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Mutations of N-Terminal Regions Render the Retinoblastoma Protein Insufficient for Functions in Development and Tumor Suppression

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To assess biological roles of the retinoblastoma protein (RB), four independent transgenic mouse lines expressing human RB with different deletions in the N-terminal region (RBΔN) were generated and compared with mice expressing identically regulated, full-length RB. Expression of both RB and RBΔN caused developmental growth retardation, but the wild-type protein was more potent. In contrast to wild-type RB, the RBΔN proteins were unable to rescue $Rb^{-/-}$ mice completely from embryonic lethality. Embryos survived until gestational day 18.5 but displayed defects in the terminal differentiation of erythrocytes, neurons, and skeletal muscle. In $Rb^{+/-}$ mice, expression of the *RBΔN* transgenes failed to prevent pituitary melanotroph tumors but delayed tumor formation or progression. These results strongly suggest that N-terminal regions are crucial for embryonic and postnatal development, tumor suppression, and the functional integrity of the entire RB protein. Furthermore, these transgenic mice provide models that may begin to explain human families with low-penetrance retinoblastoma and mutations in N-terminal regions of RB.

The retinoblastoma protein (RB) serves as a gatekeeper for negatively regulating progression through G_1 phase of the mammalian cell cycle (20). It works in part by binding and sequestering key transcription factors, like E2F-1 (46), required in S phase (for a review, see reference 51). More recent evidence suggests that RB also positively regulates other transcription factors (myoD, CAATT enhancer binding proteins [C/EBPs], and NF-IL6) and is important for the terminal differentiation of several key cell types, including neurons, myocytes, monocytes, and adipocytes (9–11, 21, 37, 53).

Human RB (hRB) is composed of 928 amino acids. It migrates in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at 110 kDa when hypophosphorylated and at positions between 110 and 116 kDa when phosphorylated (6, 8, 38). Murine Rb (mRb) has ~95% sequence homology with hRB but can be differentiated in SDS-PAGE by its faster migration at 105 kDa in the hypophosphorylated state (2, 4). Biochemical analysis has shown RB to be comprised of discrete structural domains (25). Recombinant protein from *Escherichia coli* transformed with the full-length h*RB* forms protease-resistant globular domains (N, R, A, and B) separated by linker regions, as well as an acidic, more linear C terminus (25). A more abundant, N-terminally truncated recombinant protein, $p56^{RB}$, translated in *E. coli* from an internal methionine, has been used in several studies to assess the role of RB in cell cycle regulation (20). In these and similar studies (26), $p56^{RB}$ functioned equivalently when compared with $p110^{RB}$.

The very C-terminal region of RB has nonspecific DNA binding activity, and the two globular (A and B) domains are required for binding to most known cellular and viral RB-associated proteins, including E2F-1, simian virus 40 T antigen, adenovirus E1A, and papillomavirus E7 (9, 31, 32, 49). To date, the majority of mutations in tumor specimens, tumor cell lines, and families with inherited retinoblastoma map to the simian virus 40 large-T-antigen binding domains (for a review, see reference 5). A single point mutation (Cys^{706}→Phe) in the B domain results in a nonfunctional RB incapable of binding to any of these proteins (3, 35). Clearly, then, C-terminal portions of RB are crucial for functional integrity. In contrast, the biological role of the N terminus of RB has not been clearly demonstrated. In fact, in vitro and cell culture studies have presented evidence to suggest that N-terminally truncated p56 and p94 recombinant RB proteins are equivalent or stronger tumor suppressors than full-length RB (18, 50, 57, 58). By implication, then, the N-terminal portion of RB may not have crucial functions, although a convincing biological assay to assess such a function has not yet been developed.

To address this issue, transgenic animals deficient in endogenous Rb and overexpressing transgenic hRB have been used. Mice lacking any functional Rb protein die in utero by day 14.5 of gestation (E14.5), with characteristic abnormalities in erythrocytes, hindbrain, and dorsal root ganglia (12, 34, 36, 37). Erythrocytes in $Rb^{-/-}$ embryos fail to enucleate; neurons in the hindbrain and dorsal root ganglia fail to exit cell cycle properly, continue to divide aberrantly, and die in excessive numbers by apoptosis (37). Mice heterozygous for the disrupted *Rb* allele ($Rb^{+/-}$ mice) develop normally in utero and postnatal life but succumb to pituitary melanotroph tumors with nearly 100% penetrance (30). The melanotroph tumor cells lose the remaining copy of wild-type *Rb* early during carcinogenesis and progress synchronously to end-stage tumors (47). Rb-deficient mice thus demonstrate the importance of Rb in embryonic development and cell-type specific differentiation, as well as in tumor suppression, and serve as excellent models for exploring the functions of Rb in vivo.

Genetic rescue of both the embryonic lethal phenotype in $Rb^{-/-}$ mice and melanotroph carcinogenesis in $Rb^{+/-}$ mice can be accomplished by breeding $Rb^{+/-}$ mice with mice expressing h*RB* under transcriptional control of its own promoter (4). Mice overexpressing RB are dwarves compared with their

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littermates (4). The degree of growth retardation, which is evident in embryonic stages of development and persists throughout the lives of the animals, is inversely related to the amount of total RB protein expressed. Rescue of the lethal phenotype in $Rb^{-/-}$ embryos is also dependent on the dose of RB protein and requires a threshold amount greater than or equal to the amount expressed by $Rb^{+/-}$ mice (7). Prevention of pituitary melanotroph tumors in $Rb^{+/-}$ mice, in contrast, depends not on absolute protein amount but on the number of independent genetic loci from which functional RB protein is expressed (7). Together, these studies demonstrate that an appropriately regulated h*RB* transgene can substitute for the essential functions of m*Rb* in vivo.

To explore the role of the RB N-terminal region, in vivo systems identical to those used previously in mice expressing full-length *RB* transgenes were employed to generate transgenic lines carrying different RB N-terminal deletion mutants under transcriptional control of the *RB* promoter (4, 7). The role of the RB N-terminal domain in growth retardation, normal embryonic development, and tumor suppression was then assessed. This experimental approach closely mimics the effects of RB in a natural host setting.

MATERIALS AND METHODS

Generation of the h*RB*Δ*N* transgene and production of transgenic mice. In the hRB protein, amino acid residues 195 to 224 in the N-terminal globular domain and 319 to 340 in the R globular domain (25) were found to be significantly homologous to similar regions in human p107 and p130, the two other members of the RB family of proteins (44). To aid in deleting these two sequences in the *RB* cDNA, two oligonucleotides [RBΔN(1) sense (44-mer, 5′-GCATTGGTGC TAAAAGTTTCTTATTTTATTAAACTCTCACCTCC-3′)] and RBΔN(2) antisense (46-mer, 5′-CTATAGAATCAGTCTGAAGAGTTTTAGAAAGATTT TCAACCTCTGG-3′) were synthesized and used in a series of PCRs. First, we took advantage of a unique *Eco*RI site in the full-length *RB* cDNA (2.8 kb) from the expression plasmid pRB44-2 (55). The 5′ *Bam*HI-*Eco*RI (0.9 kb) and 3′ *Eco*RI-*Bam*HI (1.9 kb) fragments from pRB44-2 were excised and subcloned individually into pGEM-3Z (Promega, Madison, Wis.) and pBSK(SK+) (Stratagene, San Diego, Calif.), respectively. Both vectors contain vector T3, T7, and SP6 sequences for use in PCR cloning.

The resulting plasmids were designated pGEM-RB(0.9) and pBSK-RB(1.9). To generate deletion mutations in the N-terminal domain of RB, pGEM-RB(0.9) was used as the template DNA for PCR, using RBΔN(1) and T7 primers. The PCR was performed with 35 cycles (94°C for 30 s, 57°C for 1 min, 72°C for 3 min) and final extension at 72°C for 10 min. The resulting PCR product (~250 bp) was then used as primer in combination with SP6 primer in a second PCR. The product resulting from this reaction (0.9 kb) was digested with *Hin*dIII and *Eco*RI and subcloned into pBSK-RB(1.9) to generate pBSK-RBΔN1. To construct the deletion mutant in the Rb R domain, plasmid pBSK-RB(1.9) was used as template DNA along with RBΔN(2) and T7 primers in another PCR. The resulting PCR product (~100 bp) was then used as primer, in combination with T3 primer, in a second PCR. The product resulting from this reaction was digested with *Nde*I and *Eco*RI and then subcloned into pGEM-RB(0.9) to generate pGEM-RBΔN2. The 0.9-kb *Bam*HI-*Eco*RI RB cDNA fragment from pGEM-RBΔN2 was excised and replaced with the mutant fragment (0.83 kb) excised from pBSK-RBΔN1 to generate pGEM-RBΔN3. The final RB N-terminal deletion mutant plasmid, pBSK-RBΔN4, which has a deletion of codons 76 to 181 from the *RB* cDNA, was simply modified from a previously constructed, in-frame deletion mutant, N-HE (15, 25), by subcloning into the pBSK vector.

To generate the four *RB* N-terminal deletion mutant transgenes, plasmid pBR-RBproRB, containing the full-length h*RB* cDNA flanked by the 1.6-kb h*RB* promoter sequence (29) and the 1.6-kb bovine β-globin gene polyadenylation sequence (4), was first subcloned into the pBSK vector. The entire 2.8-kb, full-length *RB* cDNA was then excised by *Bam*HI digestion and replaced in separate reactions with each of the four *RB* cDNA deletion mutants to generate four final transgene constructs, pRBproRBΔN1pA, pRBproRBΔN2pA, pRBproRBΔN3pA, and pRBproRBΔN4pA. All of the DNA fragments generated by PCR were confirmed to be free of additional, unintended mutations, either by DNA sequencing or by subcloning and replacing wild-type sequences outside the deleted regions.

*Sst*I-*Hin*dIII fragments containing each of the four *RB*Δ*N* transgenes were isolated, purified, and microinjected into fertilized eggs of (C57BL/6J × BALB/c) F_1 mice according to established methods (27).

Genomic Southern blotting. Transgenic founder mice were identified initially by genomic Southern hybridization using the full-length *RB* cDNA (2.8 kb, excised by *Bam*HI digestion from the transgene vectors) as probe (4). The same technique was used with selected animals from subsequent generations (see Fig. 2) to assess differences in the integration sites of various founder lines, using the entire transgene (6.0 kb) as probe. The full-length *RB* cDNA (2.8 kb) alone was used to quantify relative cDNA copy number.

Genotype analysis by PCR. The genotypes of offspring from crosses between the transgenic founders, littermate wild-type mice of the same strain, and m$Rb^{+/-}$ (m$Rb^{20neo+/-}$ [36]) mice were determined by PCR with specific sets of primers. Mouse genomic DNA was extracted from tail or digit biopsies. The primer sets for distinguishing between mutant (exon 20 *neo*) [*20neo*]) and wild-type m*Rb* genes have described previously (47). The presence of a *neo* cassette in the m*Rb* gene disrupted by gene targeting results in amplification of a PCR product 236 bp long, whereas the same primers in identical conditions amplify a fragment 151 bp long from the wild-type gene. To discriminate between animals with and without h*RB* cDNA sequences, two sets of PCR primers were synthesized: for endogenous m*Rb*, mRb,ex15,5′ (5′-CGGAAGAAGAACGTTTGTCC ATTCA-3′) and mRb,ex16,3′ (5′-CGCTGCTATACGTAGCCATTACAAC-3′); for the h*RB*Δ*N* transgene, hRB,ex15,5′ (5′-CGGAAG-AAGAACGATTAT CCATTCA-3′) and hRB,ex16,3′ (5′-CGCTGCTATATGTGGCCA-TTA-CAA C-3′). The PCR was performed with 35 cycles (94°C for 30 s, 60°C for 1 min, 72°C for 2 min) and final extension at 72°C for 10 min. An 87-bp intron between exons 15 and 16 in the endogenous m*Rb* gene results in amplification of a fragment (196 bp) larger than the corresponding fragment (109 bp) amplified from h*RB* cDNA; mice carrying the h*RB* or *RB*Δ*N* transgene were identified when both 109- and 196-bp PCR products were detected (see Fig. 5B).

Quantification of RB and RBΔN protein expression by immunoprecipitation and Western blot analysis. The procedures have already been described in detail (4, 7, 23). The only modification in the experiments reported here was the use of a different antibody to immunoprecipitate and recognize both hRB and mRb. Rabbit anti-RB peptide antibody C-15 (Santa Cruz Biochemicals, Santa Cruz, Calif.) recognizes hRB amino acids 914 to 928. Mouse monoclonal antibody (MAb) 11D7 recognizes specifically hRB. For immunoprecipitation, both MAb 11D7 and C-15 were used at a concentration of 1 μg/mg of total protein lysate in a 1-ml total volume. The antibody dosage was calculated to be in significant molar excess compared with Rb protein. Neither hRB nor mRb was detectable by Western blotting after reprecipitation from supernatants collected after the first round of immunoprecipitation. As an internal loading control in each Western blot, the abundant and constitutively expressed nuclear matrix protein, p84, was detected by direct immunoblotting with MAb 5E10 (15), at a concentration of 1 μg/mg of total protein lysate/ml. Primary antibodies on immunoblots were developed by using appropriate goat anti-rabbit or rabbit anti-mouse immunoglobulin G conjugated to alkaline phosphatase (Promega). Bound proteins were visualized by using 5-bromo-4-chloro-3-indolylphosphate toluidinium and nitroblue tetrazolium (Promega). Densitometry allowed quantification of relative protein amounts compared to the internal standards.

Histology and in situ apoptosis analysis. Mouse embryos (E18.5) collected from h*RB*Δ*N*$^{+/-}$ m$Rb^{+/-}$ intercrosses were processed for histological examinations as described previously (42). The in situ apoptosis TUNEL (terminal deoxynucleotide transferase-mediated dUTP-biotin nick end labeling) assay (19), its slight modification, and bromodeoxyuridine (BrdU) labeling have also been described (47).

Statistical analyses. χ^2 analyses were performed to compare observed and expected numbers of offspring according to Mendelian ratios. For comparisons of life spans, means, medians, and standard errors were computed, and then log-rank (Mantel-Haenzel) tests were performed. Mean values were compared by two-tailed analysis of variance, with significance assigned to P values of <0.05. All statistical analyses were performed by using InStat or Prism 2.0 software (GraphPad, Inc., San Diego, Calif.).

RESULTS

Generation of RBΔN constructs and transgenic mice. In designing the *RB* N-terminal deletion mutant constructs, strategic regions for mutation were sought. Both small sequences and large regions were deleted in four different constructs (Fig. 1A). The large deletion, RBΔN4 (amino acids 76 to 181), has previously been shown to be important for interacting with p84 (15). Smaller deletions, RBΔN1 (amino acids 195 to 224) and RBΔN2 (amino acids 319 to 340), were made based on sequence homology between RB and the related proteins p107 and p130 (17, 22, 41, 44) (Fig. 1B). The RBΔN2 deletion does not fall within the N-terminal globular domain but rather is within the smaller and more distal R globular domain (25). The RBΔN3 construct combines both the ΔN1 and ΔN2 deletions. None of the RBΔN mutations removed a consensus cdc2 phosphorylation site (40) from the resultant protein. All *RB*Δ*N* cDNA constructs were sequenced and cloned into a vector containing the same 1.6-kb human *RB* 5′ flanking sequence

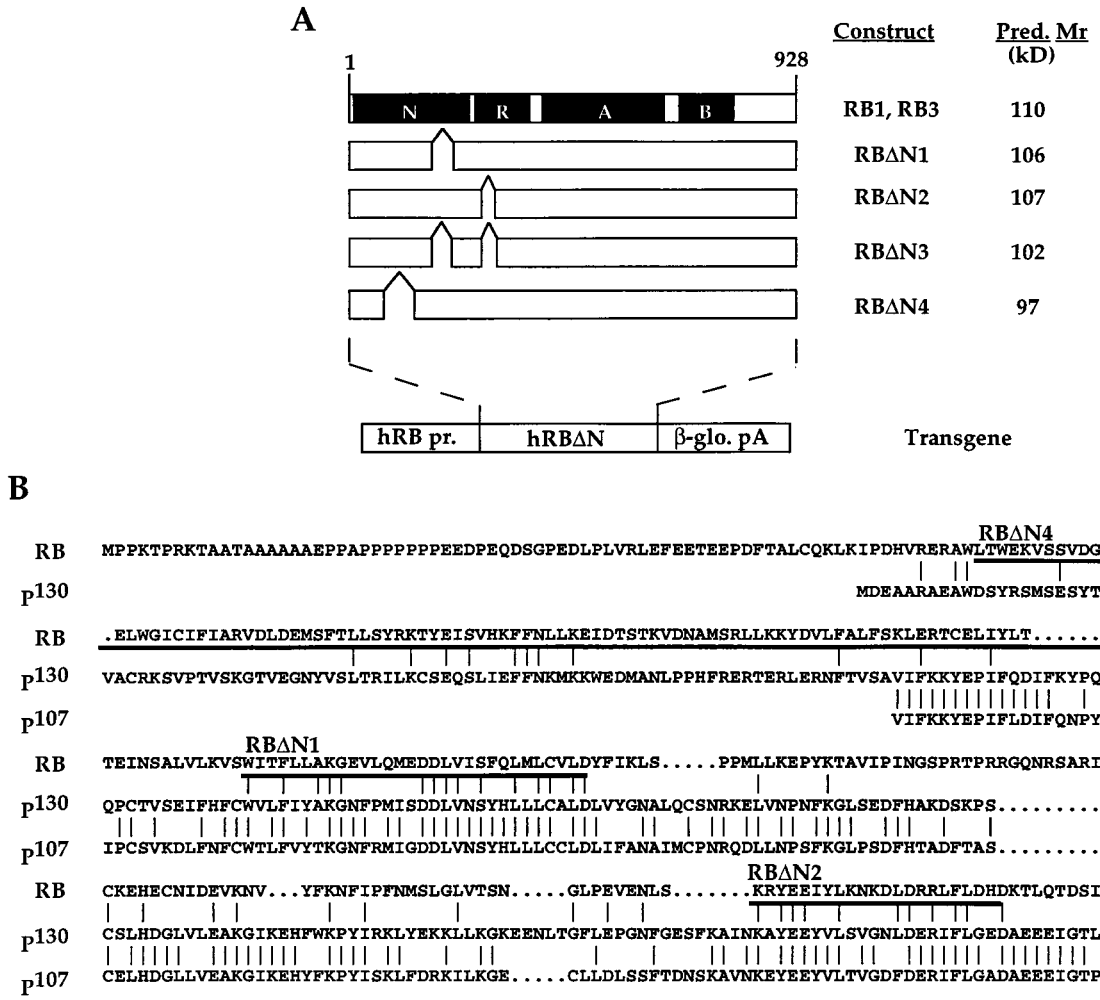

FIG. 1. RB amino-terminal deletion mutant transgene constructs. (A) *RBΔN* transgenes and predicted molecular masses (Mr) of the encoded proteins. The full-length RB protein (pr.) can be divided into four protease-resistant, globular domains (N, R, A, and B) and a negatively charged C terminus (25). β-glo. pA, β-globin polyadenylation sequence. (B) Comparison of the primary N-terminal sequences of members of the hRB family of proteins. Vertical lines indicate conserved sequences. Underscored residues show the deletions made to produce the RBΔN1, RBΔN2, and RBΔN4 constructs. In the RBΔN3 construct, the sequences shown for RBΔN1 and RBΔN2 were both deleted.

(29) and 3′ human β-globin polyadenylation sequence used to generate transgenes expressing full-length hRB (4). Except for the various N-terminal deletions, *RBΔN* transgenes were thus designed to be identical to full-length h*RB* transgenes. To generate transgenic F_0 founders, purified DNA was then injected into fertilized ova of C57BL/6× BALB/c mice.

Multiple copies of *RBΔN* transgenes integrate into host DNA at different sites. From all transgenic animal lines produced, two founders were selected for each *RBΔN* construct. The offspring of these mice were then compared with their wild-type littermates and with the offspring of two founder mice carrying stable, full-length h*RB* transgenes [RB3 and RB1-(1)] (4). To determine the copy numbers and chromosomal integration sites in the founder lines, equivalent amounts of *Bam*HI-digested genomic DNA were subjected to Southern blotting analysis. The blots were probed either with the entire (6.0-kb) *RB* transgene (Fig. 2A) or with the *RB* cDNA (2.8 kb) alone (Fig. 2B). The representative blot in Fig. 2A shows that different founder lines integrated the same transgenes at distinct chromosomal sites (notice different bands flanking the *RB* coding sequences in each lane). Densitometric analysis of the blot in Fig. 2B and similar blots from other animals in the same founder lines allowed quantitation of the copy number. RBΔN mice were compared with the RB3 and RB1-(1) animals carrying three copies and one copy of the human *RB* cDNA, respectively (4, 7). All founder lines chosen for subsequent analysis carried several copies of RBΔN cDNA (range, 4 to 28) and transmitted them stably to their offspring.

RB proteins with N-terminal deletions are expressed in mouse tissues. Expression of wild-type RB protein from h*RB* transgenes, with few exceptions, roughly correlates with *RB* cDNA copy number and directly correlates with the degree of growth retardation in the whole animal (4). To allow appropriate comparisons between the phenotypes of animals expressing identically regulated full-length RB and RBΔN mutant proteins, relative protein expression was measured in tissues from all transgenic mice (Fig. 3). RB protein in mice carrying identically regulated, full-length transgenes has been shown to be expressed ubiquitously (4). Brain and lung tissues express RB primarily in its hypophosphorylated form and therefore are the best tissues from which to quantitate RB protein and compare different founder lines (4, 7). We compared total (hRB plus mRb) and hRB protein amounts by immunoprecipitation, Western blotting, and densitometric

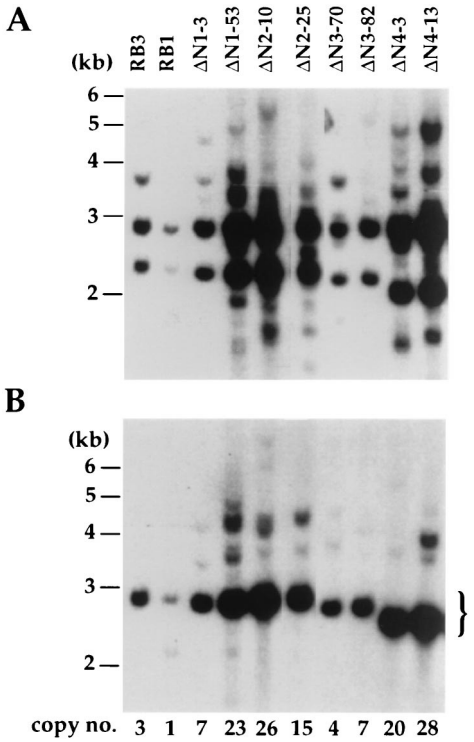

FIG. 2. Quantification of h*RB*Δ*N* cDNA copy number in transgenic founder lines by genomic Southern blot analysis. (A) Genomic DNA cut with *Bam*HI and probed with the entire, full-length *RB* transgene (1.6-kb *RB* promoter, 2.8-kb *RB* cDNA, and 1.6-kb bovine β-globin gene polyadenylation sequence [4]). (B) DNA digested with *Bam*HI and probed with human *RB* cDNA alone. Numbers indicate quantification of *RB* cDNA copy number by densitometry, relative to the internal controls RB3 and RB1-(1) (4, 7). Copy numbers represent the means from two separate blots from different sets of animals in the same founder lines.

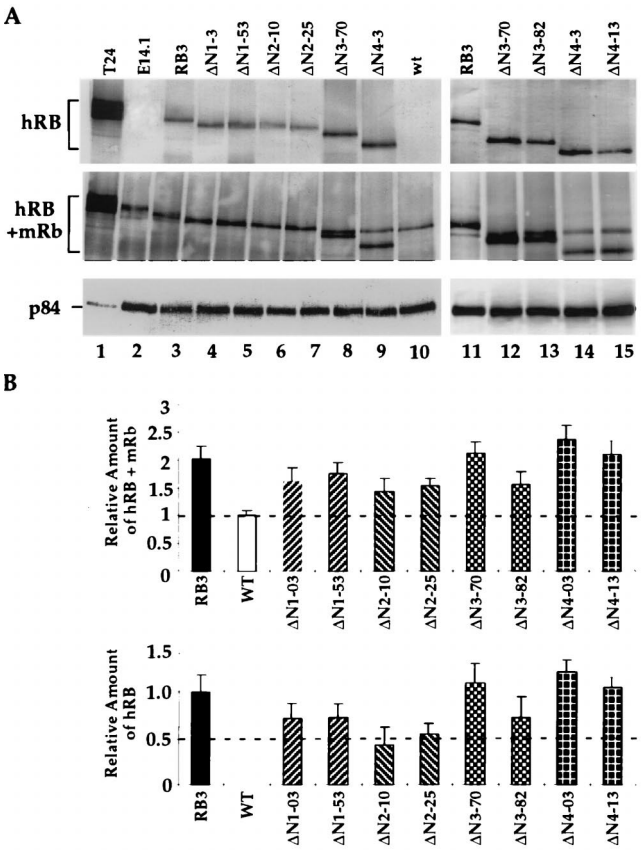

FIG. 3. Expression of the transgenic RBΔN proteins. (A) Protein extracts from the lungs of adult animals were immunoprecipitated by MAb 11D7 (upper panels), which recognizes a C-terminal hRB epitope specifically (7), or by rabbit polyclonal antibody C-15 (middle panel), which recognizes with approximately equal affinity epitopes in the carboxyl-terminal portions of both hRB and mRb. Immunoprecipitates in both cases were then separated by SDS-PAGE, transferred quantitatively to membranes, and immunoblotted with antibody C-15. The constitutively expressed protein p84 (15) served as an internal control for protein loading (lower panel). T24 human bladder carcinoma cells demonstrated the migration positions of hypo- and hyperphosphorylated forms of hRB, and mouse embryonic stem cells (E14.1) demonstrate the migration positions of mRb. Representative blots are shown. (B) Relative RB protein expression in the various transgenic lines. Three to five separate blots from three different sets of animals and two different organs (brain and lung) were analyzed and quantitated by densitometry to obtain relative protein expression (mean ± standard deviation). For total (hRB plus mRb; upper panel) RB expression, tissues from wild-type (wt) mice serve as the normals. Additional RB expression relative to normal (above the dotted line) was then compared among the various founder lines. For hRB expression (lower panel), RB3 animals, which can rescue m*Rb*$^{-/-}$ mice from embryonic lethality (7), express transgenic, full-length RB protein at maximum amounts (2× normal [4, 7]). The dotted line here then represents the minimum relative amount of full-length, transgenic protein required for rescue of the embryonic lethal phenotype (7).

analysis in representative tissues from several different founder lines. This method has been proven to be reproducible and reliable in previous studies (4, 7). Examination of several blots from separate animals and different tissue types gave similar results, from which histograms and standard errors could be derived (see Materials and Methods) (Fig. 3B). The blots demonstrate that the RBΔN proteins are indeed expressed in vivo from the transgenes, in amounts comparable to the amounts of full-length protein expressed by the previously described RB3 line. The RB3 line expresses sufficient protein to completely rescue the embryonic lethal phenotype in *Rb*$^{-/-}$ embryos (4, 7) and served in the present studies as an internal control.

Stunted growth in mice expressing *RB*Δ*N* transgenes. Previous observations in transgenic mice overexpressing the full-length hRB showed significant growth retardation compared with wild-type littermates (4). The degree of dwarfism was directly dependent on the relative amount of RB protein expressed, with the founder lines expressing RB most abundantly (RB3 mice, expressing RB at levels about twice normal) being ~70% of the size of their nontransgenic littermates. Using these RB3 mice for comparison, we examined the relative body mass of the RBΔN transgenic mice in the *Rb*$^{+/+}$ background. Representative growth curves of male RB3 and RBΔN-70 mice and wild-type, sex-matched littermates are illustrated in Fig. 4A. Although all mice expressing the *RB*Δ*N* transgenes were dwarves (Fig. 4B), the degree of dwarfism observed in these transgenic mice was not as pronounced as in the RB3 mice. This attenuation of the growth retardation occurred despite nearly equivalent relative RB protein amounts from the transgenes with larger N-terminal deletions and from RB3 (Fig. 3). Therefore, the compromised growth retardation in the RBΔN transgenic mice reflects the effects of the RB N-terminal deletions and suggests that an intact N-terminal region is essential for growth regulation by RB in the whole animal (4).

***RB*Δ*N* transgenes cannot rescue embryonic lethality in m*Rb*$^{-/-}$ mice.** To test whether any of the RBΔN mutants could rescue *Rb*$^{-/-}$ embryos, we determined the genotypes of the offspring (9 to 21 days old) from F_1 h*RB*Δ*N*$^{+/-}$ m*Rb*$^{+/-}$ intercrosses. No live offspring carrying the genotype h*RB*Δ*N*$^{+}$ m*Rb*$^{-/-}$ were found from such matings in any of the four RBΔN lines (Table 1). Pregnant females were examined daily

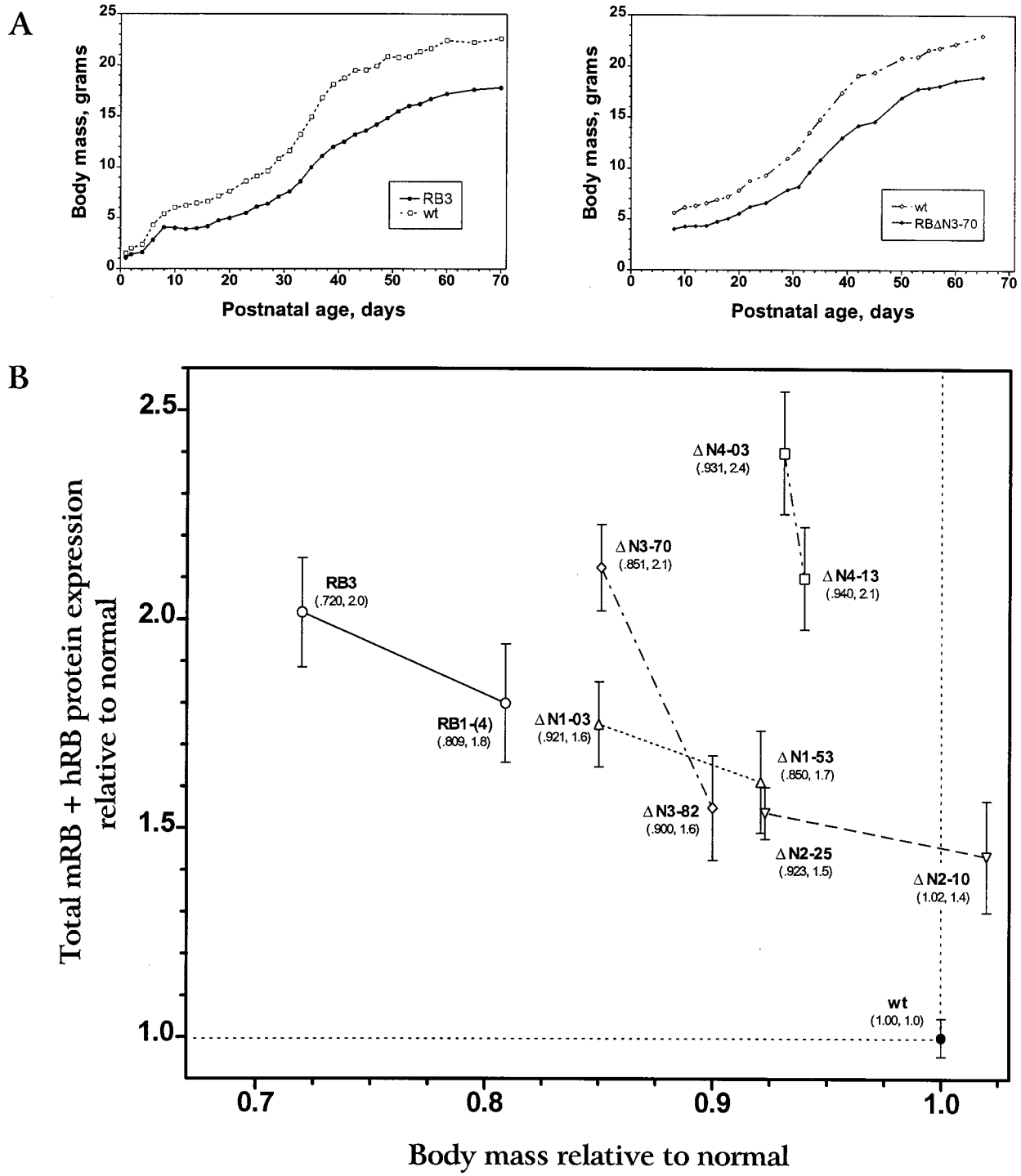

FIG. 4. Growth retardation in mice expressing full-length h*RB* and the four h*RBΔN* transgenes. (A) Representative growth curves comparing transgenic and wild-type (wt) littermates, matched for sex. (B) Body mass as a function of total (hRb plus mRb) RB expression relative to normal (wild-type) littermates. Plotted values and bars represent means ± standard deviation. Mean values for total mRb plus hRB protein expression relative to normal and body mass relative to normal are shown in parentheses for each datum point. $n = 3$ to 5 for relative total Rb expression; $n = 9$ to 33 for body mass relative to age- and sex-matched, wild-type littermates.

so that the number of offspring in particular litters could be counted within 24 h after birth. In this way, animals that died perinatally were not omitted from analyses. These data indicate that the N-terminal region of RB is required for complete mouse embryonic development.

To determine whether any of the embryos with the genotype h$RB\Delta N^{+}$ m$Rb^{-/-}$ could survive to late embryonic stages, after E14.5, when m$Rb^{-/-}$ embryos die (36), we examined the embryos from several F_1 h$RB\Delta N^{+/-}$ m$Rb^{+/-}$ intercrosses. A representative example of one litter at E18.5 is illustrated in Fig. 5A. The

TABLE 1. Genotypes from F_2 offspring from intercrosses of F_1 mice with the genotype h*RB*$^{+/-}$ m*Rb*$^{+/-}$

Founder	Total	h*RB*$^{-/-}$ m*Rb*$^{-/-}$	h*RB*$^{+/-}$ or h*RB*$^{+/+}$ m*Rb*$^{-/-}$	h*RB*$^{-/-}$ m*Rb*$^{+/-}$	h*RB*$^{+/-}$ or h*Rb*$^{+/+}$ m*Rb*$^{+/-}$	h*RB*$^{-/-}$ m*Rb*$^{+/+}$	h*RB*$^{+/-}$ or h*RB*$^{+/+}$ m*Rb*$^{+/+}$	Significance by χ^2 analysis analysis (P)[a]
No transgene	247	0[b]		166		81		
hRB1-(4)[c]	122	0	17	22	51	14	18	NS
hRB3[c]	76	0	10	11	33	8	14	NS
hRBΔN1-03	65	0	0	12	31	7	15	0.0049
hRBΔN2-25	50	0	0	6	28	4	12	0.0174
hRBΔN3-82	84	0	0	9	48	5	22	0.0005
hRBΔN4-03	39	0	0	6	19	5	9	0.0432
hRBΔN4-13	60	0	0	12	28	6	14	0.0077
Expected no.	15	0[b]	3	2	6	1	3	

[a] Observed numbers compared with expected numbers.
[b] The h*RB*$^{-/-}$ m*Rb*$^{-/-}$ genotype results in an embryonic lethal phenotype.
[c] Some data published previously (7) were combined with current data.

fetuses partially rescued by the h*RB*Δ*N1* transgene (Fig. 5A5) showed abnormal histology in several cell types, including dorsal root ganglion neurons and erythrocyte precursors (Fig. 6), the same types found to be abnormal in the *Rb*$^{-/-}$ embryos that die earlier during development (36). Fetuses with the genotype h*RB*Δ*N*$^{+}$ m*Rb*$^{-/-}$ also had abnormal striated muscles (Fig. 7). Although myoblasts fused and differentiated into myotubes in these fetuses, the structure of the muscles was distinctly abnormal: the fibers were shorter, fewer in number, and less dense. Some of the nuclei in the myotubes were larger in h*RB*Δ*N*$^{+}$ m*Rb*$^{-/-}$ embryos than in normal littermates (Fig. 7A2 and C2 and data not shown). The abnormal myocytes also died in excessive numbers by apoptosis (Fig. 7A4, B4, and C4). Results similar to those illustrated for h*RB*Δ*N1*$^{+}$ m*Rb*$^{-/-}$ embryos were also observed in litters from h*RB*Δ*N3* founder lines at E17.5 and E18.5 (data not shown). These results indicate that *Rb*$^{-/-}$ embryos expressing hRBΔN, in amounts equivalent to those expressed by similar mice expressing full-length RB protein, are unable to complete normal embryonic development. They therefore suggest the importance of the RB N-terminal region for the structural integrity and function of the entire protein during embryogenesis.

***RB*Δ*N* transgenes partially suppress pituitary melanotroph tumors in m*Rb*$^{+/-}$ mice.** F_1 mice with the genotype *RB*Δ*N*$^{+/-}$ m*Rb*$^{+/-}$ were also monitored throughout their lives for the development of tumors. Mice with the genotype *Rb*$^{+/-}$ and no transgene develop pituitary melanotroph tumors with 100% penetrance and die at a mean age of about 350 days (24, 51, 52, 56). Animals carrying the full-length h*RB* transgene in addition to a single allele for the wild-type, endogenous m*Rb* gene (h*RB3*$^{+/-}$ m*Rb*$^{+/-}$ mice), in contrast, are completely free of tumors and live as long as their wild-type counterparts (>700 days). On the other hand, mice with the genotype h*RB*Δ*N*$^{+/-}$ m*Rb*$^{+/-}$ developed the same melanotroph tumors, but the tumors progressed with a much longer lag time (Table 2). Some animals with these same genotypes, like those with the genotype *Rb*$^{+/-}$ (56), also developed thyroid medullary carcinomas (data not shown).

Interestingly, tumors in the mice with the genotype h*RB*Δ*N*$^{+/-}$ m*Rb*$^{+/-}$ lose the remaining copy of the wild-type *Rb* gene but retain the h*RB*Δ*N* transgene (Fig. 8A). In contrast, the melanotroph tumors from mice rescued from embryonic lethality by full-length h*RB* transgenes [i.e., mice with the genotypes h*RB3*$^{+/-}$ m*Rb*$^{-/-}$ (Fig. 8A) and h*RB1*-(*4*)$^{+/-}$ m*Rb*$^{-/-}$ (data not shown)] lose the entire transgene (7). To determine whether the *RB*Δ*N* transgene was expressed in the tumor cells, the medullary thyroid carcinomas, which were massive in a few mice, were examined for transgenic RBΔN protein expression. In the same tumors analyzed by PCR for loss of the wild-type allele of m*Rb*, hRBΔN protein expression could be demonstrated (Fig. 8B) but endogenous mRb protein could not (data not shown). These results suggest that the hRBΔN proteins do not completely prevent tumor formation. Figure 9 summarizes the different genotypes in mice and tumors that develop because of deficiency in functional hRB or mRb. Tumors are suppressed only when genes encoding fully functional mouse or human RB are present on at least two independent genetic loci.

DISCUSSION

N-terminal regions of RB are important for functional integrity in vivo. The studies reported here demonstrate the importance of an intact RB N-terminal region, not only for functions during mouse embryogenesis and development but also for tumor suppression in vivo. All four different hRBΔN transgenic proteins, in eight different founder lines, resulted in similar phenotypes. Subtle quantitative differences in developmental growth at the level of the organism were found with the different deletions, but in other biological assays, the RBΔN proteins functioned equivalently: none was sufficient for total rescue of the embryonic lethal phenotype in *Rb*$^{-/-}$ mouse embryos or for complete tumor suppression. Furthermore, mice from the same strain carrying inactivating m*Rb* mutations but no h*RB* transgenes (m*Rb*$^{+/-}$ and m*Rb*$^{-/-}$), and mice carrying full-length h*RB* transgenes expressed by an identical h*RB* promoter (h*RB3*$^{+/-}$ m*Rb*$^{+/-}$ and h*RB*Δ*N*$^{+/-}$ m*Rb*$^{-/-}$), always served as controls. Thus, all studies were performed with cohorts of animals whose natural history has been carefully studied (4, 7, 37, 47). The multiple independent lines also served to control the experiments, and so the effects observed when we compared mice expressing h*RB*Δ*N* transgenes with mice expressing full-length h*RB* transgenes were unlikely to have resulted solely because of different transgene chromosomal integration sites or because of subtle differences in transgene expression. The differences occurred mainly because of the N-terminal deletions. The results presented contrast sharply with reports based on cell culture and in vitro assays, which have suggested that N-terminally truncated RB proteins are equivalent or superior to wild-type RB in tumor and growth suppression (18, 50, 57, 58). Unlike the present experiments, however, these previous studies were performed largely in systems that may not have mimicked complex protein-protein interactions and regulatory processes as they work in vivo.

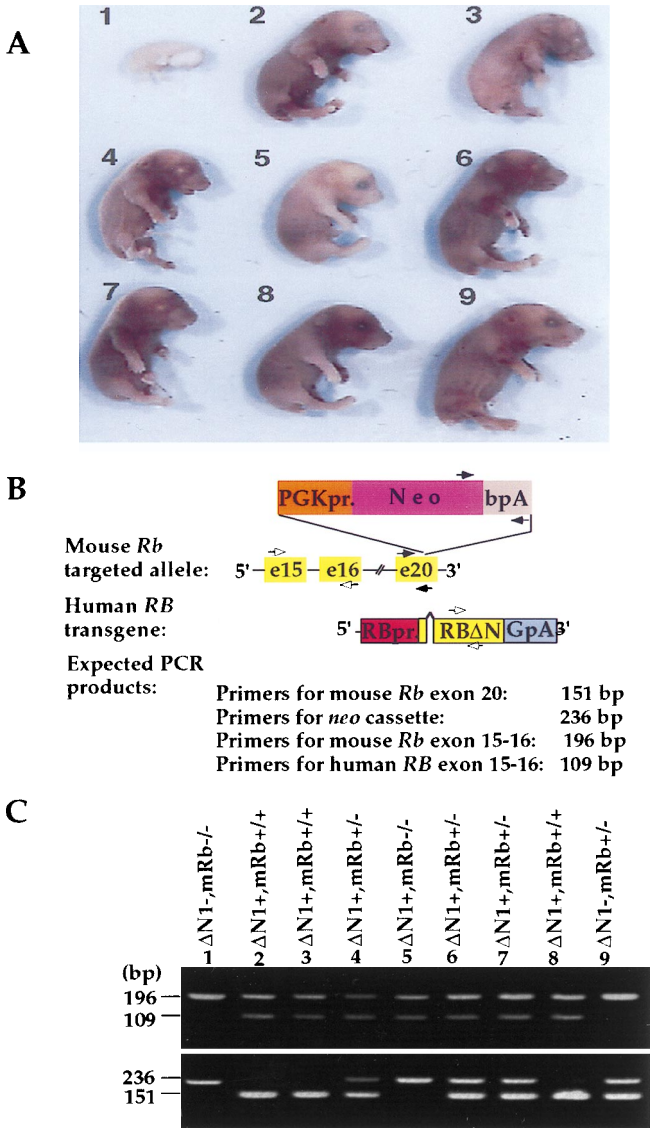

FIG. 5. Abnormal development in h*RB*Δ*N1*$^{+/-}$ m*Rb*$^{-/-}$ embryos at E18.5. (A) Whole-mounted littermates from an intercross between F_1 mice with the genotype h*RB*Δ*N1*$^{+/-}$ m*Rb*$^{+/-}$. Embryo 1, with the genotype h*RB*Δ*N1*$^{-/-}$ m*Rb*$^{-/-}$, is small, pale, and already largely reabsorbed. All other embryos are grossly normal except for embryo 5, which is recognizably pale and has an abnormally straightened nuchal region. (B and C) Analysis of genotypes by PCR. (B) In the PCRs, specific oligonucleotide primers were used to discriminate between the wild-type exon 20 (151 bp) and the mutant exon 20*neo* (236 bp) in the m*Rb* gene (47). Different oligonucleotide primers were used to determine the presence or absence of an 87-bp intron between exons 15 and 16 and thus discriminate between fragments amplified from the endogenous m*Rb* gene (196 bp) and from the h*RB* cDNA (109 bp). In samples containing no transgene, the 109-bp fragment is not amplified with the conditions chosen for PCR (see Materials and Methods for details). (C) Genomic DNA sequences were amplified from carefully collected biopsies of the embryos in panel A.

Growth suppression. Compared with the full-length transgenic RB, all of the RBΔN mutant proteins caused less pronounced growth retardation (Fig. 4B) when expressed in equivalent (ΔN1 and ΔN2) or greater (ΔN3 and ΔN4) amounts (Fig. 3). These results suggest that the entire N-terminal region of RB is required for developmental growth suppression of the organism and contrast with the equivalent or enhanced growth suppression reported for RB proteins with truncated N termini (20, 26, 50). It is interesting that little or no additive effect in relative growth suppression was found when the RBΔN1 and RBΔN2 mutants were compared with the RBΔN3 mutant, which is missing both N1 and N2 regions. The lack of an additive effect suggests that ΔN1 and ΔN2 mutations produce identical developmental growth responses via slightly different mechanisms or that the different mutations ultimately work through the same effectors. The mechanism by which RB overexpression causes growth retardation in vivo is not yet known. Studies of growth-retarded mice deficient for insulin-like growth factors I and II have suggested that proliferative events during ontogeny generate fewer cells in dwarf mice than in wild-type littermates and that the ultimate size of the animals depends on the number of precursor cells formed during a strictly defined period of development (1, 13, 16). Overexpression of hypophosphorylated RB might reduce the relative number of cells in the developing mouse by delaying progression through the G_1 phase of the cell cycle at a time when the cells are supposed to proliferate rapidly. Alternatively, expression of excess RB may lead to early withdrawal from the cell cycle and earlier differentiation of key cells during development, such that the cells might no longer respond normally to signals for growth and proliferation.

Terminal differentiation of several cell types during prenatal development. Besides having a role in negative regulation of progression through the G_1 phase of the cell cycle, RB positively regulates differentiation (reviewed in reference 9). Studies of Rb-deficient mice have shown directly that Rb is fundamental for the maturation and terminal differentiation of erythrocytes and neurons in the dorsal root ganglia and developing hindbrain (12, 34, 36, 37). Prolongation of development in *Rb*$^{-/-}$ embryos partially rescued by human *RB*Δ*N* transgenes extends previous studies and uncovers other cell types that require entirely intact RB for normal differentiation in late developmental stages. For example, in the last embryonic stage that can be assessed in *Rb*$^{-/-}$ mice without *RB* transgenes (E14.5 to E15.5), peripheral erythrocytes are abnormally nucleated and dorsal root ganglion neurons undergo excessive apoptosis. In *Rb*$^{-/-}$ mouse embryos extended to later developmental stages (E17.5 to E18.5) by hRBΔN, the majority of the erythrocytes remain nucleated, mature erythrocytes are relatively scarce, and the embryos are noticeably pale (Fig. 5A5 and 6B). These results strongly suggest that RB, including an intact N-terminal region, is crucial for the terminal differentiation of the erythroid lineage. Furthermore, although most of the neuronal cells in dorsal root ganglions survive to stages beyond E14.5 in the same embryos, they nonetheless develop in a disorganized manner and undergo apoptosis in *RB*Δ*N*$^{+/-}$ *Rb*$^{-/-}$ embryos (Fig. 6A); this finding suggests a different, later defect in neurons of the dorsal root ganglia. Whether RB directly participates in the process of terminal differentiation in these specific cell types or works indirectly through other cell types remains to be shown.

The role of RB in skeletal muscle differentiation has been explored more thoroughly than roles in erythrocyte and neuron differentiation, in part because the series of steps in skeletal muscle differentiation and the sequential activation of muscle-specific transcription factors have been well established (45). The abnormal skeletal muscle phenotype in m*Rb*$^{-/-}$ mouse embryos partially rescued by h*RB*Δ*N* transgenes occurs after myocytes have already fused into multinucleated myotubes by E17.5 and shows that the myotubes cannot maintain normal development thereafter. By E18.5, the skeletal muscles in embryos expressing only RBΔN protein, with their thin, fragmented fibers and large nuclei, die in excessive numbers by apoptosis (Fig. 7). The myotubes in these fibers still actively synthesize BrdU-labeled DNA at a developmental stage when

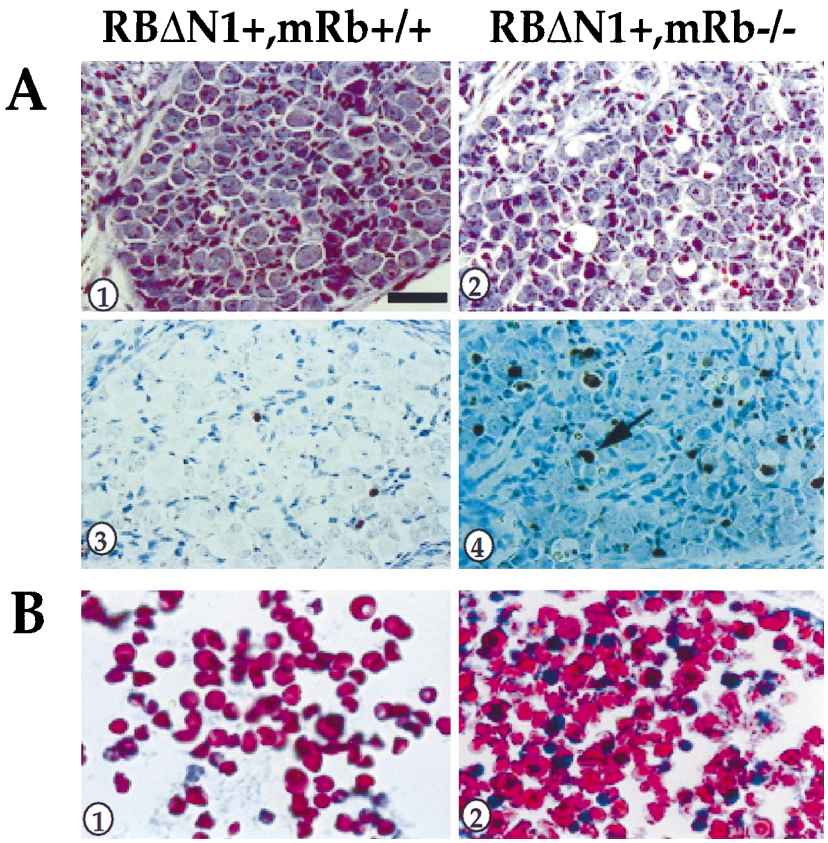

FIG. 6. Abnormal neuronal and hematopoietic tissues in m*Rb*$^{-/-}$ embryos partially rescued by h*RBΔN* transgenes. (A) Sections through the dorsal root ganglia of littermate embryos with the genotypes h*RBΔN1*$^{+/-}$ m*Rb*$^{+/-}$ (left) and h*RBΔN1*$^{+/-}$ m*Rb*$^{-/-}$ (right) at E17.5. A1 and A2, hematoxylin-and-eosin stain; A3 and A4, contiguous sections stained to reveal apoptotic cells (methyl green counterstain). (B) Peripheral blood, stained with hematoxylin and eosin. Scale bars, 50 μm (A) and 15 μm (B).

they should withdraw from the cell cycle (data not shown). These results are consistent with observations in culture systems: RB is required for activation of the transcription factors involved in the acquisition of differentiated myotube phenotype and for maintenance of the terminally differentiated state (21, 48, 53). Similar findings have been obtained for mouse embryos expressing small amounts of mRb protein from a transgene (59). RB is known to bind myoD and myogenin, but these skeletal muscle-specific transcription factors are expressed during earlier stages of muscle development (during or immediately following myoblast fusion into myotubes), not during late stages. Factors in addition to myoD or myogenin must be required for stages in myogenesis after fusion of myocytes into myotubes, and RB apparently is crucial for this process. RB may also have positive roles for terminal differentiation in cell types yet to be identified. Due to the limitations of the experimental systems, only abnormalities evident morphologically by E18.5 have been discovered so far. RB is also known to be crucial for postnatal differentiation events such as adipogenesis (11), but the roles of RB and its N-terminal regions in postnatal development and differentiation could not be assessed in vivo in embryos that lived only to E18.5.

Tumor suppression. The prevention of initiation and progression of a tumor involves not only suppression of cellular proliferation and apoptosis but aberrant differentiation as well. In our studies, mice with the genotype h*RBΔN*$^{+/-}$ m*Rb*$^{+/-}$ developed the same pituitary melanotroph tumors and medullary carcinomas of the thyroid as mice with the genotype m*Rb*$^{+/-}$ and no transgene, but the tumors progressed to end stages at much later ages in the animals rescued by the human *RBΔN* transgenes. Whether this delay in death from melanotroph tumor represents a lag in tumor initiation or in progression is not yet known. Some mice with the genotype h*RBΔN*$^{+/-}$ m*Rb*$^{+/-}$ were sacrificed at ages older than 1 year and had no histological evidence of tumors or foci of early atypical melanotroph proliferation (data not shown). Identifiable tumor foci are present in nearly 100% of mice with the genotype m*Rb*$^{+/-}$ by the age of 120 days, and loss of the remaining functional *Rb* allele in key somatic cells of mice with the genotype m*Rb*$^{+/-}$ has been shown to be an early, rate-limiting event in melanotroph carcinogenesis (47). Our observations in mice with the genotype h*RBΔN*$^{+/-}$ m*Rb*$^{+/-}$ suggest that expression of RBΔN transgenic protein delays the formation or reduces the relative number of tumor foci, rather than directly prolonging later steps in tumor progression. Counting of tumor foci in early stages of carcinogenesis will be necessary to differentiate reduced numbers of transformed clones from delayed initiation. Nonetheless, the loss of the endogenous wild-type m*Rb* gene and retention of the h*RBΔN* transgene in tumors from mice with the genotype h*RBΔN*$^{+/-}$ m*Rb*$^{+/-}$ clearly indicate that the N-terminally deleted RB proteins are insufficient for complete tumor suppression in vivo.

Mutations of RB N-terminal regions in humans. The RBΔN transgenic mice also begin to model and extend the few available cases of human retinoblastoma associated with N-termi-

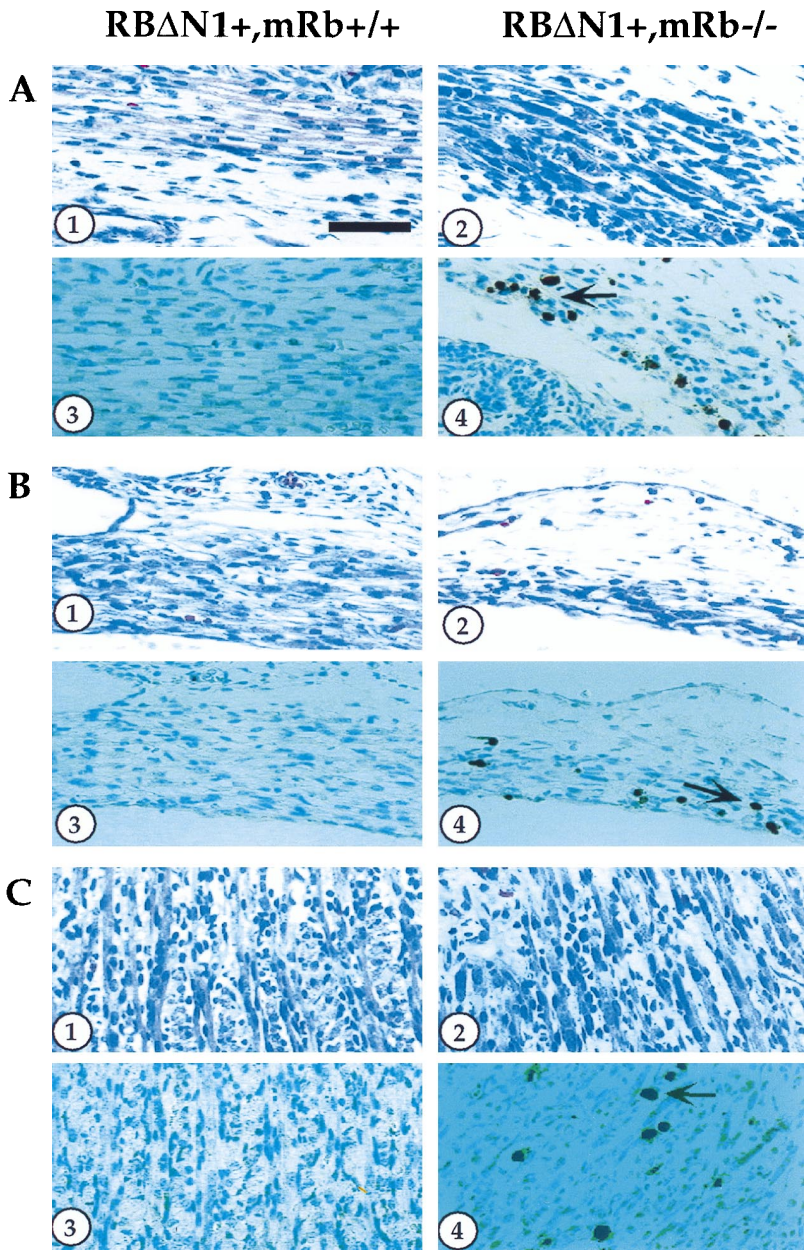

FIG. 7. Abnormal striated muscles in h*RBΔN1*$^{+/-}$ m*Rb*$^{-/-}$ embryos at E17.5. (A) Paraspinal skeletal muscle; (B) diaphragm; (C) tongue. In the partially rescued embryo (right), muscle fibers are shorter, more disorganized, and more heterogeneous than those in the normal littermate (left); some cells in the disorganized muscle fibers also contain abnormally large nuclei. In all cases, panels 3 and 4 were stained to reveal apoptotic cells in sections contiguous to the ones above them (methyl green counterstain). Scale bar, 50 μm.

nal mutations (14, 28). Some families with low-penetrance retinoblastoma carry deletions in exon 4 of *RB* (14, 43), and an exon 4 mutation has also been characterized in human tumor tissue (28). The data presented here for mice strengthen these few previous data and suggest further that the N-terminal domain of RB has a definite function in preventing tumors. Previous findings in vitro suggesting otherwise (57, 58) may not have been entirely adequate for determining structure-function relationships, due to the complicated actions of RB in vivo.

Interaction of the N terminus of RB with other regions of RB and with other proteins. The mechanism by which RB influences terminal differentiation has been elucidated in some cell lineages. Studies in cultured myoblasts and adipocyte precursors suggest that RB associates with and activates crucial, cell-type-specific transcription factors like myoD and myogenin in skeletal muscle (21, 53) and C/EBPs in adipocytes (10, 11). These transcription factors bind to the T-antigen binding domains in the C-terminal regions of RB, however, not to N-terminal regions. These transcription factors and E2F-1 bind to portions of RBΔN proteins just as well as they bind to portions of the wild-type RB in yeast two-hybrid assays (data not shown). Such in vitro studies therefore have not yet helped to define the biochemical reasons for the impaired function of RBΔN proteins in vivo. How then might N-terminal regions in RB be important for terminal differentiation, as our in vivo

TABLE 2. Prolonged life spans in F_1 mice with the genotype h*RBΔN*$^{+/-}$ m*Rb*$^{+/-}$ compared with those with the genotype m*Rb*$^{+/-}$

Genotype	Mean life span (days) ± SD[a]	No. with penetrance of pituitary tumor phenotype/no. studied
m*Rb*$^{+/-}$	346 ± 53	123/123
m*Rb*$^{+/+}$	743 ± 28	0/10
m*Rb*$^{-/-}$ h*RB1-(4)*$^{+/-}$	357 ± 29	12/12
m*Rb*$^{-/-}$ h*RB3*$^{+/-}$	361 ± 60	33/33
m*Rb*$^{+/-}$ h*RB1-(4)*$^{+/-}$	762 ± 30	0/8
m*Rb*$^{+/-}$ h*RB3*$^{+/-}$	757 ± 27	0/10
m*Rb*$^{+/-}$ h*RBΔN1-03*$^{+/-}$	584 ± 90	10/22
m*Rb*$^{+/-}$ h*RBΔN1-53*$^{+/-}$	453 ± 43	5/6
m*Rb*$^{+/-}$ h*RBΔN1-10*$^{+/-}$	477 ± 72	8/11
m*Rb*$^{+/-}$ h*RBΔN2-25*$^{+/-}$	482 ± 92	4/16
m*Rb*$^{+/-}$ h*RBΔN3-70*$^{+/-}$	500 ± 70	4/9
m*Rb*$^{+/-}$ h*RBΔN3-82*$^{+/-}$	612 ± 99	11/13
m*Rb*$^{+/-}$ h*RBΔN4-03*$^{+/-}$	509 ± 38	3/12
m*Rb*$^{+/-}$ h*RBΔN4-13*$^{+/-}$	511 ± 101	16/18

[a] Mean life spans of the animals that had died at the time of analysis; at the time of the end of this study, some mice were still living at age >365 days.

experiments clearly indicate? The most likely explanation is that N-terminal regions are integral components of the proper, overall conformation of the intact RB protein. In vitro evidence suggests that RB forms head-to-tail oligomers (25); such oligomers may allow subcompartmentalization within the nucleus in response to various signals (39). If this experimental finding in vitro is applicable in vivo, then N-terminal portions of RB would indeed be crucial. Alternatively, the N-terminal regions may be directly important for association of RB with the nuclear compartment and with other binding proteins. Pathways in development and differentiation may require interaction between several proteins, in a coordinated fashion, such that the N-terminal region of RB is required to allow simultaneous interaction between transcription factors that bind primarily to the T-antigen binding domain and to other

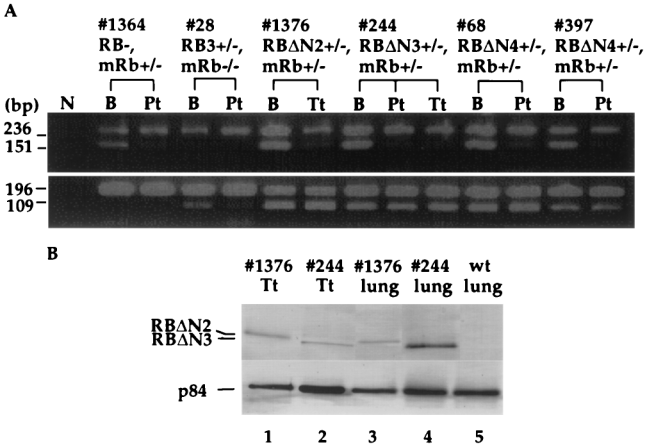

FIG. 8. Loss of the wild-type m*Rb* allele in tumors from animals with the genotype h*RBΔN*$^{+/-}$ m*Rb*$^{+/-}$. (A) Genomic DNA was isolated from the normal brains (B), pituitary melanotroph tumors (Pt), and thyroid medullary carcinomas (Tt) of moribund animals with different genotypes, as shown. DNA from each sample was then subjected to PCR analysis using the sets of primers detailed in Fig. 5B. (B) hRBΔN protein expression in thyroid medullary carcinomas from selected animals with the genotype h*RBΔN*$^{+/-}$ m*Rb*$^{+/-}$. Truncated hRB protein is expressed in the tumors analyzed. In normal lung tissues from the same transgenic mice, hRBΔN is also expressed, but lung tissues from a wild-type mouse expressed no transgenic protein (lane 5). Procedures were identical to those described for Fig. 3A, upper panel.

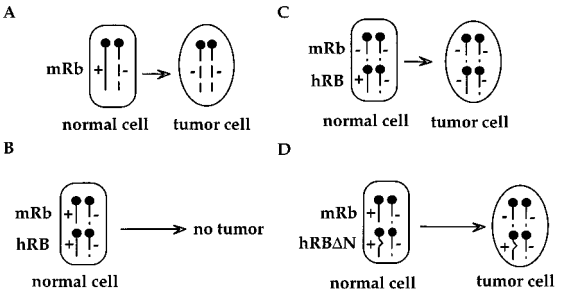

FIG. 9. Loss of remaining allele encoding fully functional RB protein in tumor cells. (A) In tumors from mice heterozygous for endogenous *Rb*Δ^{ex20}, the remaining wild-type m*Rb* allele is lost; (B) in mice with the genotype h*RB3*$^{+/-}$ m*Rb*$^{+/-}$, both functional *RB* alleles are never lost and no pituitary melanotroph tumors develop; (C) in tumors from mice with the genotype h*RB3*$^{+}$ m*Rb*$^{-/-}$, the entire human *RB* transgene is deleted; (D) in tumors from mice with the genotype h*RBΔN*$^{+}$ m*Rb*$^{+/-}$, the remaining wild-type m*Rb* allele is lost and the h*RBΔN* transgene is retained.

proteins that interact primarily with N-terminal domains. To date only three such proteins have been reported: an 84-kDa protein which associates with nuclear matrix and colocalizes to sites of RNA splicing (15), a 70-kDa heat shock cognate protein (33), and a kinase apparently active in G_2 and M phases (54). Functions of these proteins and the significance of their interactions with RB are unknown. None of these proteins fits directly into a pathway for the differentiation of erythrocytes, neurons, or late-stage myotubes. Other cellular proteins which are crucial for this process and bind specifically to N-terminal regions of RB must be sought.

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Daniel J. Riley and Chia-Yang Liu contributed equally to this work.

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