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Structure and expression of the murine retinoblastoma gene and characterization of its encoded protein

(recessive oncogene/DNA sequence analysis/leucine zipper)

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ABSTRACT **We have isolated a cDNA clone of the murine homologue of the human retinoblastoma (Rb) susceptibility gene. DNA sequence analysis reveals a high degree of conservation with the human Rb sequence, both in the coding and in the noncoding regions. The predicted amino acid sequence of the mouse Rb protein shows 91% identity to that of the human protein. Both proteins were found to contain a peptide sequence reminiscent of a leucine-repeat motif ("leucine-zipper") that is also found in the *myc*, *fos*, and *jun* oncogenes. Synthetic peptide antiserum directed against a portion of the mouse Rb protein detects three proteins of 104–110 kDa in cells that were transiently transfected with a mouse Rb gene expression construct. In the mouse embryo the expression of Rb mRNA was ubiquitous, with maximal expression being observed around 13 days of gestation. In the embryo, the highest level of expression was observed in liver and brain. In contrast, the Rb gene was found to be expressed at a very low level in adult mouse liver with high levels being found in lung, thymus, and spleen. A shorter Rb transcript was detected in mouse testes.**

Within the past decade, evidence has accumulated pointing to the involvement of negative regulators of cell growth in the cancer process. Thus, the homozygous inactivation of genes mapping to several distinct loci has been associated with a variety of different cancers (1).

We (2) and others (3, 4) have isolated a cDNA clone that corresponds to a gene with many of the properties of the sequence constituting the retinoblastoma (Rb) gene, the best studied of these tumor suppressor genes. As expected for the Rb gene, this sequence is affected by substantial structural alterations in as many as 30% of Rb tumor DNAs. Mapping of the deletions within this gene strongly implicated this sequence as the representative of the Rb locus (2–7). Subsequent work has revealed that the Rb gene specifies a nuclear phosphoprotein of 105 kDa (8, 9). Other studies have shown that the human Rb gene product is complexed by oncogene products of adenovirus, simian virus 40 (SV40), and human papillomavirus (9–11). These observations suggest that the Rb gene product functions at a central control point in the cell's growth regulatory network.

We have undertaken to develop an animal model of Rb-associated tumorigenesis. To do so, we have isolated and characterized the mouse Rb gene.** The results of this characterization are described here.

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MATERIALS AND METHODS

Southern and Northern Blot Analysis. High molecular weight DNA was prepared as described by Blin and Stafford (12). DNA (10–15 μg) was digested with restriction enzymes, electrophoresed on 0.8% agarose gels, and transferred to GeneScreen*Plus* nylon membranes (13). Filters were hybridized with 10^7 cpm of ^{32}P-labeled probe (specific activity 5×10^8 cpm/μg of DNA). The hybridization was performed at 42°C in 5× SSC/35% (vol/vol) formamide (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0). Subsequent washes were performed with ascending stringencies as indicated.

The mouse strains used in the RNA expression studies, the RNA extraction procedure, and procedures for Northern transfer and hybridization have been described (14).

Nucleotide Sequencing. DNA sequences were determined by both the method of Maxam and Gilbert (15) and by the Sanger technique (16). Sequences were analyzed using the programs developed by the University of Wisconsin Genetics Computer Group (17).

Production and Use of Anti-mouse Rb Antiserum. An oligopeptide corresponding to the sequence

CFETERTPRKNNPDEEANVVT

was synthesized. Coupling of the peptide to a keyhole limpet hemocyanin carrier was performed according to Liu *et al.* (18). New Zealand White rabbit 138 was injected subcutaneously with 200 μg of peptide conjugate in Freund's complete adjuvant. Booster injections of 100 μg of peptide conjugate in Freund's incomplete adjuvant were given subsequently every 2 weeks. After two booster injections, serum was collected and tested every other week for reactivity against *in vitro*-translated mouse Rb cRNA (RNA transcribed *in vitro* from a DNA template) as described (9). Labeling of cell lines, lysate preparation, and immunoprecipitation were performed as described (9).

Mouse Rb Gene Transfection. To construct a mouse Rb cDNA expression vector, we used the plasmid pJ3Ω (kindly provided by Jay Morgenstern, Imperial Cancer Research Fund). This plasmid is virtually identical to the pSV2 expression vector (19) with the exception that it contains a polylinker cloning site. One recombinant that had the mouse Rb

Abbreviations: Rb, retinoblastoma; SV40, simian virus 40.

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**The sequence reported in this paper has been deposited in the GenBank data base (accession no. M26391).

cDNA in the proper orientation inserted in the vector (pJ3-115) was used in the transfection experiments. Transient transfections were performed as described by Grosschedl and Baltimore (20).

RESULTS

Conservation of the Rb Gene. To investigate the extent to which homologues of the human Rb are detectable in closely and distantly related organisms, we performed Southern blot analysis on DNA isolated from the following organisms: *Branchiostoma lanceolatum* (amphioxus), *Lampetra planeri* (brook lamprey), *Scylliohinus caniculus* (cat-shark), *Xiphophorus helleri* (swordtail), chicken, mouse, and human. As a probe we used the 3.8-kilobase (kb) *Eco*RI fragment of the human Rb cDNA clone at low stringency.

The results of this experiment (Fig. 1) indicate that all DNA samples, except for the DNA from *B. lanceolatum* (data not shown), contain sequences that crosshybridize with the human Rb gene probe. We conclude that this or similarly structured genes are present in genomes of all chordates. The presence of related genes in other phyla has not been demonstrable to date.

Isolation of the Mouse Rb Gene. Because of the broad phylogenetic distribution of the Rb gene, it became possible to characterize this gene in an organism that is available to experimental manipulation, namely the mouse. Accordingly, we isolated the mouse homologue of the human Rb gene. A cDNA library of the BALB/c mouse pre-B-cell line 70Z-3 (21) was screened at low stringency with a human Rb cDNA fragment. One of the phages identified by plaque hybridization, λmrb115, carried an insert of ≈4.6 kb. Each strand of this cDNA was sequenced an average of three times.

The complete sequence of the λmrb115 insert is shown in Fig. 2. The length of the cDNA insert is 4592 base pairs and has 84% similarity to the human Rb cDNA. Remarkably, the degree of conservation in the coding region (88.7%) is not much higher than the degree of conservation in the 3′ untranslated region of the cDNA (77.3%). The 3′ untranslated region of both the mouse and the human Rb cDNA contain a total of 12 ATTA motifs, the presence of which was shown to be associated with a relative instability of mRNAs (22).

The mouse Rb cDNA contains a single large open reading frame from nucleotide 99 to a stop codon at nucleotide 2865. It encodes a 921-amino acid protein with a calculated molecular weight of 105,337, which is 821 less than the predicted molecular weight of the human protein. Comparison of the predicted amino acid sequence of the mouse Rb protein with that of human Rb protein (Fig. 3.) shows that the human Rb protein has a total of 7 additional amino acids. Others have noted the presence in the human Rb protein of two putative nucleic acid-binding "zinc-fingers" (4, 23). In the mouse Rb protein, one of the histidines found in one of the putative zinc fingers of the human protein is not present, leaving only one domain having a zinc finger-like structure. We did notice, however, a sequence that is reminiscent of another structural motif that is found to be conserved between a number of nuclear phosphoproteins: at amino acid residues 655, 662, 669, and 676 of the mouse Rb protein (and at amino acids 662, 669, 676, and 683 of human), leucine residues are found, each separated by 6 amino acids. This motif, referred to as the "leucine zipper," is thought to play a role in dimerization of DNA-binding proteins (24).

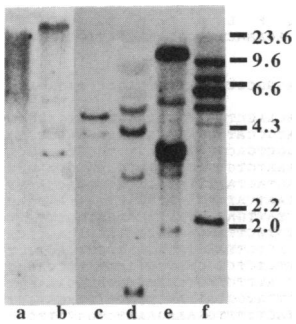

FIG. 1. Southern blot analysis of *Hin*dIII-digested total genomic DNA. DNA was from the following animals. Lanes: a, *L. planeri* (lamprey); b, *S. caniculus* (shark); c, *X. helleri*; d, chicken; e, mouse; f, human. Blots were probed with the 3.8-kb *Eco*RI fragment of the human Rb gene. Hybridization was performed at 42°C in 35% formamide/5× SSC. Filters were washed at 55°C in 1× SSC (lanes a and b) or at 63°C in 0.5× SSC. *Hin*dIII-digested phage λ DNA was used as molecular size markers as indicated in kb to the right.

In total, 82 of 921 amino acids are different between mouse and human Rb proteins, making the two proteins 91% identical. When conservative changes are disregarded, these proteins have 95% sequence similarity.

Expression of the Rb Gene in Mouse. The absence of a functional Rb gene is associated in humans with a number of malignancies. The reasons for the tissue specificity of tumor formation are not known. One possible explanation is that the Rb gene is only expressed in a limited number of tissues in which its expression represents a critical regulator of cell proliferation. Consequently, lack of a functional Rb gene would only affect these tissues. This provoked a systematic survey of mouse tissues. We began by preparing RNA from mouse embryos at various times of gestation. Expression of the Rb gene was detected using Northern blot analysis.

As can be seen in Fig. 4*A*, the Rb gene is expressed as early as 9.5 days of gestation with maximal expression reached at 12.5–14.5 days. We also determined where in the mouse embryo the Rb gene was expressed. To do this, 12.5-day mouse embryos were dissected and RNA was extracted from the various tissues. As before, Rb expression was detected by Northern blot analysis. These data (Fig. 4*B*) indicate that the expression of the Rb gene is quite variable within the embryo with liver and neuronal tissues having the highest level of Rb mRNA.

A different result was found when tissues from adult mice were tested for expression of the Rb gene. As can be seen in Fig. 5*A*, the adult liver, in contrast to the fetal liver, has relatively low levels of Rb mRNA. Brain, kidney, spleen, thymus, and lung were found to have high levels of Rb mRNA. Poly(A)-selected RNA from mouse blood was found to consist mainly of globin mRNA. Consequently, the signals obtained with the Rb and actin probes were relatively weak. When total RNA derived from mouse blood was analyzed for Rb expression, it was found also to have relatively high levels of Rb mRNA (data not shown). Interestingly, a second shorter Rb transcript was found in RNA derived from adult mouse testes (Fig. 5*B*). The appearance of the 2.8-kb Rb transcript coincides with the appearance of spermatids in the testes. The significance and structure of this shorter transcript is at present unknown. We conclude from the analysis of Rb gene expression in the mouse that there is no correlation between the tissues in which the Rb gene is expressed and the sites at which Rb loss leads to tumorigenesis in humans.

Mouse Rb Protein. To determine whether the mouse Rb cDNA that we isolated could direct the synthesis of functional mouse Rb protein, we first generated polyclonal rabbit antiserum 138 against a synthetic oligopeptide corresponding to a portion of the mouse Rb protein that was predicted to be hydrophilic and divergent between the human and mouse Rb proteins. After several booster injections with immunogen, antiserum was collected and tested for reactivity by immunoprecipitation of radiolabeled *in vitro* translations of *in vitro*-synthesized human and mouse Rb cRNAs. Serum 138 specifically precipitated proteins translated from *in vitro*-

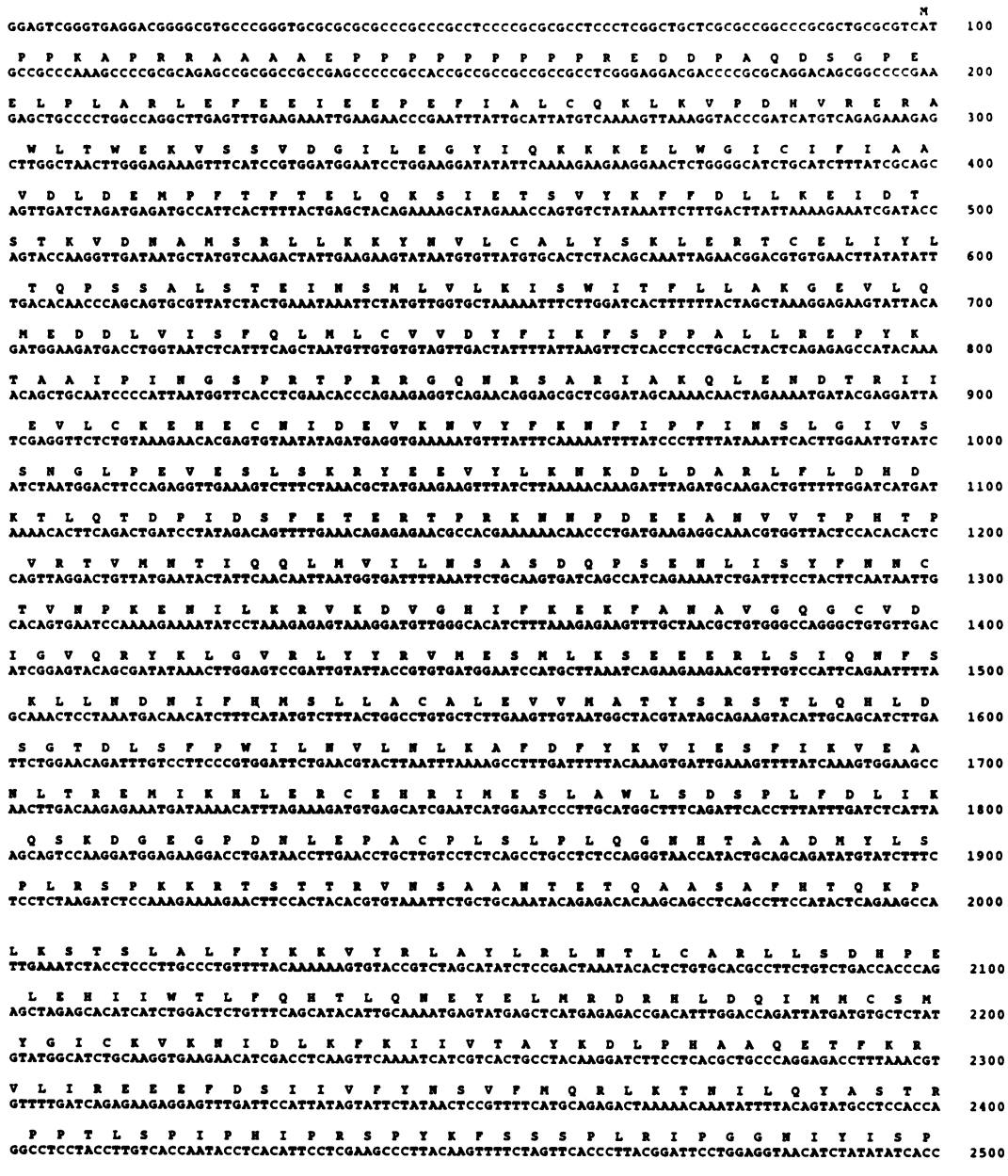

FIG. 2. Organization and nucleotide sequence of the murine Rb cDNA. The single-letter amino acid sequence of the large open reading frame is indicated above the DNA sequence. Both strands were sequenced an average of three times.

synthesized mouse Rb cRNA and not those synthesized from human Rb cRNA (data not shown).

To determine the size of the protein encoded by the mouse Rb cDNA, we linked the insert from λmrb115 to the SV40

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Mouse       MPPKAPRR.....AAAAEPPPPPPPPPREDDPAQDSGPEELPLARLEFEEIEEPEFIALCQKLKVPDHVRERAWLTWEKVSSVDGILEGYIQKKKELWG
Human       MPPKTPRKTAATAAAAAAEPPAPPPPPPPEEDPEQDSGPEDLPLVRLEFEETEEPDFTALCQKLKIPDHVRERAWLTWEKVSSVDGVLGGYIQKKKELWG
              1                                                                                                100

Mouse       ICIFIAAVDLDEMPFTFTELQKSIETSVYKFFDLLKEIDTSTKVDNAMSRLLKKYNVLCALYSKLERTCELIYLTQPSSALSTEINSMLVLKISWITFLL
Human       ICIFIAAVDLDEMSFTFTELQKNIEISVHKFFNLLKEIDTSTKVDNAMSRLLKKYDVLFALFSKLERTCELIYLTQPSSSISTEINSALVLKVSWITFLL
            101                                                                                              200

Mouse       AKGEVLQMEDDLVISFQLMLCVVDYFIKFSPPALLREPYKTAAIPINGSPRTPRRGQNRSARIAKQLENDTRIIEVLCKEHECNIDEVKNVYFKNFIPFI
Human       AKGEVLQMEDDLVISFQLMLCVLDYFIKLSPPMLLKEPYKTAVIPINGSPRTPRRGQNRSARIAKQLENDTRIIEVLCKEHECNIDEVKNVYFKNFIPFM
            201                                                                                              300

Mouse       NSLGIVSSNGLPEVESLSKRYEEVYLKNKDLDARLFLDHDKTLQTDPIDSFETERTPRKNNPDEEANVVTPHTPVRTVMNTIQQLMVILNSASDQPSENL
Human       NSLGLVTSNGLPEVENLSKRYEEIYLKNKDLDARLFLDHDKTLQTDSIDSFETQRTPRKSNLDEEVNVIPPHTPVRTVMNTIQQLMMILNSASDQPSENL
            301                                                                                              400

Mouse       ISYFNNCTVNPKENILKRVKDVGHIFKEKFANAVGQGCVDIGVQRYKLGVRLYYRVMESMLKSEEERLSIQNFSKLLNDNIFHMSLLACALEVVMATYSR
Human       ISYFNNCTVNPKESILKRVKDIGYIFKEKFAKAVGQGCVEIGSQRYKLGVRLYYRVMESMLKSEEERLSIQNFSKLLNDNIFHMSLLACALEVVMATYSR
            401                                                                                              500

Mouse       STLQHLDSGTDLSFPWILNVLNLKAFDFYKVIESFIKVEANLTREMIKHLERCEHRIMESLAWLSDSPLFDLIKQSKDGEGP.DNLEPACPLSLPLQGNH
Human       STSQNLDSGTDLSFPWILNVLNLKAFDFYKVIESFIKAEGNLTREMIKHLERCEHRIMESLAWLSDSPLFDLIKQSKDREGPTDHLESACPLNLPLQNNH
            501                                                                                              600

Mouse       TAADMYLSPLRSPKKRTSTTRVNSAANTETQAASAFHTQKPLKSTSLALFYKKVYRLAYLRLNTLCARLLSDHPELEHIIWTLFQHTLQNEYELMRDRHL
Human       TAADMYLSPVRSPKKKGSTTRVNSTANAETQATSAFQTQKPLKSTSLSLFYKKVYRLAYLRLNTLCERLLSEHPELEHIIWTLFQHTLQNEYELMRDRHL
            601                                                                                              700

Mouse       DQIMMCSMYGICKVKNIDLKFKIIVTAYKDLPHAAQETFKRVLIREEEFDSIIVFYNSVFMQRLKTNILQYASTRPPTLSPIPHIPRSPYKFSSSPLRIP
Human       DQIMMCSMYGICKVKNIDLKFKIIVTAYKDLPHAVQETFKRVLIKEEEYDSIIVFYNSVFMQRLKTNILQYASTRPPTLSPIPHIPRSPYKFPSSPLRIP
            701                                                                                              800

Mouse       GGNIYISPLKSPYKISEGLPTPTKMTPRSRILVSIGESFGTSEKFQKINQMVCNSDRVLKRSAEGGNPPKPLKNVRFDIEGADEADGSKHLPAESKFQQK
Human       GGNIYISPLKSPYKISEGLPTPTKMTPRSRILVSIGESFGTSEKFQKINQMVCNSDRVLKRSAEGSNPPKPLKKLRFDIEGSDEADGSKHLPGESKFQQK
            801                                                                                              900

Mouse       LAEMTSTRTRMQKQRMNESKDVSNKEEK
Human       LAEMTSTRTRMQKQKMNDSMDTSNKEEK
            901                      928
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FIG. 3. Comparison of the amino acid sequences of the Rb proteins of mouse and man. The single-letter amino acid sequence of the mouse and human Rb proteins is represented. A vertical bar represents amino acid identity between the two proteins. Dots indicate gaps to achieve maximal alignment between the two proteins.

early promoter and transfected the resulting pJ3-115 construct into monkey COS-7 cells (25). Sixty hours after transfection, the transfected cells were analyzed for their content of mouse Rb protein by immunoprecipitation.

Three species of mouse Rb protein of 104–110 kDa were detected in lysates of [^{35}S]methionine-labeled transfected cells immunoprecipitated with mouse Rb-specific antiserum (Fig. 6). Two of these three species were detected when ^{32}P-labeled extracts were used for immunoprecipitation (Fig. 6). Immunoprecipitates of untransfected COS-7 cells with antiserum 138 did not precipitate monkey Rb protein in parallel experiments (data not shown). The transiently transfected mouse Rb protein is functional by two criteria: (*i*) it coprecipitated a small quantity of SV40 large tumor antigen, a viral oncoprotein that has been shown to complex with

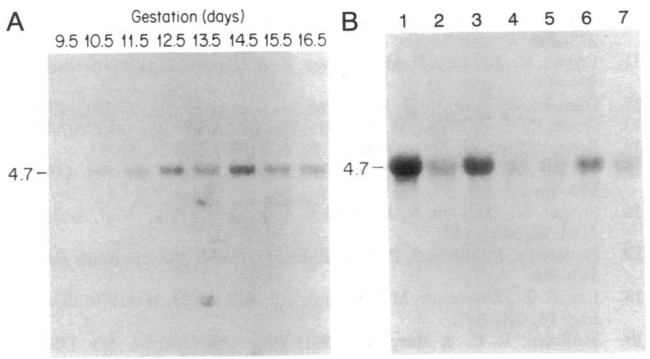

FIG. 4. Expression of the Rb gene in mouse embryos. (*A*) RNA was isolated from whole mouse embryos at various times of gestation as indicated by lane labels in days. Total RNA (25 μg) was fractionated on an agarose gel and probed with the mouse Rb cDNA. (*B*) Mouse embryos (12.5 days) were dissected. RNA was isolated from the following tissues. Lanes: 1, liver; 2, viscera; 3, brain; 4, head (without brain); 5, limbs; 6, spinal column, 7, carcass. Each lane contains 25 μg of RNA.

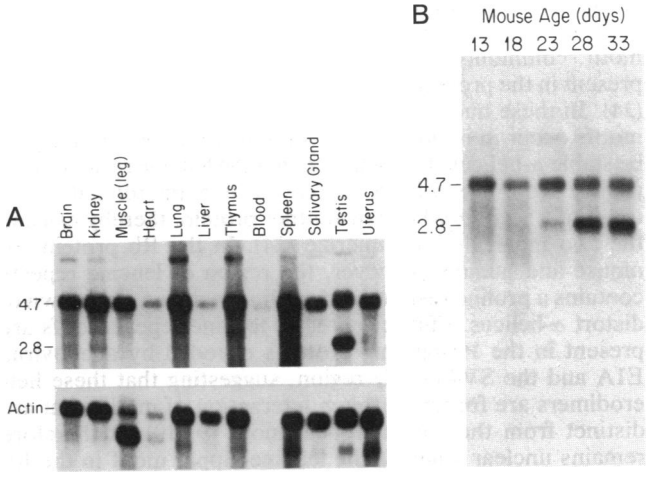

FIG. 5. Expression of the Rb gene in adult mice. (*A*) RNA was isolated from organs of adult mice as indicated. (*Upper*) Poly(A)-selected RNA (10 μg) was used for Northern blot analysis; the probe was the mouse Rb cDNA. (*Lower*) Reprobing of the same filter with an actin cDNA probe. (*B*) RNA was isolated from the testes of mice from the indicated ages. Poly(A)-selected RNA (10 μg) of each sample was used for Northern blot analysis. The probe was the Rb cDNA. The sizes of the transcripts in kb are indicated.

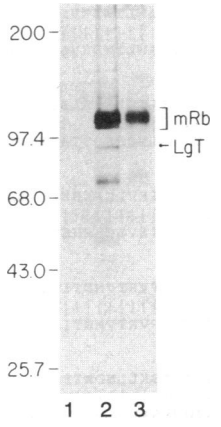

FIG. 6. Immunoprecipitation of mouse Rb proteins. Immunoprecipitation of mouse Rb proteins from transiently transfected cells. COS-7 cells were transiently transfected with pJ3-115 by using the DEAE-dextran technique (12). Sixty hours after transfection, cells were incubated with [^{35}S]methionine (lanes 1 and 2) or [^{32}P]orthophosphate (lane 3) and cell lysates were prepared. Cell lysates were precipitated with either preimmune serum (lane 1) or immune rabbit antiserum 138 (lanes 2 and 3) and precipitates were electrophoresed on an 8% SDS/polyacrylamide gel. The gel was processed for fluorography and exposed to x-ray film for 4 hr at −70°C. Mouse Rb proteins, coprecipitated SV40 large tumor antigen (LgT), and molecular masses of proteins in kDa are indicated.

human Rb proteins (10), and (*ii*) we have found that the mouse Rb proteins overexpressed in COS-7 cells exhibit an associated DNA-binding activity described for the human Rb protein (data not shown and ref. 8). We conclude from these data that the mouse Rb cDNA described here can direct the synthesis of intact mouse Rb protein.

DISCUSSION

Like other critical human genes, the Rb gene sequence is represented in very similar form in other mammalian species. We presume that it represents a centrally important regulator of mammalian cell growth and morphogenesis. Indeed, the wide tissue distribution of Rb transcripts suggests that its role is played out in a far wider arena than just the developing retina.

Our data indicate that the human and mouse Rb proteins have sequences reminiscent of leucine-repeat motifs. This motif, commonly referred to as the leucine zipper, is also present in the proteins encoded by the *myc*, *fos* and *jun* genes (24). In these nuclear oncogene proteins, the leucine-repeat motifs occur in regions of the proteins that are presumed to be stable α-helices. It is believed that the leucine residues that project from the α-helix of one protein interdigitate with those of a second α-helix in another protein, thereby causing the two molecules to dimerize (24). In the Rb proteins of mouse and human, however, the region of leucine repeats contains a proline residue, and proline residues are known to distort α-helices. Furthermore, no leucine-repeat motifs are present in the Rb-binding proteins encoded by adenovirus E1A and the SV40 early region, suggesting that these heterodimers are formed through interaction of motifs that are distinct from the leucine-repeat motif (9, 10). It therefore remains unclear whether the leucine-zipper motif in the Rb proteins plays a role in protein–protein interaction.

We show here that cells are transiently transfected with a mouse Rb cDNA expression construct synthesize proteins of 104–110 kDa that are specifically precipitated with a mouse Rb antiserum (Fig. 6), which corresponds well to the predicted molecular weight of the mouse Rb protein (105,337). In metabolically labeled mouse F9 teratocarcinoma cells, a protein of the same molecular weight is detected with the mouse Rb antibody, but a larger species of 132 kDa is also found (R.B. and J.M.H., unpublished observations).

Our present data highlight two major puzzles, neither of which is resolved here. First, in humans Rb inactivation is associated to date largely with retinoblastoma and sarcoma tumors. This idiosyncratic subset of tissues represented by these tumors contrasts with the wide expression of the Rb gene demonstrated here and suggested by earlier studies (2, 4). We are left with the presumption that while Rb expression may contribute to the physiology of many cell types, Rb inactivation appears to trigger cancer in only a few of these.

A second puzzle concerns the species distribution of retinoblastoma tumors. Although 1 in 20,000 newborn humans suffers from retinoblastoma, to our knowledge, the disease has never been observed in any other mammalian species, including those domestic, agricultural, or laboratory species that present ample opportunity for observation. We are reluctant to believe that the basic genetic mechanisms of growth control differ between mammalian species and consider it possible that Rb inactivation will be associated with a range of tumors in mice that is quite distinct from that seen in humans.

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