 1995). Fifty micrograms of total protein was loaded per lane.

Vectors Expressing pRB

In order to further analyze the function of Δ24–25, we made plasmid vectors, SVhRBHA and SVhRBHA$^{\Delta 24-25}$, expressing pRB from the SV40 promoter. SVhRBHA was built in two steps. First, the C-terminal coding portion of the *RB* cDNA was amplified by use of primers (O*RB*1, 5′-GGC CAA GCT TCT CCG GCT AAA TAC ACT T; and O*RB*2, 5′-CCC GAA TTC CCA TTT CTC TTC CTT GTT T), which introduced a *Hin*dIII site and an *Eco*RI site at the 5′ and 3′ end of the fragment, respectively. The 3′ primer also changed the stop codon to TGG. The *Hin*dIII/*Eco*RI fragment was subcloned into *Hin*dIII/*Eco*RI-digested pECE-HA (a gift from P. Hamel), thereby adding a hemagluttinin (HA) tag onto the 3′ end of the *RB* sequence. The insert was checked for PCR errors by sequencing. The *RB* fragment, together with the SV40 poly-A tail present in pECE-HA, was excised by *Dra*III/*Bam*HI digestion and was subcloned into *Dra*III/*Bam*HI-digested SVh*RB* (Bremner et al. 1995). The same strategy was used to generate SVhRBHA$^{\Delta 24-25}$, except that Δ24–25 cDNA was used as a template for the initial PCR. To build the +nuclear localization–signal (NLS) versions of these plasmids, the parent plasmid was partially digested with *Eco*RI, and an oligomer, encoding the SV40 large T NLS, was inserted between the *RB* cDNA and the HA tag: sense strand, 5′-AA TTC ATC GAT AAG AAA AAG CGG AAG GTC G; and antisense strand, AA TTC GAC CTT CCG CTT TTT CTT ATC GAT G. Constructs were confirmed by sequencing.

Glutathione-S-transferase (GST) expression vectors were constructed by use of pGEX-2T (Pharmacia). A GST-*RB* plasmid containing exons 19–27 of human *RB* cDNA (R. Bremner, unpublished data) was used as a template for amplification with primer *RB776* (5′-GGC GGA ATT CCC CCT ACC TTG TCA CCA), the last 18 bp of which hybridizes to codons 776–781 at the start of exon 23, and primer GEX3 (5′-GAG CTG CAT GTG TCA GAG GT), which hybridizes to a region 3′ of the pGEX-2T multiple cloning site. The resulting PCR fragment was cut with *Eco*RI and ligated into *Eco*RI-digested pGEX-2T to generate pGST*RB*23–27. The same 5′ primer was used to construct pGST*RB*23–27Δ24–25, but the template was SVhRBHA$^{\Delta 24-25}$, and the 3′ primer (O*RB*6) hybridized at the 3′ end of the *RB* cDNA (5′-G CCG GAA TTC TCA TTT CTC TTC CTT GTT). The PCR fragment was digested with *Eco*RI and cloned into *Eco*RI-digested pGEX-2T. Inserts were checked by sequencing.

Nuclear Localization

Immunostaining using horseradish peroxidase (HRP)–conjugated secondary antibody followed by DAB/H_2O_2 reaction was as described elsewhere (Zacksenhaus et al. 1993). The monoclonal anti-pRB (14001A) recognizes an epitope in the N-terminus of pRB (amino acids 300–380; PharMingen). HRP-conjugated goat anti-mouse secondary antibody was from Bio-Rad. The expression vectors (10 μg) were transfected in duplicate into Saos-2 cells and were stained for localization of pRB.

Repression of E2 Promoter Elements

The pE2(−80/−70)CAT reporter plasmid, which consists of two E2F sites upstream of a TATA box and the chloramphenicol actyltransferase (CAT) gene (Bremner et al. 1995), was transfected into $RB^{-/-}$ C33A cells (cervical carcinoma), together with varying amounts of the Δ21, SVhRBHA, or SVhRB$^{\Delta 24-25}$HA plasmids. The resultant promoter activity was assessed by measurement of CAT levels, as described elsewhere (Bremner et al. 1995).

MDM2 Binding

GST fusion proteins were prepared as described elsewhere (Zacksenhaus et al. 1993). ^{35}S-labeled in vitro translated (IVT) human MDM2 was generated by use of the TNT reticulocyte system (Promega) and the plasmid pHDM1A (a gift from A. Levine). Twenty microliters of IVT MDM2 was incubated with 1 μg of GST or GST fusion protein in 0.5 ml of binding buffer (0.5% NP-40, 20 mM Tris pH 8.0, 100 mM NaCl, and 0.5 mM EDTA) at 4°C for 1 h. Twenty microliters of glutathione-Sepharose–bead slurry (Pharmacia), prewashed in binding buffer, was added, and the suspension was rocked at 4°C for 30 min. Bead-protein complexes were spun at 2,000 *g* in a microfuge, washed four times with binding buffer, boiled in a 30-μl sample buffer, and analyzed by SDS-PAGE. The presence of equal amounts of different GST proteins was confirmed by Coomassie staining. IVT MDM2 was detected by autoradiography and was quantified by use of a Molecular Dynamics phosphorimager.

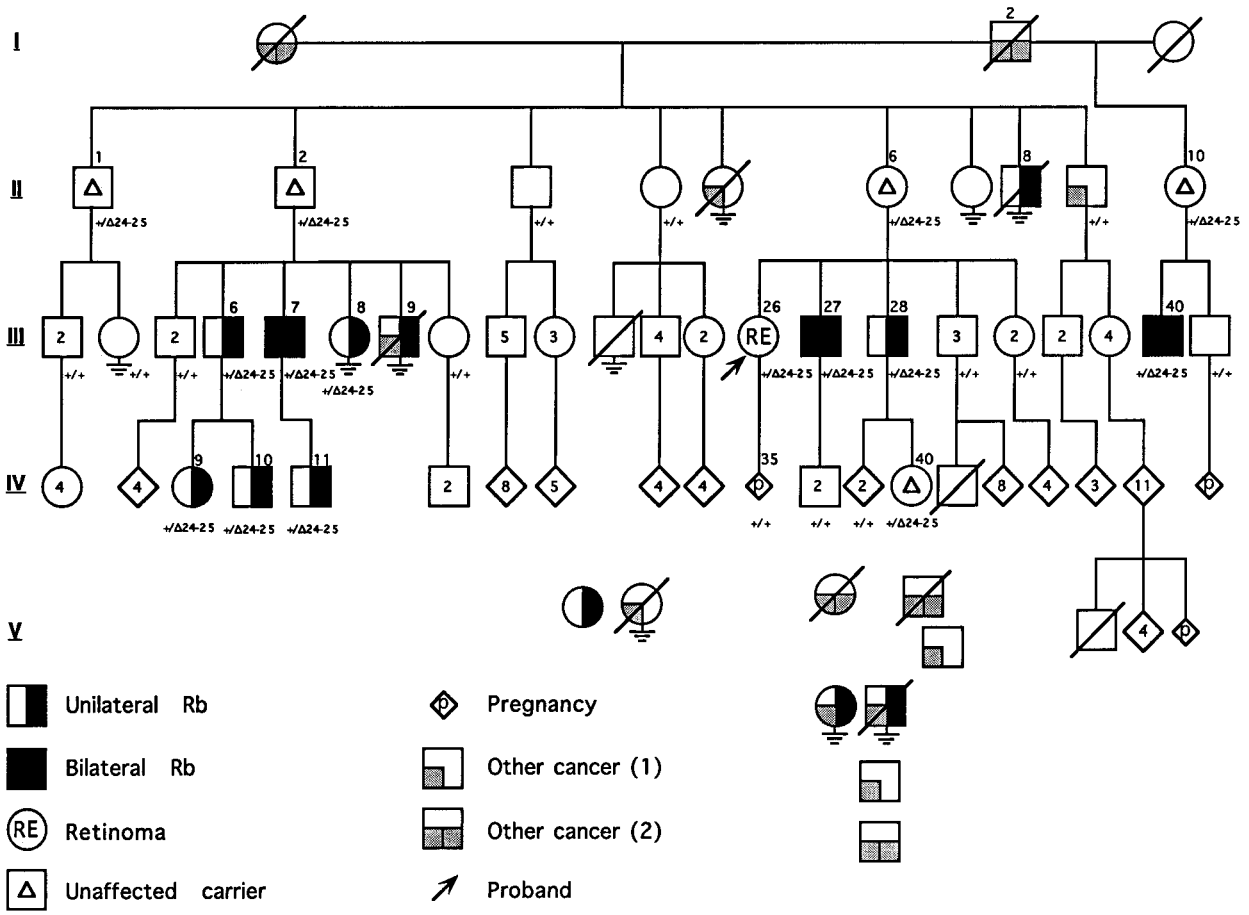

Figure 1 RBF65 pedigree. Only blood relatives are shown. In selected cases, numbers assigned to different individuals are shown above the symbol. Some symbols represent two or more family members; this is indicated by a number within the relevant symbol. Genotypes that were determined by fragment analysis are indicated below the symbols.

Saos-2 Growth-Suppression Assay

Four 60-mm dishes of Saos-2 cells were transfected with 10 μg of the *RB* expression vector, together with 1 μg of pBABEpuro, which confers puromycin resistance, and 0.5 μg of cytomegalovirus β-galactosidase (CMVβgal), which expresses β-galactosidase. Transfection efficiency was assessed by measurement of β-galactosidase activity on one of the four plates 2 d after transfection. Cells on the other plates were treated with puromycin for 18 d, and flat cells were counted and normalized to β-galactosidase activity.

Results

Penetrance and Expressivity of the RBF65 Pedigree

The large Canadian family, RBF65, has been described elsewhere, indicating linkage of retinoblastoma to chromosome 13q14 (Connolly et al. 1983). The pedigree now includes >120 blood relatives over four affected generations (fig. 1). The first-generation carrier (I-2) was implicated because, although only one child (II-8) developed unilateral *RB*, 17 of his descendants are known, by clinical analysis or by the molecular testing described below, to carry a mutant *RB* allele. Only 11 of the 18 developed retinoblastoma (61% penetrance), only 3 had bilateral tumors (low expressivity), and 1 had retinoma (Gallie et al. 1982*a*, 1982*b*). Thus, 14/36 (39%) of at-risk eyes developed retinoblastoma.

The diseased-eye ratio (DER) is defined as the mean number of diseased eyes per carrier (Lohmann et al. 1994) and represents a combination of penetrance and expressivity that is particulary useful in retinoblastoma, in which the two eyes develop independent tumors. Additional data on the precise number of tumors in an individual is not usually available and, in any case, would be less clearly interpreted than the number of eyes affected. Families with the usual, high-penetrance retinoblastoma have DER values >1.0 and, in most pedigrees, close to 2.0 (Lohmann et al. 1994). The DER for RBF65 is .78, typical of LP retinoblastoma families (Lohmann et al. 1994).

Deletion of Exons 24 and 25 in an Individual from Family RBF65, Detected by Fragment Analysis

The *RB* mutation of family RBF65 was found by analysis of blood from patient IV-9 (unilateral retinoblastoma) (fig. 1), by quantitative multiplex PCR of exon sets (fragment analysis), which efficiently identifies deletions, insertions, and changes in copy number of exons or the promoter. More than 30% of *RB* mutations are detected by fragment analysis (authors' unpublished data). A multiplex set comparing copy number with that of a control fragment (C4; chromosome 15) detected two copies of exons 3, 9, 13, 20, and 23 but only one copy of exon 24 (data not shown). A second multiplex set detected two copies of exon 26 but only one copy of exon 25 (data not shown). These data suggested that IV-9 carried a heterozygous deletion of exons 24 and 25 (Δ24–25).

Deletion of Exons 24 and 25 in Family RBF65: Segregation with the Disease

Molecular testing by fragment analysis was performed on 35 members of family RBF65. Ten individuals who had clinical evidence of an *RB* mutation, as well as the three unaffected second-generation relatives who were predicted to be carriers because they had affected descendants (fig. 1), all showed heterozygosity for Δ24–25 ($RB^{+/\Delta 24-25}$). Significantly, among the other 22 family members studied, two previously unsuspected individuals were found to carry Δ24–25: II-1 has three unaffected children who do not have Δ24–25, and IV-40 has no descendants (fig. 1). The remaining 20 at-risk relatives who were shown not to have Δ24–25 have a total of 90 descendants, all free of retinoblastoma or retinoma. Thus, carriers of Δ24–25 do not necessarily develop retinoblastoma, but those with retinoblastoma or retinoma in this family always have Δ24–25. These findings are convincing evidence that, in the RBF65 pedigree, Δ24–25 is the allele that causes LP retinoblastoma.

Melanoma Tumor: No LOH for Δ24–25

No retinoblastoma tumor is available for study from this family, since at-risk individuals are clinically monitored for tumors from birth, allowing small tumors to be treated without removal of the eyes. The large metastatic melanoma that arose in individual III-9 was studied by use of DNA prepared from paraffin-embedded, formalin-fixed tumor tissue and showed both wild-type and Δ24–25 alleles, with no evidence of LOH (data not shown). It is unlikely that the wild-type allele detected in this assay was derived from contaminating normal tissue, since histological analysis revealed only tumor cells in the area used to prepare DNA.

Prenatal Diagnosis of an Unaffected Fetus

Identification of the causative mutation allowed us to offer prenatal diagnosis to III-26, who had retinoma and was positive for Δ24–25. Fragment analysis of DNA from amniotic fluid revealed that the fetus, IV-35, was unaffected (fig. 1). Prior to identification of Δ24–25, 25 children in this family had been undergoing intensive clinical screening for tumors; the molecular test showed that only two children were at risk and needed the clinical screening for tumors. Each of these children developed one tumor in one eye, each treated successfully with only laser. This has resulted in a significant decrease in health-care costs for this family (Noorani et al. 1996).

A 4-kb Genomic Deletion Encompassing Exons 24 and 25

To characterize the extent of the deletion, we performed Long PCR (Barnes 1994; Cheng et al. 1994) on genomic DNA from affected and unaffected individuals. Amplification of normal DNA with primers in introns 23 and 26 generated the predicted fragment of 12 kb (fig. 2, lanes 2, 14, 16, 20, and 21). An additional fragment of ~8 kb was detected in whole blood of Δ24–25 carriers (fig. 2, lanes 3–13 and 17–19). This result suggests that Δ24–25 individuals carry a heterozygous 4-kb deletion encompassing the 3′ end of intron 23, the whole of exon 24, introns 24 and 25, and the 5′ end of intron 25 (fig. 2).

Expression of the Δ24–25 Allele

Deletion of exons 24 and 25 predicts in-frame splicing of exons 23 and 26, leading to a transcript 174 bp shorter than wild type and to a stable, internally deleted, protein. Most mutated *RB* transcripts, unlike normal transcripts, are not detectable in EBV-transformed lymphocytes (Dunn et al. 1989), presumably because of instability of mRNA undergoing premature termination of translation (Schneider et al. 1994).

EBV-transformed $RB^{+/\Delta 24-25}$ lymphocytes from IV-9 and control normal ($RB^{+/+}$) lymphocytes were used to prepare total RNA. Samples were reverse-transcribed with random hexanucleotides and were amplified with primers specific for exons 20 and 27 of *RB*. Only the wild-type 420-bp fragment was detected by use of RNA obtained from the normal individual (fig. 3*A*, lane 1). In contrast, both the wild-type 420-bp fragment and a 246-bp fragment were detected in RNA from the $RB^{+/\Delta 24-25}$ cells (fig. 3*A*, lane 3). The truncated fragment was subcloned; in three of the resultant plasmids, accurate splicing between exons 23 and 26 was documented by sequencing (fig. 3*B* and *C*).

Δ24–25 predicts in-frame loss of the 58 codons for amino acids 830–887 (fig. 3*C*). The resultant protein, $pRB^{\Delta 24-25}$, should be ~6 kD shorter than wild-type pRB. To test this prediction, cell lysates from EBV-transformed $RB^{+/+}$ and $RB^{+/\Delta 24-25}$ lymphocytes were analyzed by western blotting using an anti-pRB monoclonal antibody. Only the wild-type 110-kD unphosphorylated and slowly migrating hyperphosphorylated forms of

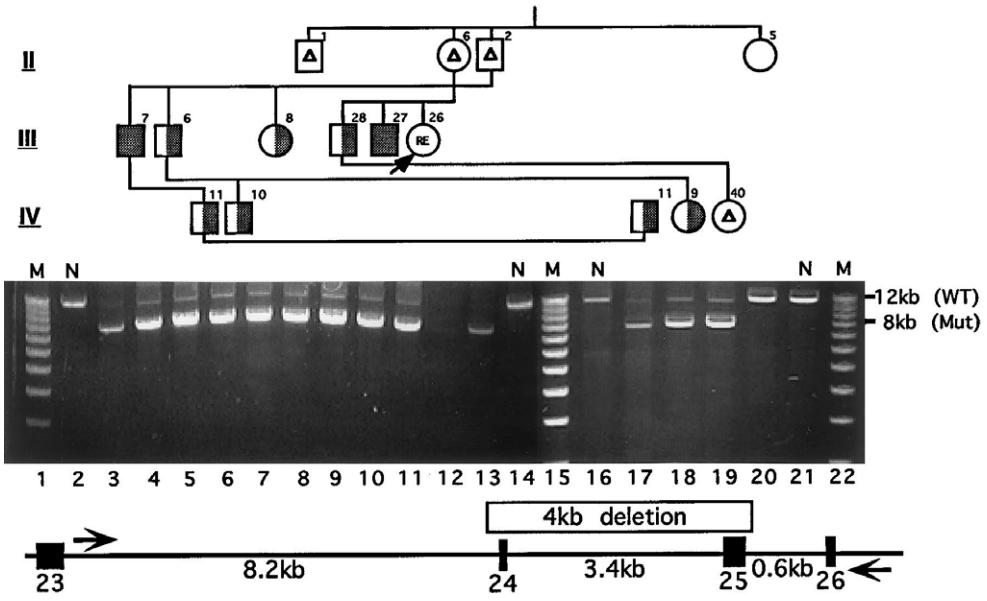

Figure 2 Identification of a 4-kb deletion in Δ24–25 carriers, by long PCR. DNA from 14 members of the pedigree was analyzed by long PCR. Pedigree symbols are as shown in figure 1. Normal controls (N) were run in lanes 2, 14, 16, and 21. A 1-kb ladder was used as a marker (M). The wild-type (WT) 12-kb and mutant (Mut) 8-kb bands are indicated. A schematic diagram of the amplified region is shown below the gel. Primers used in the PCR are represented by arrows; and exons are represented by blackened boxes. The approximate site of the 4-kb deletion is indicated.

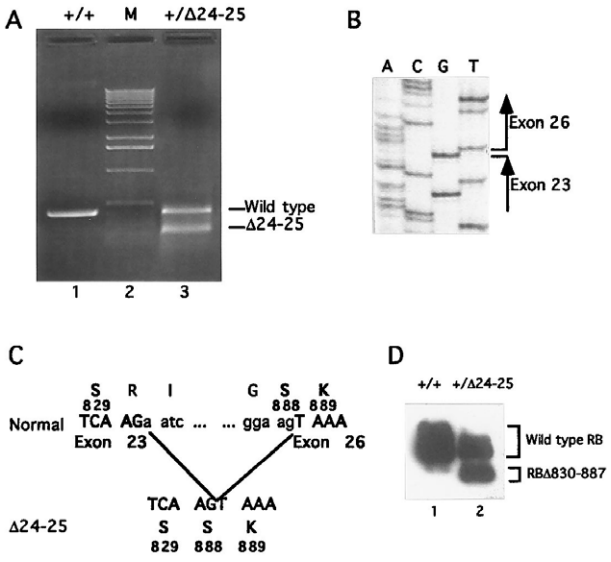

Figure 3 Expression of Δ24–25 at the RNA and protein levels. *A*, RT-PCR analysis of RNA from EBV-transformed normal (lane 1) or heterozygous (lane 3) lymphocytes. The 1-kb ladder was used as a marker (M). Wild-type 420-bp and Δ24–25 246-bp fragments are indicated. *B*, Sequence of the Δ24–25 fragment amplified in panel *A*. The end of exon 23 and the start of exon 26 are shown. *C*, Diagram showing consequences of the Δ24–25 mutation. The reading frame is not altered; fusion of the last two bases in exon 23 with the first base in exon 26 regenerates codon 888; and codons 830–887 are deleted. *D*, Western analysis of protein from EBV-transformed normal (lane 1) or heterozygous (lane 2) lymphocytes. Wild-type and mutant proteins are indicated. Another EBV-transformed line, from a separate individual, gave the same result (data not shown).

pRB (Buchkovich et al. 1989; Chen et al. 1989; DeCaprio et al. 1989) were detected in $RB^{+/+}$ lysates. However, in lysates of $RB^{+/\Delta 24-25}$ cells, an additional lower band was detected at ~104 kD (fig. 3*D*). Thus, in EBV-transformed lymphocytes, the Δ24–25 allele is expressed at both RNA and protein levels.

"Weak-Allele" Characteristics of Δ24–25

The simplest type of weak allele is one that partially impairs one or more essential pRB functions. We discovered two pRB properties that are impaired but not ablated by Δ24–25.

1. Nuclear localization.—Previously, we had mapped the pRB NLS to a 17-amino-acid motif encoded within exon 25 (Zacksenhaus et al. 1993). In the absence of the NLS, pRB is distributed between cytoplasm and nucleus. When the pocket domain is also mutated, pRB localizes exclusively to the cytoplasm (Zacksenhaus et al. 1993). Because immunostaining for pRB in $RB^{+/\Delta 24-25}$ lymphocytes was inconclusive, we transfected the plasmids SVhRBHA and SVhRB$^{\Delta 24-25}$HA (expressing pRB and pRB$^{\Delta 24-25}$, respectively) into Saos-2 cells, followed by immunostaining with the pRB monoclonal antibody. Wild-type pRB was found exclusively in the nucleus (fig. 4*A*), whereas pRB$^{\Delta 24-25}$ was both nuclear and cytoplasmic (fig. 4*B*), resembling the distribution of pRB$^{\Delta NLS}$ that lacked the NLS (Zacksenhaus et al. 1993).

2. E2F repression.—The pRB pocket alone binds free E2F, but both the pocket and the C-domain are required to bind E2F on DNA (Hiebert 1993). Therefore, we tested the ability of pRB$^{\Delta 24-25}$ to interact with E2F in a

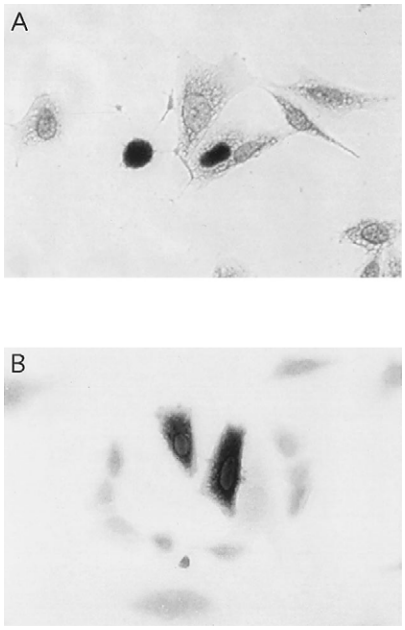

Figure 4 Localization of pRB$^{\Delta 24-25}$, shown to be partially aberrant. Saos-2 cells were transfected with vectors expressing wild-type pRB (*A*) or pRB$^{\Delta 24-25}$ (*B*). Forty-eight hours later the subcellular location of these proteins was determined by immunostaining with an anti-pRB monoclonal antibody.

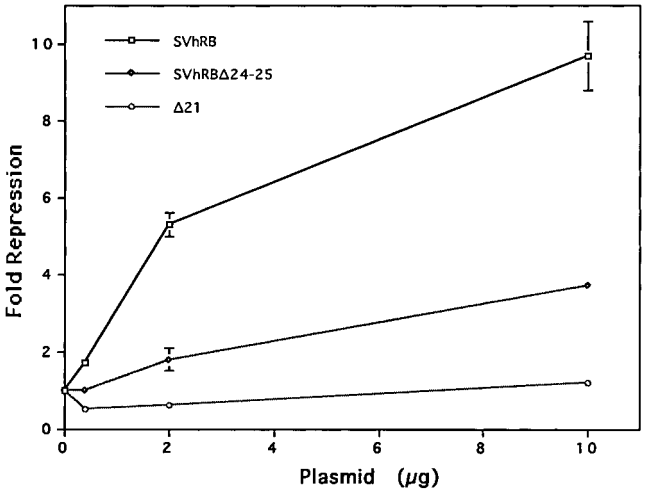

Figure 5 Repression of E2F by pRB$^{\Delta 24-25}$: partially reduced relative to wild-type pRB but not eliminated. C33A cells were transfected with 2 μg of the reporter plasmid pE2(−80/−70)CAT together with either a control vector SVLuc (Bremner et al. 1995) or various amounts of the indicated *RB* plasmids, and CAT activity was measured. Fold repression is relative to the CAT activity obtained with the control vector. An equivalent number of moles of SV40 promoter was used in each transfection, by including, where necessary, SVLuc as a filler plasmid.

function assay. The E2-CAT reporter plasmid was transfected into *RB*$^{-/-}$ C33A cells, together with varying amounts of the plasmids expressing wild-type pRB, pRB$^{\Delta 24-25}$, or Δ21 (expressing a mutant version of pRB, which lacks exon 21 and does not bind E2F). The resultant promoter activity was assessed by measurement of CAT levels. pRB$^{\Delta 24-25}$ retained some ability to repress E2F-mediated transcription—unlike Δ21, which did not affect E2F-mediated transcription (fig. 5).

MDM2: Failure to Bind to pRB$^{\Delta 24-25}$

*Mdm*2 is a proto-oncogene that was originally isolated from a spontaneously transformed mouse cell line (Fakharzadeh et al. 1991). It cooperates with activated *ras* to transform primary rat-embryo fibroblasts (Finlay 1993) and is amplified in 30%–40% of human sarcomas (Oliner et al. 1992; Ladanyi et al. 1993; Leach et al. 1993; Reifenberger et al. 1993; Cordon-Cardo et al. 1994). Expression of an MDM2 transgene in the lactating mammary inhibits gland development and promotes polyploidy and tumorigenesis (Lundgren et al. 1997). The tumorigenic effects of MDM2 may be due, in part, to its ability to bind and inactivate pRB (Xiao et al. 1995). Since this interaction is mediated by residues 792–928 of the pRB C-domain (Xiao et al. 1995), we tested whether it is disrupted by Δ24–25.

IVT MDM2 was incubated with either GST alone, GST fused to the C-domain of pRB (GST23–27), or the C-domain lacking amino acids 830–887 (GST23–27$^{\Delta 24-25}$). Resultant complexes were purified on glutathione-Sepharose beads, and MDM2 binding was assessed by PAGE and autoradiography. MDM2 bound GST23–27 (fig. 6, lane 3) but showed negligible interaction with GST23–27$^{\Delta 24-25}$, similar to the result obtained

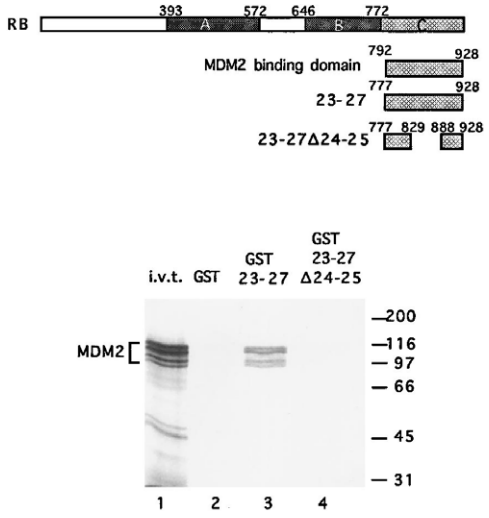

Figure 6 Δ24–25 ablation of interaction of pRB with MDM2. The schematic diagram indicates the position of the MDM2-binding domain (Xiao et al. 1995), the exon 23–27 portion of pRB (present in GST23–27), and the same region with the Δ24–25 mutation (present in GST23–27Δ24–25). IVT MDM2 was incubated with the indicated GST protein, and bound complexes were purified and analyzed by SDS-PAGE and autoradiography.

with GST alone (fig. 6, compare lanes 2 and 4). Quantitation by Phosphorimager confirmed that interaction of GST23–27$^{\Delta 24-25}$ with IVT MDM2 was equivalent to background levels (data not shown).

Ablation of MDM2 binding by Δ24–25 is surprising, given that this mutation is a weak, not null, allele. Possible explanations, including new models of LP retinoblastoma, are outlined in the Discussion.

pRB$^{\Delta 24-25}$: Failure to Suppress the Growth of Saos-2 Cells

Saos-2 cells express a truncated, nonfunctional pRB (Shew et al. 1990). Overexpression of wild-type RB in Saos-2 causes growth arrest in G1, manifested by flattening and enlargement of transfected cells (Goodrich et al. 1991; Templeton et al. 1991; Qian et al. 1992; Qin et al. 1992). To assay biological activity, Saos-2 cells were transfected with the SVhRBHA or SVhRB$^{\Delta 24-25}$HA expression vectors and pBABEpuro, which confers resistance to the drug puromycin, and by CMVβgal, to control for transfection efficiency. Transfected cells were selected in puromycin and were analyzed microscopically.

As expected (Buchkovich et al. 1989; Chen et al. 1989; DeCaprio et al. 1989; Templeton et al. 1991; Hinds et al. 1992; Qian et al. 1992; Qin et al. 1992; Zacksenhaus et al. 1993; Zhu et al. 1993), expression of wild-type pRB resulted in many enlarged flat cells (fig. 7*A*) and no colonies. In contrast, expression of pRB$^{\Delta 24-25}$ did not induce flat cells (fig. 7*A*), similar to the results seen with the null allele, pRB$^{\Delta 21}$ (fig. 7*A*) (Horowitz et al. 1989, 1990), and a similar number of colonies grew on plates transfected with either Δ21 or Δ24–25 (data not shown).

Restoration of Nuclear Localization of pRB$^{\Delta 24-25}$: No Recovery of Saos-2 Growth Suppression

Since pRB$^{\Delta 24-25}$ partially represses E2F activity (fig. 5), a reduction in the number of flat Saos-2 cells was expected. Instead, the effect of Δ24–25 was as dramatic as that of a null mutation. Thus, functions other than E2F repression must be required for blocking of Saos-2 cell growth. One possibility is that the combined effects of Δ24–25 on both E2F regulation and nuclear localization severely impair growth suppression. Alternatively, the loss of other C-domain functions may be more critical.

To address the role of nuclear targeting in growth suppression by pRB$^{\Delta 24-25}$, the coding region for large T-antigen NLS was fused in-frame to the end of the coding sequence in SVhRB$^{\Delta 24-25}$HA. The new plasmid, SVh*RB*$^{\Delta 24-25}$NLS, expressed a protein, pRB$^{\Delta 24-25NLS}$, that was targeted exclusively to the nucleus (fig. 7*B*). Repression of E2F activity was slightly improved (1.7-fold) by this modification (data not shown). However, despite this enhancement and the nuclear location, growth suppression was not rescued or even improved (fig. 7*A*). The low activity of pRB$^{\Delta 24-25NLS}$ was not due to an aberrant effect of the viral NLS, since wild-type pRB modified in the same way suppressed growth, albeit at a slightly lower level (2.5-fold) than wild-type pRB (fig. 7*B;* SVh*RB*^{NLS}). These data further support the idea that E2F regulation alone is insufficient to suppress Saos-2 cell growth and demonstrate that nuclear targeting of pRB$^{\Delta 24-25}$ does not restore its ability to suppress Saos-2 cells. Other functions that are affected by Δ24–25, such as interaction with MDM-2, must explain why pRB$^{\Delta 24-25}$ cannot suppress the growth of these cells. However, the clinical LP phenotype suggests that the Δ24–25 allele *can* weakly suppress development of retinoblastoma tumors.

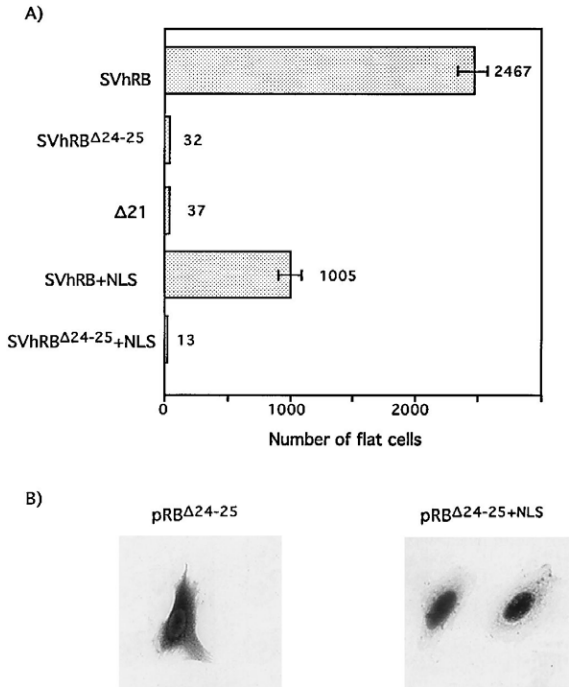

Figure 7 Δ24–25 ablation of suppression of Saos-2 cell growth by pRB. *A,* Saos-2 growth assay. Four 60-mm dishes of Saos-2 cells were transfected with 10 μg of the indicated *RB* plasmids, together with 1 μg of pBABEpuro, which confers puromycin resistance, and 0.5 μg of CMVβgal, which expresses β-galactosidase. Transfection efficiency was assessed by measurement of β-galactosidase activity on one of the four plates 2 d after transfection. Cells on the other plates were treated with puromycin for 18 d, and flat cells were counted, normalized for β-galactosidase activity, and plotted as shown; bars indicate SD. Similar results were obtained in another experiment (data not shown). *B,* Targeting pRB$^{\Delta 24-25}$ to the nucleus, by use of a viral NLS. Saos-2 cells were transfected with plasmid vectors expressing the indicated proteins. Cellular localization of pRB was determined by immunostaining.

Discussion

Clinical Impact of Identification of LP Mutations

Mutation identification permits accurate genetic counseling, without which the developing retinoblas-

toma tumors might be missed in children of LP families who are screened clinically on the basis of conventional risk estimates. In the family reported here, the closest affected relative of IV-1, IV-2, IV-3, and IV-4, on the basis of clinical diagnosis alone, was their grand-uncle, II-2, so distantly related that conventional screening recommendations would not have required repeated clinical examination. Fortunately, II-1 did not transmit the Δ24–25 allele. Thus, identification of the RB mutation is particularly significant for LP families, where the preponderance of unaffected carriers might result in an underestimate of risk.

In-frame mutations may be polymorphisms and might require either clinical confirmation that the DNA change is causative (not present in the normal cells of either unilateral cases or the parent of bilateral cases) or function studies showing that the altered allele has deficient activity. In the large family RBF65, linkage of the Δ24–25 allele to disease is very clear, since no one without Δ24–25 had retinal alterations or transmitted the predisposition to retinoblastoma. However, many individuals with Δ24–25 were asymptomatic, so, in the absence of further function studies, the formal possibility would exist that this mutation could be a polymorphism linked to the real mutation, despite the important functions attributed to this region of pRB. Our function studies strongly support the conclusion that Δ24–25 is the disease-causing allele in RBF65. Similarly, the exon 4 deletion that removes 40 amino acids from the N-domain of pRB and is associated with LP retinoblastoma (Dryja et al. 1993) could be a polymorphism, but separate studies suggest that large deletions in this region alter pRB function (Hamel et al. 1990; Qian et al. 1992).

Germ-line RB mutations increase the risk of developing osteosarcoma as well as retinoblastoma. Given that $pRB^{\Delta 24-25}$ fails to suppress the growth of the Saos-2 osteosarcoma cell line, Δ24–25 may behave like a high-penetrance mutation in bone. No RBF65 family members have developed osteosarcoma, but this does not negate the hypothesis, since, in one study, only 1/45 (2.2%) of patients with hereditary retinoblastoma who did not receive radiation developed osteosarcoma by 18 years of age (Draper et al. 1986), and since there are only 18 Δ24–25 carriers in RBF65. However, until additional tumor data are obtained, the possibility that some LP mutations behave as null alleles in nonretinal tissues remains highly speculative.

Functional Importance of the pRB C-Domain

The identification and characterization of weak *RB* mutations is likely to generate new insight into the function of this important growth-regulatory gene. The common types of clinical mutation causing retinoblastoma lead to premature termination of translation and to undetectable protein (Horowitz et al. 1990), so function information has come almost exclusively from in vitro mutagenesis studies. Many *RB* mutations in other tumor types are also null alleles (Harbour et al. 1988; Yokota et al. 1988; Wadayama et al. 1994). A few mutations have been observed that cause single-amino-acid changes or in-frame deletions, both in retinoblastoma (Kato and Wakabayashi 1988; Canning and Dryja 1989; Yandell et al. 1989; Hashimoto et al. 1991; Onadim et al. 1992; Blanquet et al. 1993; Dryja et al. 1993; Hogg et al. 1993; Cowell et al. 1994; Kato et al. 1994; Lohmann et al. 1994) and in other tumor types (Horowitz et al. 1989, 1990; Bookstein et al. 1990; Kaye et al. 1990; Shew et al. 1990; Scheffner et al. 1991). In many of these cases, expression of the defective protein has been documented in tumor and/or constitutional cells (Horowitz et al. 1989, 1990; Bookstein et al. 1990; Kaye et al. 1990; Shew et al. 1990; Hashimoto et al. 1991; Scheffner et al. 1991; Kato et al. 1994; Kratzke et al. 1994). Significantly, virtually all these mutations disrupt the pocket domain. Δ24–25 is the first example of an in-frame deletion both affecting the pRB C-domain and shown to be expressed at the protein level, proving that this region of pRB is functionally important in the retina. A large deletion encompassing exons 24 and 25 has also been observed in a case of leukemia (Hansen et al. 1990). However, it was not clear whether exon 23 was also affected, nor was expression of the mutant allele verified.

Domain-Specific Patterns of LP Mutations

Two large deletions have been identified that show the LP phenotype: Δ24–25, which removes 58 residues, and deletion of exon 4 (Δ4), which removes 40 amino acids (Dryja et al. 1993). Both these sizable defects affect regions outside the pocket domain. In contrast, identified pocket LP mutations only affect one amino acid (Onadim et al. 1992; Lohmann et al. 1994), suggesting that in the critical pocket the types of mutations that can cause LP retinoblastoma are limited. Both small deletions and many substitutions of one or a few amino acids in the pocket disrupt binding to E1A and to the large T antigen (Hu et al. 1990; Kratzke et al. 1992; Stirdivant et al. 1992), consistent with the idea that this domain is very sensitive to perturbation.

Why Δ24–25 Is a Low-Penetrance Mutation

We identified several defects associated with Δ24–25, each or all of which may explain why it causes LP retinoblastoma.

1. Nuclear localization.—Previously, we had identified a bipartite NLS within exon 25 of *RB* (human codons 860–876, mouse codons 853–869) (Zacksenhaus et al. 1993). However, nuclear localization of pRB requires both the NLS and the pocket domain; mutation of either region only partially disrupts nuclear localization (Zacksenhaus et al. 1993; Aguzzi et al. 1995). Expression of Δ24–25 in Saos-2 cells clearly showed cyto-

plasmic and nuclear staining, consistent with an intact pocket domain allowing partial translocation into the nucleus with E2F, as we have shown elsewhere for pRB (Zacksenhaus et al. 1996). Functionally, reduced nuclear localization may be similar to reduced expression of *RB,* which is the consequence of LP mutations in the *RB* promoter (Sakai et al. 1991).

2. E2F repression.—Binding of pRB to E2F inhibits the transcriptional activation domain (Flemington et al. 1993; Hagemeier et al. 1993*b*) and maintains in a repressed state various genes required for S-phase (La Thangue 1994; DeGregori et al. 1995; Duronio and O'Farrell 1995; Ohtani and Nevins 1995). Although the pocket domain is sufficient to bind free E2F, C-terminal amino acids 793–869 are required for binding E2F on DNA (Hiebert 1993). Deletion of amino acids 841–850 or 841–909 disrupts binding of pRB to DNA-bound E2F and reduces—but does not abrogate—repression of the adenovirus E2 promoter by pRB (Hiebert 1993). Δ24–25 removes amino acids 830–887, so the residual repression of E2F-mediated transcription by $pRB^{\Delta 24-25}$ is probably mediated by binding to free E2F, either (*a*) in the cytoplasm, in the absence of the NLS, or (*b*) more efficiently, in the nucleus, when an NLS is provided ($pRB^{\Delta 24-25NLS}$). We have shown that repression of transcription from a simple promoter requires only binding of pRB to E2F (which would be intact for $pRB^{\Delta 24-25}$) but that silencing of a complex promoter requires pRB to bind to E2F on DNA (which would be deficient for $pRB^{\Delta 24-25}$), where it presumably interacts with other transcription factors (Zacksenhaus et al. 1996). The partial effect of Δ24–25 on E2F repression is consistent with the idea of a weak allele that, when duplicated by LOH, still blocks tumorigenesis.

3. MDM2 binding.—MDM2 is a widely expressed protein (de Oca Luna et al. 1996) that binds to the C-domain of pRB, between amino acids 792 and 928 (Xiao et al. 1995). Δ24–25, which deletes residues 830–887, ablated interaction of pRB with MDM2, even in a low-stringency binding assay.

Abrogation of any function by an LP mutation raises an interesting dilemma. According to the weak-allele hypothesis, a homozygous LP mutation is nontumorigenic, and tumors form only if the second hit is a distinct null mutation. Duplication of Δ24–25 would generate a retinal cell in which pRB cannot bind and regulate either MDM2 or other proteins that bind to the same domain, such as c-ABL (Welch and Wang 1993). If these interactions were essential for tumor suppression in the retina, Δ24–25 would be a high-penetrance mutation. One explanation is that these interactions exist only to inhibit pRB, in which case their loss would potentiate, rather than impair, pRB function. However, if it is assumed that pRB also negatively regulates MdM2 and/or additional C-domain–binding proteins, other hypotheses, outlined below, may explain why abrogation of these interactions does not cause high-penetrance retinoblastoma.

First, MDM2-binding may be a nonessential function. "Nonessential" could mean either that this activity is redundant or that it is irrelevant. In this case, other defects must explain the LP phenotype associated with Δ24–25. However, since MDM2 is clearly important in the regulation of cell division and tumorigenesis (Fakharzadeh et al. 1991; Oliner et al. 1992, 1993; Finlay 1993; Ladanyi et al. 1993; Leach et al. 1993; Reifenberger et al. 1993; Cordon-Cardo et al. 1994; Lundgren et al. 1997), it seems unlikely that its deregulation in the retina would have no effect.

Second, the simplest type of weak allele is one in which an *essential* function is *partially* impaired, but it is also possible that an LP mutation could *completely* inactivate a *semiessential* function. Loss of a semiessential activity might contribute to tumorigenesis only if other pRB functions were also diminished—that is, when the normal *RB* allele sustained a null mutation.

Third, some LP mutations may create "death alleles" rather than weak alleles: in this model, duplication of the LP mutation by LOH would result in cell death, potentially because of the conflicting signals generated by a partially functional pRB molecule. The retina would still be protected from cancer in this scenario. Presumably, a null mutation of the normal allele would alter the balance toward tumorigenesis rather than toward apoptosis. Mice may also be protected from retinoblastoma because loss of the *RB* gene leads to apoptosis (Maandag et al. 1994), so it is possible that, under certain conditions, this is also the outcome in the human retina.

Finally, in a "three-hit" model, abrogation of a pRB function, such as MDM2-binding, would not lead to tumorigenesis but would only prime the cell so that a third mutation, perhaps at an oncogene, would initiate retinoblastoma. Tumor frequency would be low because of the requirement for an additional event. All retinoblastomas have, besides *RB* defects, additional genetic changes, such as the iso(6p) chromosomal rearrangement (Squire et al. 1984) or the K-ras mutation (Bautista et al. 1996), supporting the idea that other mutations facilitate retinoblastoma.

Although $RB^{+/-}$ mice are resistant to retinoblastoma, 100% develop pituitary middle-lobe tumors at 2–11 mo of age, and the tumors always show LOH for the *RB* null allele (Jacks et al. 1992; Hu et al. 1994; Harrison et al. 1995). The tumor frequency suggests that, as in human retinoblastoma, two hits are rate-limiting for tumor development (Hu et al. 1994). Thus, at the genetic level, human retinoblastoma and mouse pituitary middle-lobe tumors bear a striking resemblance. Some insight into LP retinoblastoma may come, therefore, from analysis of the effect of LP RB mutations on mouse pituitary tumorigenesis. For example, a homozygous

weak allele or death allele should not generate tumors, but in the three-hit model a small proportion of tumors are predicted to be homozygous for the LP allele. Generation of mice homozygous for an LP mutation may help distinguish between weak alleles and death alleles, on the basis of the level of apoptosis in different tissues.

Several groups have shown that pRB is cleaved by a member of the caspase (interleukin-1β-converting enzyme–related) family of proteases during apoptosis (An et al. 1996; Janicke et al. 1996; Chen et al. 1997; Tan et al. 1997). Digestion occurs at a caspase consensus cleavage-recognition site (DEADG) between positions 883 and 887 in the pRB C-terminal domain (Janicke et al. 1996; Chen et al. 1997; Tan et al. 1997). Mutation of this site blocks caspase-mediated cleavage of pRB and enhances resistance to tumor necrosis factor α–induced apoptosis (Janicke et al. 1996; Tan et al. 1997). This site is deleted in $pRB^{\Delta 24-25}$, and could render retinal cells more resistant to apoptotic stimuli. Duplication of such an allele may cause retinoblastoma only if, subsequently, another locus is mutated (the three hit hypothesis). Again, insight into this issue may be gained from determining the in vivo effect of mutation of the mouse pRB DEADG sequence.

Multiple pRB Growth-Suppression Domains?

Δ24–25 behaved like a null allele in the Saos-2 growth assay, consistent with the idea that growth suppression in the retina, where Δ24–25 has some antitumorigenic activity, may involve protein domains different than those involved in other tissues. Two other observations are consistent with this idea. First, the LP mutation, Arg661Trp, which has the same retinal phenotype as does Δ24–25, behaves like wild-type pRB in suppressing the growth of a lung-carcinoma cell line (Kratzke et al. 1994). Thus, in three different cell types, osteosarcoma, lung carcinoma, and retinal cells, LP mutations have quite different effects. Second, although a stop codon in *RB* exon 3 renders mice susceptible to both thyroid and pituitary tumors, a similar defect in either exon 19 or exon 20 is associated only with pituitary tumors (Hu et al. 1994; Maandag et al. 1994; Williams et al. 1994; Harrison et al. 1995). Small amounts of truncated pRB in the latter case (Lee et al. 1992) appear to be sufficient for tumor suppression in the thyroid but not for tumor suppression in the pituitary. The pRB "growth suppression domain" was first defined in Saos-2 cells (Templeton et al. 1991; Qian et al. 1992; Qin et al. 1992), but it clearly needs to be studied in other cell types.

The Mechanism of Growth Suppression in Saos-2 Cells

Partial regulation of E2F activity by $pRB^{\Delta 24-25}$ did not correlate with an intermediate effect on the growth of Saos-2 cells, suggesting that E2F regulation alone is insufficient for growth suppression in this cell type. In agreement, Sellers et al. (1995) have recently shown that a heterologous repression domain fused to E2F-1 shuts down expression of E2F-regulated genes but does not suppress the growth of Saos-2 cells. Additional effects of pRB are therefore implicated. We have shown that targeting $pRB^{\Delta 24-25}$ to the nucleus did not improve growth suppression. However, interaction of pRB with MDM2 was completely ablated by Δ24–25, suggesting that perhaps this or a related interaction is essential for growth suppression in Saos-2 cells. c-ABL, like MDM2, binds the pRB C-domain (Welch and Wang 1993), and it is likely that this interaction is also ablated by Δ24–25. Wang's group has shown that suppression of Saos-2 cell growth requires the assembly of multiple proteins in one complex by different pRB-binding domains (Welch and Wang 1995*a,* 1995*b*). This "matchmaker" function may not be as critical in the retina, where Δ24–25 behaves as an LP allele, as it is in Saos-2 cells.

Retinoblastoma and Homozygous LP Alleles

The weak-allele hypothesis predicts that a homozygous LP allele is nontumorigenic (Sakai et al. 1991). Few tumors are available from LP families to determine whether this assumption is correct. In one example, a tumor was shown to be heterozygous, and the second allele had sustained a distinct null mutation (Dryja et al. 1993). In another family, LOH was observed in the retinoblastoma, but quantitation was not possible, so it was uncertain whether LOH was due to hemi- or homozygosity (Lohmann et al. 1994). No retinoblastoma tumor was or is likely to be available from the RBF65 family, since tiny tumors are cured without surgery. The melanoma from III-9 did not show LOH and may have arisen from a separate null-*RB* second hit, or it could be unlinked to the *RB* mutation. Melanoma is a common second tumor in individuals with *RB* mutations (Eng et al. 1993). The issue of whether homozygous LP mutations are ever tumorigenic remains unsolved. In the three-hit hypothesis for LP retinoblastoma, discussed above, LOH would be observed in some tumors.

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