Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 2775–2779, April 1990
Biochemistry

Frequent inactivation of the retinoblastoma anti-oncogene is restricted to a subset of human tumor cells

(recessive oncogene/retinoblastoma protein/splicing)

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Contributed by Robert A. Weinberg, December 18, 1989

ABSTRACT **We have used polyclonal anti-synthetic peptide serum to study the role of retinoblastoma gene (*RB*) inactivation in a variety of human tumor cell lines. Our analysis indicates that inactivation of the RB protein, p105-Rb, is universal in retinoblastoma cells, vindicating the predictions of the Knudson "two-hit" hypothesis. In addition, our analysis has shown that inactivations of the *RB* gene are nearly as frequent in a more common human tumor, small cell lung carcinoma. One-third of bladder carcinomas surveyed also carry altered or absent p105-Rb. Other human tumors by contrast demonstrate only infrequent inactivation of the *RB* gene. These results suggest that inactivation of the *RB* gene is a critical step in the pathogenesis of a subset of human tumors.**

The recent isolation of molecular clones of the retinoblastoma gene (*RB*) has made it possible to evaluate the role of *RB* gene inactivation in the genesis of a variety of human tumors (1–3). These molecular analyses have confirmed and extended earlier karyotypic and genetic studies that had implied that both copies of this gene suffer inactivation during the formation of retinoblastomas and several types of sarcoma (4–10). Since the inactivation of *RB* function appears to trigger tumorigenesis, the *RB* gene has been termed a "tumor suppressor" gene, whose expression is required to constrain normal cellular proliferation.

Analysis of the protein product encoded by the *RB* gene, p105-Rb, has shown it to be a nuclear phosphoprotein having an affinity for DNA (11, 12). This protein may be involved in growth regulation in a wide variety of cell types. Thus, expression of p105-Rb has been detected in all human cells examined except certain types of tumor cells that have suffered *RB* gene inactivations (ref. 11; J.M.H., unpublished observations). Recent evidence has shown that the *RB* gene and its encoded protein are also involved in a second, quite distinct, mechanism of tumor formation: oncoproteins specified by three DNA tumor viruses [adenovirus, simian virus 40 (SV40), and human papillomavirus] complex with p105-Rb (13–16). This complex formation is apparently central to the ability of these oncoproteins to transform primary cells (14, 17).

In the initial studies designed to detect inactivation of chromosomal copies of the *RB* gene, cloned segments of the *RB* gene were used as probes in Southern blot analysis of tumor DNAs (1–3, 18–21). These studies revealed alterations in the *RB* gene in as many as 30% of retinoblastomas and heritable osteosarcomas. Unanswered was the genetic status of the remaining 70% of tumors belonging to these classes. Their *RB* genes could have suffered subtle alterations in structure of the sort that escape detection by Southern blotting analysis. Indeed, several recent reports have documented subtle genetic changes in the *RB* gene that could lead to its functional inactivation (12, 22). However, it is also possible that the majority of retinoblastomas and sarcomas carry intact *RB* alleles and that these particular tumors arise because of alterations in other, as yet uncharacterized, genes. To address this problem, we have employed a more sensitive analysis of *RB* inactivation by analyzing the *RB*-encoded p105 protein in 18 independent retinoblastoma tumor cell lines. In addition, we have extended this strategy to an analysis of the frequency of *RB* gene inactivation in several additional types of human tumors.

MATERIALS AND METHODS

Preparation of Cell Lysates and Immunoprecipitation of the RB Protein from Human Tumor Cells. Tumor cell cultures were incubated for 3–5 hr with [^{35}S]methionine, and cell lysates were prepared as described (13). Immunoprecipitations were performed with rabbit serum no. 147, 144, or 140 (13), and precipitates were resolved by electrophoresis for 15 hr on SDS/polyacrylamide gels, processed for fluorography, and exposed for 1–3 days at −70°C. Bladder, colon, and breast carcinoma cultures were acquired from the American Type Culture Collection as were two retinoblastoma cultures (Y79 and WERI-1). The derivation of all other retinoblastoma cultures and small cell lung carcinoma (SCLC) cultures has been described (1, 23, 24). Melanoma tumor cells were a kind gift of Nick Dracopoli of the Massachusetts Institute of Technology.

Amplification of Tumor Cell mRNA by Using the Polymerase Chain Reaction (PCR) Technique and DNA Sequence Analysis. RB mRNA was specifically amplified by using oligonucleotide primers from the RB cDNA sequence and the PCR as described (12). Amplified fragments were cloned into M13 and sequenced on both strands by the dideoxy chain termination technique (25). Sequences from each culture were confirmed by analysis of several independent phage clones produced from two independent RNA amplification reactions.

Construction of Mutant RB cDNA Constructs, *in Vitro* Transcription and Translation, and Coprecipitation Analysis.

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Abbreviations: SV40, simian virus 40; PCR, polymerase chain reaction; SCLC, small cell lung carcinoma(s).

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Mutant RB cDNAs were generated by replacing wild-type RB cDNA sequences bracketing exons 21 and 22 with PCR-amplified fragments from NCI-H69C and NCI-H1436 cells. Wild-type and mutant RB cDNAs were then transcribed and translated as described (13). Coprecipitations were performed by incubating [^{35}S]methionine-labeled *in vitro* translation products with nonradioactive human 293 cell lysates containing adenovirus E1A prior to immunoprecipitation with a monoclonal antibody against E1A (M73; ref. 26). Direct immunoprecipitations of *in vitro* translation products were performed by using rabbit serum no. 147 (13). Precipitates were resolved by electrophoresis and processed for fluorography. Autoradiography was for 3 days at −70°C.

Preparation of Genomic DNA and Southern Blot Analysis. Genomic DNA was prepared by standard methods, cleaved with *Hin*dIII, and resolved by electrophoresis through a 0.7% agarose gel. After transfer to nitrocellulose, human RB cDNA probe 3.8R (1) was radiolabeled by the oligo-labeling technique (27) and used as a hybridization probe. Following a high-stringency wash [68°C; 0.1% SDS/0.1× SSC (1× SSC = 0.15 M sodium chloride/0.015 M sodium citrate, pH 7)], the nitrocellulose filter was exposed for 3 days at −70°C with a DuPont Lightning Plus intensifying screen.

RESULTS

The *RB* Gene Is Inactivated in All Retinoblastomas. We reasoned that genetic lesions in the *RB* gene that escape detection by Southern blot analysis might nevertheless affect the structure or amount of *RB*-encoded protein expressed in tumor cells. Retinoblastoma cells were incubated with [^{35}S]methionine, and cell lysates were prepared for immunoprecipitation with polyclonal rabbit anti-synthetic peptide serum no. 147 (13). As shown in Fig. 1*A*, we failed to detect any RB protein in all 18 different retinoblastoma samples that we analyzed. This contrasts with the substantial amount of p105-Rb present in normal retinoblasts and in a variety of other cell types including other neuroectodermal cells (ref. 11; J.M.H., unpublished observations). These results were corroborated by immunoprecipitations with two other polyclonal sera, nos. 140 and 144, produced against two distinct synthetic peptides (13). In each instance no RB-related proteins were detected (data not shown).

We note that the majority (13/18) of these retinoblastomas were characterized as cell cultures grown for relatively short periods of time (5–10 passages) following explantation of tumors from patients. This suggests that the absence of detectable RB protein is not an artifact of extensive passaging and adaptation to tissue culture. Although the majority of retinoblastoma tumor cell lines did not express detectable levels of wild-type RB messenger RNA, four retinoblastoma tumor cell cultures (RBLA-10, RBLA-18, RBLA-20, and RBLA-22) expressed detectable levels of intact RB mRNA as judged by Northern blot analysis with an RB cDNA probe (data not shown). We presume that the RB mRNA in these cultures contain mutations that specify aberrant versions of p105-Rb, which do not accumulate to steady-state levels readily detectable by immunoprecipitation.

Although some data has suggested that inactivation of the *RB* gene is only involved in a small minority of retinoblastomas (20), we conclude from the present results that *RB* gene inactivation is universal in retinoblastomas and that earlier analyses of *RB* gene structure have led to underestimates of the frequency of involvement of this gene in the pathogenesis of these tumors. Our data create a puzzle, since many of the mutations capable of inactivating *RB* gene function should specify defective proteins that are readily detectable in tumor cells (e.g., see below). We are left with the speculation that even marginally defective, aberrant RB proteins are not tolerated in retinoblastomas and are rapidly degraded in these tumor cells.

The *RB* Gene Is Inactivated in Most SCLC. Encouraged by the utility of p105-Rb analysis, we extended our study to SCLC; an earlier report showed structural abnormalities in the *RB* gene in 18% of SCLC tumor cell lines and the absence of RB mRNA in as many as 60% of these lines (23). Here once again analysis of protein proved more sensitive and decisive than nucleic acid analysis (Fig. 1*B*). We studied p105-Rb in

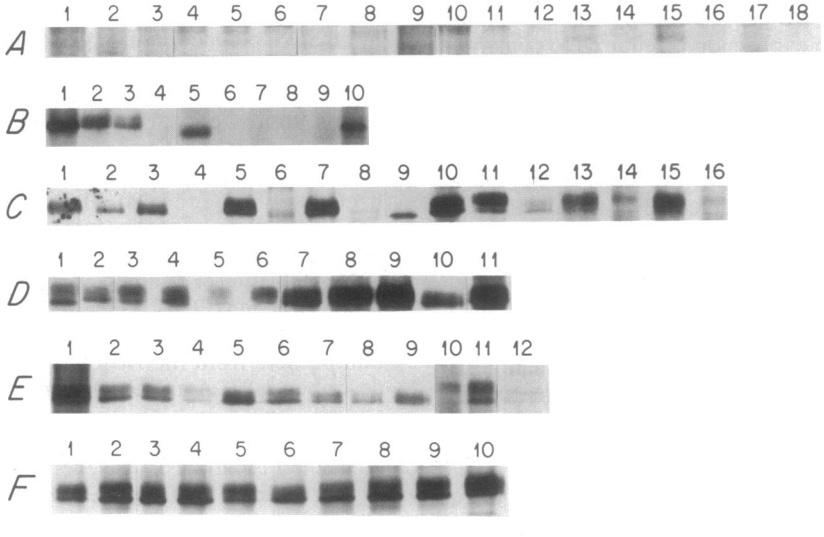

FIG. 1. Immunoprecipitation of tumor cell cultures by using polyclonal anti-RB peptide serum. (*A*) Retinoblastoma cultures. Lanes: 1, RBLA-9; 2, RBLA-29; 3, RBLA-30; 4, RBLA-19; 5, RBLA-20; 6, RBLA-22; 7, RBLA-1; 8, RBLA-10; 9, RBLA-18; 10, RBLA-12; 11, RBLA-13; 12, RBLA-28; 13, Y79; 14, Rb355; 15, WERI-1; 16, RBLA-8; 17, WERI-24; 18, WERI-27. (*B*) Small cell and large cell lung carcinoma cultures. Lanes: 1, NCI-H209; 2, NCI-H841; 3, NCI-H460; 4, NCI-H510; 5, NCI-H1436; 6, NCI-H711; 7, NCI-H1092; 8, NCI-H1184; 9, NCI-H1105; 10, NCI-H69C. (*C*) Bladder carcinoma cultures. Lanes: 1, SW1710; 2, SW800; 3, AY-2; 4, 5637; 5, A1663; 6, DTB-5; 7, EJ; 8, MGHU-5; 9, J82; 10, SW780; 11, SW1738; 12, HT1197; 13, SCaBER; 14, TCCSUP; 15, UM-UC-3; 16, HT1376. (*D*) Colon carcinoma cultures. Lanes: 1, LoVo; 2, DLD-1; 3, SW480; 4, HCT-15; 5, SW1116; 6, Colo320HSR; 7, WiDR; 8, Colo321; 9, Colo205; 10, HT29; 11, SW403. (*E*) Breast carcinoma cultures. Lanes: 1, HBRC; 2, T-47D; 3, SK-BR-3; 4, BT474; 5, Cama-1; 6, MCF-7; 7, MDA-MB453; 8, SW613; 9, MDA-MB157; 10, MDA-MB436; 11, MDA-MB361; 12, MDA-MB468. (*F*) Melanoma cultures. Lanes: 1, ML853.2; 2, WM35; 3, RANDA 220; 4, WM9; 5, Johnson; 6, Wolfe; 7, Jimi WO2; 8, WM88; 9, WM278; 10, MENO.

nine SCLC cell lines, all of which were previously characterized as expressing intact RB mRNA (23). Only one of these lines, NCI-H841, expressed levels of intact p105-Rb equivalent to those found in control cell lines (e.g., non-SCLC lung neoplasias; see below). The RB protein was fully absent from five tumor cell lines (NCI-H510, NCI-H711, NCI-H1092, NCI-H1105, and NCI-H1184) and present as an aberrantly migrating species in the remaining three (NCI-H209, NCI-H69C, and NCI-H1436). The RB proteins of cell lines NCI-H69C and NCI-H1436 migrate in SDS/polyacrylamide gels with apparent molecular masses 3–4 kDa less than that of wild-type p105-Rb (Fig. 1*B*). In contrast, the electrophoretic migration of the RB protein precipitated from cultures of NCI-H209 cells approximates that of the unphosphorylated form of wild-type p105-Rb. However, unlike the wild-type protein, it is not detected in immunoprecipitates from NCI-H209 tumor cells labeled with [^{32}P]orthophosphate (data not shown). Thus, the RB protein detected in NCI-H209 cells represents an additional class of aberrantly modified and presumably defective RB proteins. As mentioned above, such aberrant and ostensibly defective forms of p105-Rb were not observed in retinoblastomas.

In consonance with the further observations of Harbour *et al.* (23), p105-Rb is infrequently inactivated in cell lines derived from non-SCLC pulmonary tumors. We analyzed two pulmonary large cell carcinomas (NCI-H460 and LX-1), a pulmonary adenocarcinoma (A549), and a pulmonary carcinoid (NCI-H727), all of which expressed intact RB message; each was found to express intact p105-Rb (Fig. 1*B*; data not shown). Yokota *et al.* (28) have recently reported frequent loss of RB protein expression in nine additional independent SCLC cell lines. Summing the present results with those of previous reports (23, 28), we note that 1 out of 32 SCLC cultures has proven positive for intact p105-Rb protein. In contrast, the vast majority of other types of pulmonary tumors that have been examined have uniformly proven to produce readily detectable levels of intact RB mRNA and protein (refs. 23 and 28; data not shown).

Two Aberrant RB Proteins Identified in SCLC Tumor Lines Result from Splicing Mutations. We have examined the mutations in two SCLC cell lines that encode an aberrantly migrating RB protein, using a protocol that includes PCR amplification of RB mRNA and genomic DNA sequences (29, 30). Reminiscent of our previous results with the J82 human bladder carcinoma cell line, both of these mutations affect splicing of the RB mRNA precursor, leading to the elimination of an exon and to the truncation of RB mRNA and resulting protein (12). In one mutant, NCI-H69C, the mRNA encoding exon 21 of the *RB* gene is fused in-frame directly to exon 23, eliminating 38 amino acids of the exon 22 coding sequence from the RB message and the resulting protein. This is consistent with the observed increased mobility of this *RB* gene product on polyacrylamide gels (Fig. 1*B*). PCR amplification and direct sequencing of genomic DNA flanking and including exon 22 in this cell line shows a single point mutation that simultaneously creates a stop codon and a novel splice donor site 30 nucleotides into exon 22 (Fig. 2). As only mutant exon 22 sequences were detected in the genomic DNA of NCI-H69C, we presume this mutation to be hemi- or homozygous in the genome of this cell line. Since this mutation results in the biogenesis of an RB mRNA that lacks exon 22, we presume that this mutation influences utilization of the normal splice acceptor site immediately upstream of exon 22 or destabilizes full-length RB mRNA, resulting in deletion of the entire exon. The effect of this mutation in NCI-H69C cells is not absolute, as a minority (5–10%) of PCR-amplified mRNAs produce fragments of wild-type size (data not shown). Translation of these full-length, but point-mutated, messages would be predicted to give rise to a truncated RB protein of 747 amino acids.

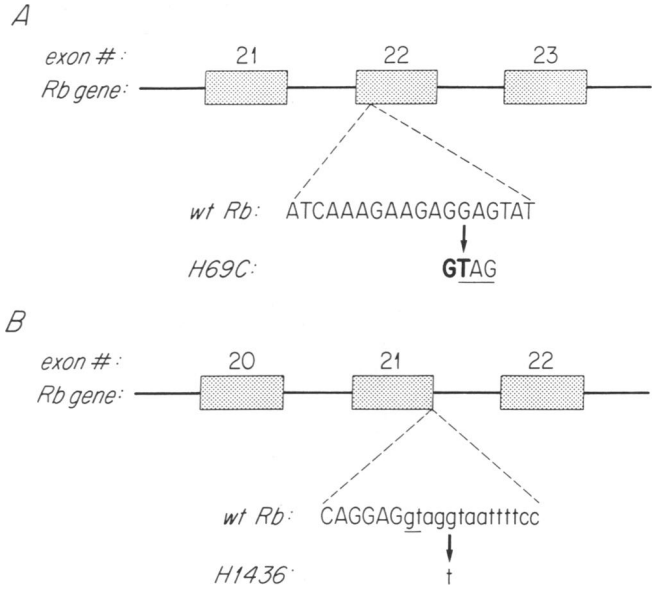

FIG. 2. Sequence of point mutations associated with *RB* splicing defects in SCLC NCI-H69C and NCI-H1436. (*A*) NCI-H69C. Shown are exons 21–23 of the *RB* gene and the wild-type sequence (wt Rb) 30 nucleotides into exon 22. The point mutation simultaneously generates a stop codon (underlined), and a novel splice donor shown in boldface lettering within exon 22. (*B*) NCI-H1436. Shown are exons 20–22 of the *RB* gene and the wild-type sequence about the 3′ end of exon 21. Exon sequences are capitalized; intron sequences are shown in lowercase lettering. The exon 21 splice donor sequence is underlined.

Synthesis of such a defective RB product has not been detected by our procedures in these cells.

To determine whether loss of exon 22 sequences results in a functionally defective RB product, we have assessed the ability of this truncated protein to bind adenovirus E1A and SV40 large tumor antigen. We reconstructed a wild-type RB cDNA clone, replacing those sequences flanking and including exon 22 by a DNA fragment lacking exon 22 sequences; the latter was obtained by PCR amplification of RB mRNA from NCI-H69C cells. *In vitro* transcription of this mutant construct and translation of the resulting transcripts leads to the synthesis of RB proteins slightly smaller in size than those synthesized from a wild-type cDNA template (Fig. 3). In contrast to wild-type RB proteins, these mutant RB proteins do not coprecipitate when mixed with cell lysates containing adenovirus E1A or SV40 large tumor antigen and incubated with monoclonal antibodies specific for each viral oncoprotein (Fig. 3; data not shown). We presume from these results that the deletion of amino acids encoded by exon 22 leads to the synthesis of an RB protein that is defective in other aspects of function within the cell.

PCR amplification of RB mRNA from another SCLC mutant line, NCI-H1436, shows that it also carries a splicing defect that leads in this instance to the elimination of exon 21 from its translated RB product. Here 35 amino acids are deleted from the RB protein, leading to a truncated protein that also does not complex with the adenovirus E1A and SV40 large tumor antigen viral oncoproteins (Fig. 3). Loss of this same exon by a similar splicing mutation has previously been documented in the J82 bladder cell line (12). Sequence analysis of 400 nucleotides of genomic DNA flanking and including exon 21 of NCI-1436 cells shows a single hemi- or homozygous point mutation within intron sequences five nucleotides downstream from the 3′ end of exon 21 (Fig. 2). This mutation falls within a region near the splice donor site known to be well conserved among other such sites (31). We

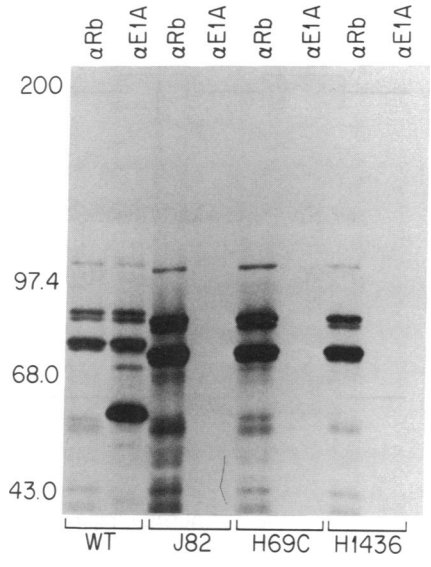

FIG. 3. *In vitro* translation of mutant and wild-type RB cDNAs and coprecipitation with adenovirus E1A. Indicated at the top of each gel lane are the antisera used for direct precipitation of RB [anti-Rb (no. 147)] or indirect precipitation [anti-E1A (M73)]. At the bottom, the cell lines from which the cDNAs were prepared are indicated. Wild-type RB cDNA was isolated as described (1). RB cDNA prepared from J82 bladder carcinoma cells was translated and run in parallel as a control (12). Molecular mass markers (in kDa) are shown on the left.

presume that this point mutation prevents incorporation of exon 21 sequences into RB mRNA by an as yet unknown mechanism. Taken together, we have identified three splicing mutations, within the *RB* genes of tumor cells, that lead to the production of truncated and presumably nonfunctional RB proteins. Each is apparently unable to associate with two viral oncoproteins due to the deletion of sequences contained within a 73-amino acid region of p105-Rb. We presume from our results that a binding site for E1A and SV40 large tumor antigen resides within this segment of p105-Rb. Nonetheless we cannot exclude the possibility that deletions within this region affect oncoprotein binding to other domains of this protein. Whether the location of these three splicing mutations, all clustered in the exon 21–22 region, are coincidental or reflect selection for stable, nonfunctional proteins remains to be determined. Since similar splicing mutations leading to exon loss and in-frame fusion of flanking exons can be predicted to occur throughout the *RB* gene, the latter hypothesis seems likely.

Inactivations of the *RB* Genes Are Infrequently Associated with Several Other Human Tumors. In contrast with our experience with retinoblastoma and SCLC cultures, we have identified instances of *RB* gene inactivations only infrequently in several other human tumor cell types. In an earlier report, we described a defective *RB* allele in a human bladder carcinoma line, J82 (12). This result suggested the involvement of the *RB* gene in the genesis of bladder carcinomas, a possibility that we have investigated through examination of the RB protein in 16 independent human bladder tumor cell lines. In 5 of these tumor lines (MGHU-5, 5637, DTB-5, HT1376, and TCCSUP) RB protein was undetectable; the sixth was the previously studied J82 bladder carcinoma line, which carries a splicing mutation leading to the production of a slightly truncated version of the RB protein (Fig. 1*C*). The independence of all of these tumor cell lines was confirmed through use of five distinct highly polymorphic restriction fragment length polymorphism probes mapping to chromosome 13 (p68RS2.0, p88PR0.6, and p95HS0.5; ref. 32), to chromosome 2 (pYNH24; ref. 33), and to chromosome 17 (pYNZ22; ref. 33). We conclude that *RB* inactivation occurs and presumably plays an etiologic role in about one-third of bladder carcinomas or their derived cell lines. Absent from this group is the EJ/T24 bladder carcinoma, which carries a *ras* oncogene together with apparently normal *RB* alleles (34).

To determine whether the absence of the RB protein from the five bladder tumor lines was due to detectable deletions of the *RB* gene, Southern blot analysis was performed with an RB cDNA probe (Fig. 4). Of the five lines, three exhibit gross deletions of the *RB* gene (TCCSUP, MGHU-5, and DTB-5). We presume that the other two RB protein-negative cell lines carry subtle mutations, akin to those described above, that abrogate the production of stable RB protein.

These results caused us to examine whether or not *RB* inactivation is frequently involved in other types of epithelial tumors. Accordingly, we examined 11 cell lines derived from another common epithelial tumor, human colon carcinoma (Fig. 1*D*). All 11 colon tumor lines showed normal levels of p105-Rb that in all instances comigrated with RB protein precipitated from control cultures. The independence of these tumor lines was again confirmed using the restriction fragment length polymorphism probes employed in our analysis of bladder cells. We conclude from these results that absent or aberrant RB protein is not frequently associated with the etiology of colon cancers or their derived cell lines.

To extend these results to two other common human tumor types, we examined 12 breast carcinoma cell lines and 10 melanoma cell lines for their expression of p105-Rb (Fig. 1 *E* and *F*). The *RB* alleles in primary breast tumors and breast cell lines have recently been studied by others (35, 36). All breast cell lines we examined but two, MDA-MB468 and MDA-MB436, contained normal levels of p105-Rb. These two cell lines were also found in previous studies to carry defective *RB* genes (35, 36). All 10 melanoma tumor lines we examined produced abundant levels of apparently wild-type p105-Rb. Taking the previous analyses together with our own, we conclude that *RB* inactivation is relatively infrequent in human mammary carcinoma and melanoma cell lines. In all of these analyses, we assume that the presence of normal amounts of correctly migrating p105-Rb is indicative of intact *RB* alleles. Thus, we cannot exclude the possibility that some of the ostensibly normal p105-Rb found in the mammary and colon carcinoma lines in fact carry subtle structural alterations that cripple function.

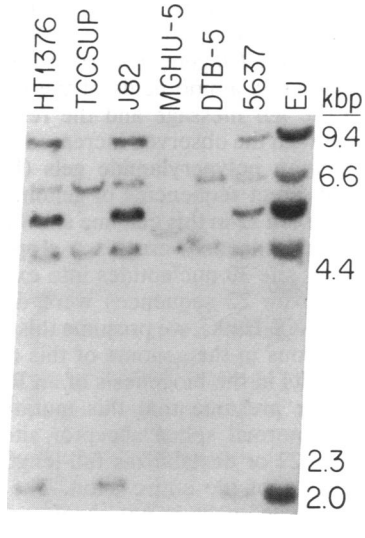

FIG. 4. Southern blot analysis of bladder carcinoma DNAs. Cell cultures from which DNAs were prepared are identified at the top of each gel lane. Shown on the right are molecular size markers from *Hin*dIII-cut λ DNA. kbp, Kilobase pairs.

DISCUSSION

Constitutional hemizygosity of *RB* predisposes children to retinoblastomas and a variety of sarcomas (4–10). In rare instances, other tumor types have been reported at increased frequency in such hemizygous individuals (37, 38). These same individuals have not been reported to have increased risk of sustaining bladder carcinomas and SCLC. The presently reported involvement of somatically inactivated *RB* alleles in these carcinomas contrasts strongly with the apparent lack of predisposition to these tumors seen in individuals constitutionally hemizygous for the *RB* gene. It is possible that this paradox is more apparent than real, in that these types of second site tumors may have been underreported in studies of children cured of early onset retinoblastomas. Alternatively, the pathogenesis of these carcinomas may involve biological mechanisms that are distinct from those triggering retinoblastomas and sarcomas. For example, *RB* gene inactivation may serve as an initiating event in the multistep pathogenesis of retinoblastomas and sarcomas but act as a late (progressional) event in the formation of these carcinomas.

The expression of the *RB* gene in a wide range of tissues contrasts with the narrow range of tissues in which *RB* inactivation appears to be involved in tumorigenesis (ref. 11; J.M.H., unpublished observations). One might presume that the *RB* gene is not critical for growth regulation in many of the cell types in which it is expressed. Such gratuitous expression of p105-Rb may occur in cell types lacking downstream effector(s) of *RB* function. Alternatively, the *RB* gene may function universally in regulating cell growth, but may often operate in the presence of other redundantly acting genes whose action may mask the effects of *RB* inactivation. Since the regulatory pathways that constrain cell proliferation are poorly understood, we are still many years away from directly addressing these possibilities.

Note Added in Proof. Recently, Bookstein *et al.* (39) have described another aberrant RB protein lacking amino acids encoded by exon 21 of the *RB* gene.

J.M.H. wishes to thank Stephen H. Friend and Nick Dracopoli for supplying melanoma lines and Drs. Y. Nakamura and R. White for providing restriction fragment length polymorphism probes. This work was supported by grants from the National Cancer Institute (CA 08131, CA 13106, and CA 39826), the National Eye Institute (EY 04950, EY 05321, and EY 07573), the American Cancer Society (JFRA-211), the American Business Foundation for Cancer Research, and the Rowland Foundation. T.P.D. is a Research To Prevent Blindness-Dolly Green Scholar, and R.A.W. is an American Cancer Society Research Professor.

1. Friend, S. H., Bernards, R., Rogelj, S., Weinberg, R. A., Rapaport, J. M., Albert, D. M. & Dryja, T. P. (1986) *Nature (London)* **323**, 643–646.
2. Lee, W.-H., Bookstein, R., Hong, F., Young, L.-J., Shew, J.-Y. & Lee, E. Y.-H. P. (1987) *Science* **235**, 1394–1399.
3. Fung, Y.-K. T., Murphree, A. L., T'Ang, A., Qian, J., Hinrichs, S. H. & Benedict, W. F. (1987) *Science* **236**, 1657–1661.
4. Knudson, A. G., Jr. (1971) *Proc. Natl. Acad. Sci. USA* **68**, 820–823.
5. Yunis, J. J. & Ramsay, N. (1978) *Am. J. Dis. Child.* **132**, 161–163.
6. Sparkes, R. S., Sparkes, M. C., Wilson, M. G., Towner, J. W., Benedict, W. F., Murphree, A. L. & Yunis, J. J. (1980) *Science* **208**, 1042–1044.
7. Sparkes, R. S., Murphree, A. L., Lingua, R. W., Sparkes, M. C., Field, L. L., Funderburk, S. J. & Benedict, W. F. (1983) *Science* **219**, 971–973.
8. Benedict, W. F., Murphree, A. L., Banerjee, A., Spina, C. A., Sparkes, M. C. & Sparkes, R. S. (1983) *Science* **219**, 973–975.
9. Godbout, R., Dryja, T. P., Squire, J., Gallie, B. L. & Phillips, R. A. (1983) *Nature (London)* **304**, 451–453.
10. Cavenee, W. K., Dryja, T. P., Phillips, R. A., Benedict, W. F., Godbout, R., Gallie, B. L., Murphree, A. L., Strong, L. C. & White, R. L. (1983) *Nature (London)* **305**, 779–784.
11. Lee, W.-H., Shew, J.-Y., Hong, F. D., Sery, T. W., Donoso, L. A., Young, L.-J., Bookstein, R. & Lee, E. Y.-H. P. (1987) *Nature (London)* **329**, 642–645.
12. Horowitz, J. M., Yandell, D. W., Park, S.-H., Canning, S., Whyte, P., Buchkovich, K. J., Harlow, E., Weinberg, R. A. & Dryja, T. P. (1989) *Science* **243**, 937–940.
13. Whyte, P., Buchkovich, K. J., Horowitz, J. M., Friend, S. H., Raybuck, M., Weinberg, R. A. & Harlow, E. (1988) *Nature (London)* **334**, 124–129.
14. DeCaprio, J. A., Ludlow, J. W., Figge, J., Shew, J.-Y., Huang, C.-M., Lee, W.-H., Marsilio, E., Paucha, E. & Livingston, D. M. (1988) *Cell* **54**, 275–282.
15. Ludlow, J. W., DeCaprio, J. A., Huang, C.-M., Lee, W.-H., Paucha, E. & Livingston, D. M. (1989) *Cell* **56**, 57–65.
16. Dyson, N., Howley, P. M., Munger, K. & Harlow, E. (1989) *Science* **243**, 934–937.
17. Whyte, P., Williamson, N. M. & Harlow, E. (1989) *Cell* **56**, 67–75.
18. Friend, S. H., Horowitz, J. M., Gerber, M. R., Wang, X.-F., Bogenmann, E., Li, F. P. & Weinberg, R. A. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 9059–9063.
19. Weichselbaum, R. R., Beckett, M. & Diamond, A. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 2106–2109.
20. Goddard, A. D., Balakier, H., Canton, M., Dunn, J., Squire, J., Reyes, E., Becker, A., Phillips, R. A. & Gallie, B. L. (1988) *Mol. Cell. Biol.* **8**, 2082–2088.
21. Higgins, M. J., Hansen, M. F., Cavenee, W. K. & Lalande, M. (1989) *Mol. Cell. Biol.* **9**, 1–5.
22. Dunn, J. M., Phillips, R. A., Becker, A. J. & Gallie, B. L. (1988) *Science* **241**, 1797–1800.
23. Harbour, J. W., Lai. S.-L., Whang-Peng, J., Gazdar, A. F., Minna, J. D. & Kaye, F. J. (1988) *Science* **241**, 353–357.
24. Bogenmann, E. & Mark, C. (1983) *J. Natl. Cancer Inst.* **70**, 95–104.
25. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.
26. Harlow, E., Franza, B. R., Jr., & Schley, C. (1985) *J. Virol.* **55**, 533–546.
27. Feinberg, A. P. & Vogelstein, B. (1983) *Anal. Biochem.* **132**, 6–13.
28. Yokota, J., Akiyama, T., Fung, Y.-K. T., Benedict, W. F., Namba, Y., Hanaoka, M., Wada, M., Terasaki, T., Shimosato, Y., Sugimura, T. & Terada, M. (1988) *Oncogene* **3**, 471–475.
29. Kawasaki, E. S., Clark, S. S., Coyne, M. Y., Smith, S. D., Champlin, R., Witte, O. N. & McCormick, F. P. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 5698–5702.
30. Yandell, D. W. (1989) in *Cancer Cells: Molecular Diagnostics of Human Cancer*, ed. Furth, M. & Greaves, M. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), Vol. 7, pp. 223–227.
31. Breathnach, R. & Chambon, P. (1981) *Annu. Rev. Biochem.* **50**, 349–383.
32. Wiggs, J., Nordenskjold, M., Yandell, D. W., Rapaport, J., Grondin, V., Janson, M., Werelius, B., Petersen, R., Craft, A., Riedel, K., Liberfarb, R., Walton, D., Wilson, W. & Dryja, T. P. (1988) *N. Engl. J. Med.* **318**, 151–157.
33. Nakamura, Y., Leppert, M., O'Connell, P., Wolff, R., Holm, T., Culver, M., Martin, C., Fujimoto, E., Hoff, M., Kumlin, E. & White, R. (1987) *Proc. Natl. Acad. Sci. USA* **235**, 1616–1622.
34. Parada, L. F., Tabin, C. J., Shih, C. & Weinberg, R. A. (1982) *Nature (London)* **297**, 474–478.
35. Lee, E. Y.-H. P., To, H., Shew, J.-Y., Bookstein, R., Scully, P. & Lee, W.-H. (1988) *Science* **241**, 218–221.
36. T'Ang, A., Varley, J. M., Chakraborty, S., Murphree, A. L. & Fung, Y.-K. T. (1988) *Science* **242**, 263–266.
37. Draper, G. J., Sanders, B. M. & Kingston, J. E. (1986) *Br. J. Cancer* **53**, 661–667.
38. DerKinderen, D. J., Nagelkerke, N. J. D., Tan, K. E. W. P., Beemer, F. A. & Den Otter, W. (1988) *Int. J. Cancer* **41**, 499–504.
39. Bookstein, R., Shew, J.-Y., Chen, P.-L., Scully, P. & Lee, W.-H. (1990) *Science* **247**, 712–715.